THE ROLE OF NEUROD1 IN PHYSIOLOGICAL AND PATHOLOGICAL NEUROGENESIS

by

REBECCA R. BRULET

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December 9th, 2016

THE ROLE OF NEUROD1 IN PHYSIOLOGICAL AND PATHOLOGICAL NEUROGENESIS

REBECCA R. BRULET

The University of Texas Southwestern Medical Center at Dallas, 2016

Supervising Professor:

JENNY HSIEH, Ph.D.

Abstract

Neurogenesis in the adult brain is a complex and highly regulated process. Under normal physiological conditions neurogenesis in the hippocampal subgranular zone (SGZ) is important for learning, memory, and mood regulation. What is not well understood, however, is whether in certain disease contexts, like epilepsy, aberrant neurogenesis can contribute to the progression of spontaneous reoccurring seizures (SRS) and associated memory decline. In this work, I present evidence that aberrant hippocampal neurogenesis is causative in the perpetuation of SRS. In an effort to target a select stage of adult neurogenesis I identified the bHLH transcription factor NeuroD1, known to be important in adult neurogenesis, as being strongly upregulated after status epilepticus (SE). Additionally, I show expression of NeuroD1 in aberrant ectopically localized granule cells suggesting a potential role for this transcription factor in the progression of epilepsy. NeuroD1 conditional knockout (cKO) in progenitor cells of the hippocampus may be sufficient to reduce the number of immature and mature neurons amongst the labeled population, however the total number of immature and mature neurons was not significantly changed aside from the immature neurons ectopically localized to the hilus. Consistent with this, the total SRS was unchanged in the NeuroD1 cKO.

Transdifferentiation, or the direct inter-lineage conversion of adult somatic cells is a powerful tool with the potential to be used in neuronal replacement strategies in certain neurological disorders or CNS injuries. Transdifferentiation of reactive astrocytes into glutamatergic neurons via retroviral targeting in the cortex can be accomplished by overexpression of the transcription factor NeuroD1. However, what is not well understood is whether the state of reactive gliosis is necessary to "prime" these cells for the transdifferentiation process. In this work I present evidence to suggest that overexpression of NeuroD1 in the absence of reactive gliosis is capable of astrocyte to neuron transdifferentiation, however the total number of converted cells is vastly lower than what was previously published, suggesting that reactive gliosis does indeed enhance and facilitate the conversion process.

Table of Contents

Prior Publications

1. Cho Kyung-Ok, Lybrand Zane R, Ito Naoki, Brulet Rebecca, Tafacory Farrah, Zhang Ling, Good Levi, Ure Kerstin, Birnbaum Shari G, Scharfman Helen E, Eisch Amelia J, Hsieh Jenny. Aberrant Hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. 2015. Nature Communications, **26**(6).
2. Mukherjee Shradha, Brulet Rebecca, Zhang Ling, Hsieh Jenny. REST regulation of gene networks in adult neural stem cells. Nature Communications, 2016.
3. Brulet Rebecca, Zhu Jingfei, Aktar Mahafuza, Hsieh Jenny, and Cho Kyung-Ok. Mice with conditional NeuroD knockout display reduced aberrant hippocampal neurogenesis but no change in epileptic seizures. 2016. Experimental Neurology, *in preparation*.
4. Brulet Rebecca, Taito Matsuda, Ling Zhang, Carlos Miranda, Mauro Giacca, Brian K. Kaspar, Kinichi Nakashima, and Jenny Hsieh. NeuroD1 instructs neuronal conversion in non-reactive astrocytes. 2016. Stem Cell Reports, *in revision.*

List of Figures

List of Abbreviations:

Ascl1: achaete-scute homolog 1

AHP: adult hippocampal progenitor

bHLH: basic helix-loop-helix

BMP: bone morphogenetic protein

BrdU: bromodeoxyuridine

BrUTP: bromouridine-triphosphate

Cdc20: cell division cycle 20

ChIP: chromatin immunoprecipitation

Cre: cre recombinase

cKO: conditional knockout

DAPI: 4',6-diamidino-2-phenylindole

Dcx: doublecortin

DG: dentate gyrus

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

Egf2: epidermal growth factor 2

EGC: Ectopic granule cell

ERT2: Estrogen-receptor variant

FACS: fluorescent activated cell sorting

FF: free-floating

Fgf2: fibroblast growth factor 2

Fl/Fl: Floxed/Floxed

GABA: γ-aminobutyric acid

GCL: Granule cell layer

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

HCN: hippocampal neural progenitor

IHC: immunohistochemical

iKO: inducible knockout

i.p.: intraperitoneally

iPSC: induced pluripotent stem cell

iQNP: induced quiescent neural progenitor

iTAP: induced transient amplifying progenitor

IRES: internal ribosome entry site

Lenti: lentivirus

M: mitosis

MEF: mouse embryonic fibroblast

ML: molecular Layer

Mms22l: methyl methanesulfonate-sensitivity protein 22-like

NeuroD1: neurogenic differentiation

Ngn2: neurogenin 2

NP: neural progenitor

Npm1: nucleophosmin 1

NSPC: neural stem/progenitor cell

NRSF: neural restrictive silencing factor

NS: neurosphere

P2A: porcine teschovirus-1

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PI: propidium iodide

Prox1: prospero Homobox 1

PVA/DABCO: polyvinyl alcohol/1,4 diazabicyclo [2.2.2] octane

QNP: quiescent neural progenitor

qPCR: quantitative PCR

Rad51: Rad51 recombinase

REST: repressor element 1-silencing transcription factor

Retro: retrovirus

RGL: radial glial-like

RNA: ribonucleic acid

RNA-seq: RNA-sequencing

Rpl4: ribosomal protein L4

Rps6: ribosomal protein S6

rRNA: ribosomal RNA

SCI: spinal cord injury

SE: status epilepticus

SEM: standard error of mean

SGZ: subgranular zone

shRNA: short hairpin RNA

SM: slide-mounted

Sox2: SRY (sex determining region Y)-box 2

SRS: spontaneous recurring seizures

SVZ: subventricular zone

TAM: tamoxifen

TAP: transit amplifying progenitor

TBI: traumatic Brain injury

TBS: tris-buffered saline

Tipin: timeless interacting protein

TSS: transcription start site

Wnt: wingless-related integration site

WT: wildtype

YFP: yellow fluorescent protein

Chapter 1

Introduction

Discovery of adult neurogenesis

A century ago dogma stated that neurons in the brain could be produced only during the embryonic and perinatal stages of development in mammals, and that once these neurons were lost, they could not be replaced (1-3). During this time period our ability to study neurogenesis was largely hampered by a lack of sufficient scientific tools, however, as new innovations to technology were made, namely the discovery that tritium labeled thymidine could be used to trace dividing cells, the study of cell proliferation in different organ systems, including the brain, took off (4).

One of the first studies to suggest that neurogenesis indeed continued on in the adult brain was the 1965 study by Dr. Joseph Altman and Dr. Gopal D. Das (5). In this work Altman and Das injected adult rats with tritiated thymidine and examined sections of the brain to determine whether any recently dividing cells could be identified (5). Fascinatingly, Altman and Das discovered many labeled cells in the hippocampal dentate gyrus (DG) , olfactory bulb, and neocortex leading to the idea that new neurons could in fact be generated in some regions of the adult brain (5). It wasn't until many years later in 1984, that Dr. JA Paton and Dr. Fernando Nottebohm presented evidence demonstrating the integration and function of these newborn neurons in the vocal control nucleus of adult songbirds, further strengthening the idea that neurons could be generated throughout adulthood, and had a critical role to play in brain function (6).

With the discovery of a thymidine analog, 5-bromo-2-deoxyuridine (BrdU), the detection of dividing cells became even more accessible. As a result many groups in the late 1980s and 1990s published work identifying newborn neurons in a variety of species including the adult primate (7-10). Further, several groups were successful in the establishment and in vitro culture of adult neural stem cells from the hippocampal dentate gyrus and lateral ventricle allowing for easier study

and experimentation with these multipotent cells (11, 12). Each of these discoveries was critical to the acceptance of the idea that multipotent stem cells are preserved in certain areas of the adult brain, and can give rise to new functional neurons throughout the life of a variety of animal species.

Overview of adult neurogenesis

The phenomenon of adult neurogenesis is now widely accepted in the field due to much effort from these pioneering scientists. Their work and others has led to the successful identification of two areas in the adult brain that serve as resident home to adult neural stem cells; the subgranular zone (SGZ) of the hippocampus, and the subventricular zone (SVZ) of the lateral ventricle (1, 5, 6, 10-13). Newborn neurons in each of these regions play critical roles in olfaction, learning and memory, and regulation of mood throughout the life of most animals. Since this thesis work will focus exclusively on neurogenesis in the SGZ of the hippocampus, I will leave more comprehensive discussion of adult neural stem cells in the SVZ region to others.

In the dentate gyrus of the hippocampus quiescent neural progenitors, or QNPs reside within the inner region of the granule cell layer, commonly referred to as the SGZ. These QNPs have a very distinct morphology, with a triangular cell body, a single process extending up through the granule cell layer, and a small bushy tuft of fine processes that extends into the molecular layer (ML)(Figure 1.1A). Theses QNPs as their name implies are maintained primarily in their quiescent state, but occasionally become activated at low rates in order to generate a cell type called the transit amplifying progenitor (TAP)(Figure 1.1B). TAPs are highly proliferative cells residing within the SGZ, with small round cell bodies, and short horizontal processes (Figure 1.1B). These cells undergo multiple rounds of amplifying division before generating the immature neuroblasts population (Figure 1.1C)(14). Neuroblasts look quite similar to the TAP cell population, but gradually lose their proliferative ability to yield immature neurons that extend elaborate cellular processes up into GCL and ML (Figure 1.1C). As these cells transition into the mature neuronal

stage, their rounded cell bodies migrate up into the GCL, their dendritic trees grow larger, and they begin to project axons out to the CA3 region of the hippocampus (Figure 1.1D,E)(14). The proliferation, differentiation, and maturation of these newborn cells typically takes between several weeks to a month after birth for successful integration into the existing brain circuitry (2, 3) .

In the hippocampus each of the cellular transitions present in the process of adult neurogenesis can be easily distinguished by immunohistochemical (IHC) staining with cell stage-specific markers in addition to cellular morphology. The radial glial like cells can, as their name suggests, be labeled by the astrocytic marker glial fibrillary acidic protein (GFAP) as well as the neuroectodermal stem cell marker Nestin (3, 14, 15). Co-labeling of GFAP/Nestin+ cells with the transcription factor SRY-box 2 (Sox2), or the cellular proliferation markers Ki67 or BrdU are most often used to distinguish the radial glial like cells, or QNPs from resident astrocytes found in the hippocampal SGZ (3, 14, 15). As QNPs transition into TAPs and eventually neuroblasts they begin to upregulate expression of several key transcription factors such as Neurogenin 2 (Ngn2), T-box brain protein 2 (Tbr2), and NeuroD1 (ND1) (3, 14-16). Coupling of these markers along with the cellular proliferation markers Ki67, or BrdU can used to successfully identify TAPs and immature neuroblasts in the SGZ. Following this stage, neuroblasts transition into immature neurons that can be labeled by the expression of the marker doublecortin (Dcx), Prospero-homeobox domain 1 (Prox1), and Calretinin (3, 14, 15). Finally, as the immature neurons begin to fully differentiate and mature they show expression of the markers neuronal nuclei (NeuN), Prox1, and Calbindin (3, 14, 15).

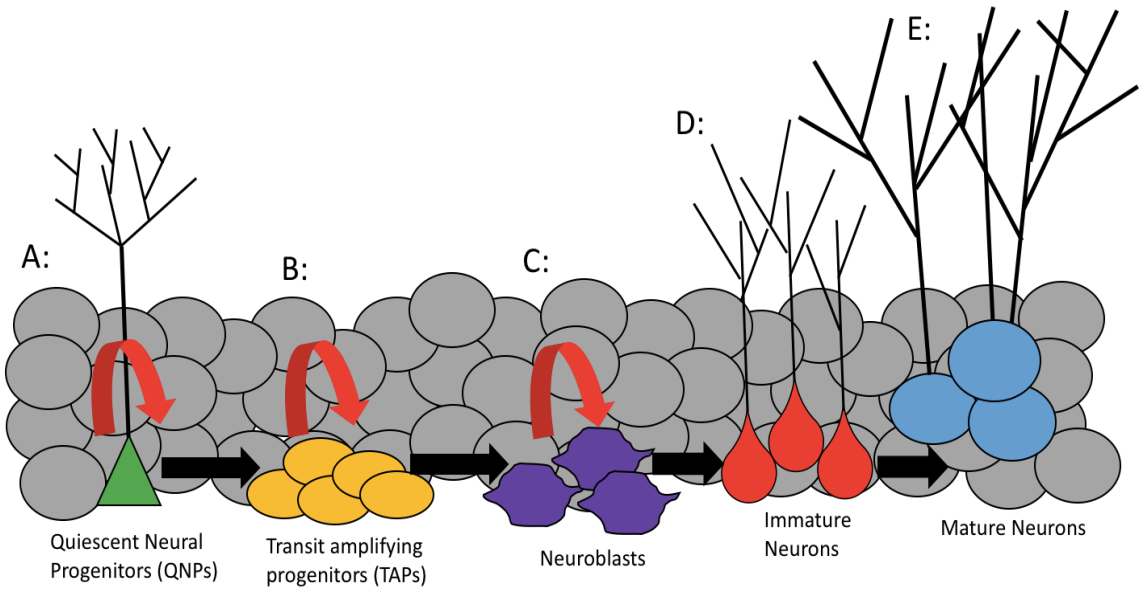

Figure 1.1 Adult hippocampal neurogenesis A: Quiescent neural progenitor cells are either maintained in their quiescent state or activated to become B: Transit amplifying progenitors (TAPs) which rapidly self-renew to expand the neural stem cell pool and then go on to give rise to C: the neuroblasts cells that then begin to differentiate into D: Immature neurons and finally E: Mature neurons.

During the amplification, differentiation, and maturation of these neural stem cells there are two key stages in which cellular death plays an important role in the pruning and selection of this cell population. The first stage of cell death is found amongst the TAP population within approximately one week after their transition from quiescence (17). Once past this stage, these cells will undergo an additional round of selection at the immature neuronal stage. This stage of cell death is largely controlled by the existing circuitry, with cells that fail to form the proper synaptic connections being selected against and removed from the dentate (18-20). Surviving cells then undergo functional integration with existing granule neurons. Once fully matured these new neurons are both morphologically and electrophysiologically similar to their previously established neighbors (21, 22).

The neurogenic potential of the neural stem cells residing within the SGZ is not constant with age. Turnover and generation of newborn neurons is highest during the juvenile period, and steadily decreases with age. In the rodent model, incorporation of BrdU and other cellular proliferation markers drops significantly between 12 to 27 months of age (23, 24). The mechanism behind the decline in neurogenesis is not currently well understood. Theories that metabolic potential might be directly linked with the retardation of neural stem cell proliferation have proven largely untrue as the density of proliferating cells remains relatively constant in other regions of the brain (24). Interestingly, evidence has suggested that neurogenic enhancement through exercise or provision for an enriched environment can enhance neurogenesis and lead to a modest, but long-lasting conservation of these proliferative cells (25-27).

Regulation of Adult Neurogenesis

Adult neurogenesis is an elaborate process that takes the coordinated effort of several major regulatory pathways in order for the successful proliferation, differentiation, and maturation of neural stem cells. In this section I will discuss some of the major pathways governing proper

neural stem cell development in the SGZ including the Wnt, Sonic Hedgehog (Shh), Notch, and Bone morphogenetic protein signaling pathways (Bmp).

The Wnt family is comprised of a large family of secreted glycoproteins that have long been known to be important in axis formation of the embryo (28). Interaction of the Wnt family members with the seven-pass transmembrane receptors of the Frizzled (Fz) family trigger activation of the Wnt pathway (29). Currently 19 members of the Wnt family of glycoproteins have been identified (30). During embryonic development Wnt signaling is critical for development of the hippocampus and cortex. Ectopic activation of the Wnt pathway during embryogenesis leads to repression of the neuronal genes Pax6, Ngn2, Tbr2, and Meis2, and ultimately disruption of neuronal organization and the cortical plate (31). In the adult brain members of the Wnt family play critical roles in neural proliferation, plasticity, and synapse formation (30). Early studies using transgenic mice showed expression of the Wnt/β-catenin pathway in the SGZ and GCL of the adult hippocampus (32). In vitro work using co-culture of adult hippocampal progenitors (AHPs) and astrocytes known to express the Wnt family member Wnt 3, demonstrated that reduction of Wnt signaling using the secreted Frizzled-related protein 2 and 3 (sFRP2/3) was capable of decreasing induction of neuronal differentiation (32). Similarly, blockage of the Wnt pathway using lentiviral expression of the secreted mutant Wnt 1 protein (dnWnt) in vivo led to a significant reduction in the number of newborn neurons, and ultimately deficits in spatial memory and object recognition (32, 33). Additionally, the Wnt pathway plays a role in the signaling mechanisms of the orphan nuclear receptor (TLX), known to be required in the regulation of neural stem cell self-renewal and maintenance (34).

In vertebrates, Shh is one of three members of the hedgehog protein family best known for its role as a morphogen in the developing central nervous system (CNS). Hedgehog signaling in cells is accomplished primarily through the interaction of two proteins; the seven-pass G-protein-

coupled receptor smoothened (SMO) and the twelve-pass membrane hedgehog receptor patched (PTC1)(35-38). Studies using loss-of-function mutations in the Shh pathway during embryonic development of the CNS have demonstrated its critical role in telecephalic patterning, development of the retinal system, cerebellum, and ventral patterning of the dorsal midbrain (37, 39-44). In the adult brain, the Shh signaling component Patched (Ptc) is expressed in the hippocampal formation indicating cellular competency for this pathway (45). Work done in vivo using ectopic activation of the Shh pathway via adeno-associated virus (AAV) has shown an increase in BrdU+ cells suggesting an increase in cell proliferation(45). Additional work done in the SGZ indicates that QNPs actively respond to Shh signaling by increasing cell proliferation after an ablation insult using the anti-mitotic AraC (46) . Despite the fact that Shh signaling plays a crucial role in both embryonic and adult development of the hippocampus, the downstream mechanistic details of how this signaling is accomplished are largely unknown at this time.

The Notch signaling pathway plays a critical role in the proliferation, differentiation, and apoptosis of cells in the developing CNS. Through Notch receptor activation of the membrane-bound ligands Delta and Jagged, the notch intracellular domain (NICD) is released and translocated to the nucleus where is it responsible for activation of the bHLH family of transcription factors (47). In the adult, various members of the Notch signaling pathway have been successfully identified in the SVZ and SGZ (48). In the SGZ, Notch1 is expressed in the radial glial like precursors and is subsequent downregulated in the TAP population suggesting that Notch signaling is important in radial glial differentiation into committed neural precursors (49). Indeed, conditional loss of Notch1 signaling in Nestin+ NSCs in the hippocampus resulted in fewer labeled cells at each stage of neurogenesis, and interestingly an overall decrease in the size of the Dcx+ immature neuron dendritic trees (49, 50). Additionally, overexpression of Notch signaling in NICD transgenic mice led to a 3-4 fold increase in proliferating cells in the DG (49).

The bone morphogenetic protein (Bmp) signaling pathway, while not as well studied as the previous three signaling pathways has been implicated in the maintenance of the NSC pool in the adult SGZ as well (51). Currently about twenty members of the Bmp protein family have been identified, constituting the largest subgroup of the transforming growth-factor beta (TGFβ) superfamily (52). Bmps are highly expressed in both the developing and adult CNS and play many roles in cell survival, proliferation, and cell fate specification (53). In areas of adult neurogenesis expression of Bmp family members serves to inhibit neuronal fate at the expense of glial differentiation (54).

As previously mentioned there are two waves of cell death that occur in the SGZ during adult neurogenesis. This selection is due in part to glutamatergic and GABAergic signaling from mature neurons found in the DG. GABAergic signaling is critical to the maturation of new neurons, as well as their successful synaptic integration into the existing circuitry. Mice that received GABAergic stimulation via injection of the GABA receptor agonist $GABA_{A\text{-}R}$ had significantly increased numbers of newborn neurons in the hippocampus (55). Similarly, conversion of GABA-induced depolarization into hyperpolarization in newborn neurons led to deficits in dendritic formation, and improper synapse development (56). Activation of glutamatergic signaling through the NMDA receptor or AMPA/KA receptor has been show to increase dentate cell proliferation (57). This indicates a critical role of glutamatergic/GABAergic signaling in the selection of newborn neurons that will eventually integrate into the established neuronal network.

Evidence has suggested a role for small RNA regulation of neurogenesis, specifically through expression of several microRNAs. MicroRNAs are ~22nt long non-coding RNAs that play important roles in posttranscriptional gene regulation. Due to their short sequence and wide range of targeting microRNAs are primary candidates for widespread control of gene expression during neurogenesis. For example, miR-9 and miR-124 are expressed in the brain in both embryonic and

adult development. In vitro expression of miR-124 in the p19 mouse neuroblastoma cell line in the absence of induction factors is sufficient to induce neuronal differentiation largely due to its ability to trigger alternative pre-mRNA splicing (58). Transfection of neural stem cells in vitro with miR-9 resulted in a significant decrease in the number of BrdU labeled cells (59). Both miR-9 and miR-124 have been demonstrated to be targets of the RE1-silencing transcription factor (REST) known for its role in restricting neuronal gene activation in adult neurogenesis (60). Expression of the microRNA miR-137 has been found to increase during neuronal differentiation indicating it is likely involved in control of this process. Indeed, overexpression of miR-137 in aNSC increased cell proliferation, while inhibition of miR-137 resulted in enhanced neuronal differentiation (61). Work from Szulwach et al. showed miR-137 control of aNSC is achieved primarily through repression of the polycomb family member Ezh2 leading to a decrease in histone H3 trimethyl lysine 27 (61). Similarly, overexpression of the micro RNA miR-184 and miR-195 in aNSCs enhanced proliferation at the expense of neuronal differentiation (62, 63). It is thought that this process is mediated in part due to ability of miR-195 to reduce expression of the NeuroD1 and GFAP promoters (62).

On a more hierarchical level, epigenetic control through histone modification, DNA methylation, and chromatin remodeling are equally important in the regulation and control of adult neurogenesis. DNA in a cell is compacted and subsequently made unavailable for gene transcription when wrapped around the histone proteins H2A, H2B, H3, and H4 (64). Tightening or loosening of this structure to expose specific genes is critical to how a cell is able to regulate gene expression. Recently work in the last century has demonstrated that modifications to the amino-terminal tails of the core histone proteins is necessary in this regulatory process for tightening/loosening of the DNA, and is accomplished by chromatin modifying enzymes. The four main chromatin-modifying enzymes are histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HdMTs)(64). Currently, eleven different HDACs have been identified in mammals, some of which have been shown to play an important role

in regulation of the aNSC pool. Treatment of adult hippocampal neural precursors with the histone deacetylase valproic acid (VPA) is capable of inducing rapid neuronal differentiation of these cells at the expense of the glial lineage (65). More specifically, treatment of NSCs with siRNA to HDAC3/5/7 has been shown to significantly reduce the proliferative capacity of aNSCs (66). Conditional removal of HDAC3 from the NSC pool in the hippocampus of transgenic mice led to a decrease in proliferation due to defects in G2/M cell cycle progression (67). While HDAC 4/5/7/9 have been shown to undergo upregulation of expression in differentiating NSCs there has not been much work on their specific role in these cells (68).

More recently work in the field has been focused on the role of the niche environment in supporting the growth and proliferation of neural stem cells. Proliferating neural stem progenitor cells found within the SGZ are capable of expression and secretion of the growth factor vascular endothelial growth factor (VEGF)(69). VEGF is an endothelial cell mitogen, and as its name suggests very important in regulation of angiogenesis (70). Previous work in the adult brain has shown that VEGF is important in the regulation of neural stem cell proliferation within the hippocampus, primarily through its control of the MEK/ERK and PI3K/Akt-dependant pathways (71). More recently, several studies have demonstrated the requirement of VEGF signaling for the neurogenic effects of antidepressant treatment (72-74). VEGF has also been reported in several studies to be required for the increase in cell proliferation observed after various pro-neurogenic stimuli such as exercise and environmental enrichment (75).

Function of Adult Neurogenesis

The birth of new neurons in the SGZ of the hippocampus and SVZ of the lateral ventricle has been linked to several important functional processes. Neural stem cells residing within the SVZ of the lateral ventricle undergo migration and subsequent differentiation into granule neurons of the olfactory bulb, and have been shown in a number of studies to be important in olfactory

discrimination (76-80). Neurogenesis in the SGZ on the other hand has been linked to processes such as learning and memory. Transgenic mice in which the pro-apoptotic gene Bax was conditionally ablated in neural stem cells of the SGZ showed improvement in functional tasks involving pattern separation (81). Enhancement of neurogenesis by voluntary exercise has been linked to improved performance in hippocampal dependent memory tasks such as the Morris Water Maze, while ablation of neurogenesis led to worsening performance (Fig 1.2 A,B) (82-84). Inhibition of WNT pathway signaling in the hippocampus using viral delivery of a dominant-negative WNT (dnWNT) led to significantly worse performance in hippocampal dependent object recognition tasks compared to control treated animals (Fig 1.2 C) (85).

Interestingly, hippocampal neurogenesis has also been linked to the beneficial effects seen by many options for the treatment of depression (Fig 1.2 D,E) (86, 87). This is primarily postulated to be due to the ability of anti-depressant medications to increase hippocampal neurogenesis. For example, chronic treatment of rats with the three antidepressants tranylcypromine, fluoxetine, and reboxetine led to a marked increase in the number of BrdU+ cells in the SGZ of the hippocampus (87). Additionally, it has been proposed that neurogenesis in the SGZ is important in mating related behavior such as mate selection. Neurogenesis stimulated in the SGZ and SVZ of female mice after exposure to pheromones from dominant male mice has been shown to correlate with female preference for selection of that partner compared to those exposed to sub-ordinate male pheromones (88).

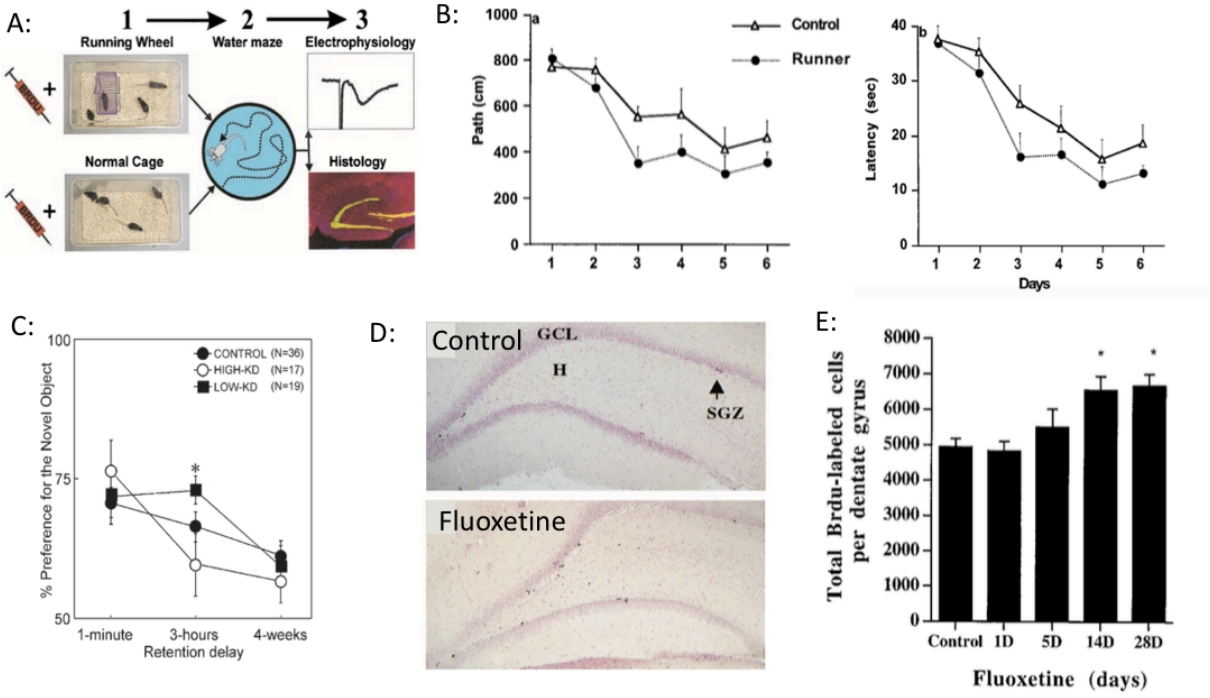

Figure 1.2 The functional roles of adult hippocampal neurogenesis A: Experimental paradigm showing experimental enhancement of neurogenesis by addition of a running wheel to home cage, compared to control mice that received no wheel. After 1 month mice were subjected to the Morris water maze task to examine the function of increased neurogenesis on learning and memory. B: Animals housed in cages with access to a running wheel had enhanced neurogenesis, and a significantly improved performance over the controls in the Morris Water Maze test as indicated by path length and latency to reach the submerged platform. C: Inhibition of hippocampal neurogenesis led to a significant worsening in performance in an object recognition memory task. D,E: Rats receiving treatment with the anti-depressant fluoxetine showed significant increases in the number of BrdU labeled cells within the hippocampal SGZ. (82, 85, 87) Copyright 1999 National Academy of Sciences

Epilepsy and Neurogenesis

Mesial Temporal Lobe Epilepsy (mTLE) is a disorder of the CNS characterized by spontaneous reoccurring seizures (SRS). Approximately 3 million people in the United States and 65 million people worldwide are affected by this disorder, making it the 4th most common neurological disorder to affect people of all ages (89). mTLE is typically diagnosed after more than one unprovoked seizure event is reported, and although there are currently many anti-seizure medications available for its treatment, there is still no cure for this disease. Approximately one-third of all patients with this disorder are refractive to anti-seizure medications (90). Those refractory to these medications often go to great lengths to alleviate their seizures sometimes have Deep Brain Stimulation (DBS) or surgical resection of affected areas of the brain (90).

The underlying cellular and molecular mechanisms of mTLE are currently not well understood. Previous work has demonstrated several important cellular changes that occur within the hippocampal formation and surrounding structures in patients with mTLE. These include astrogliosis, mossy fiber sprouting, hilar basal dendrites, ectopic migration of newborn neurons, neuronal cell death, granule cell layer dispersion, and an increase in adult neurogenesis (90-94). However, what is not well understood is which of these processes, or combination of processes, is particularly important for the SRS activity seen in this disease. Despite many observations of increased neurogenesis, ectopic migration, and aberrant changes to newborn neurons in the hippocampus, evidence to suggest aberrant neurogenesis as a driving force behind mTLE is still lacking and controversial (Fig 1.3E).

Rats subjected to experimental epilepsy using an M1 muscarinic receptor agonist called pilocarpine showed a significant increase in the total number of BrdU labeled cells 2 weeks after status (Fig 1.3A) (95). Further work in a limbic kindling model of epilepsy in rats showed similar increases in BrdU+ cells within the SGZ (96). This increase in neurogenesis is transient with

proliferation levels returning to normal within approximately 3-4 weeks after status (95). Co-labeling of these BrdU labeled cells with the markers Prox1, NeuN, and Dcx have shown that these cells are indeed neurons (95, 97). Interestingly, seizure activity also appears to increase the rate of neurogenesis within the hippocampus (98). In addition to an increase in the number of newborn neurons, a number of studies have reported an increase in the ectopic migration of these newborn cells into the hilar region, as well as dispersion of cells found within the GCL (Fig 1.3 B,C) (95, 97). As neurons mature after a seizure event, a subpopulation of these newborn cells will extend a basal dendrite down into the hilus, a process that it not normally noted under control conditions (Fig 1.3D) (99). These hilar basal dendrites (HBD) are covered in numerous spines and successfully integrate into the existing circuitry of the hippocampus leading some to suggest these cells form recurrent excitatory loops that may contribute to SRS (98-100). Even more interesting is the fact that mature neurons already present at the time of status epilepticus (SE) do not exhibit similar changes suggesting that the newborn neurons are the population most vulnerable to the effects of seizures in the hippocampus (101, 102).

Several studies would suggest that seizure-induced neurogenesis makes use of a similar profile of transcription factors as normal adult neurogenesis. Expression of the bHLH transcription factors, shown to be critical to the regulation of different stages of neurogenesis, are of particular interest. Indeed, induction of acute seizures using the glutamatergic receptor agonist Kainic Acid (KA) led to a significant upregulation in the number of Neurogenenin 2 (Ngn2) expressing cells (103). Similarly, epileptic rats showed an increase in mRNA levels of the bHLH family member Mash1 (Ascl1), while mice treated with pentylenetetrazol (PTZ) to induces seizures showed significant increases in NeuroD2 (104, 105). Given evidence that would suggest seizure effects both the proliferation, differentiation, and maturation of newborn neurons it is very likely that at least some of these bHLH factors play a causative role in aberrant neurogenesis.

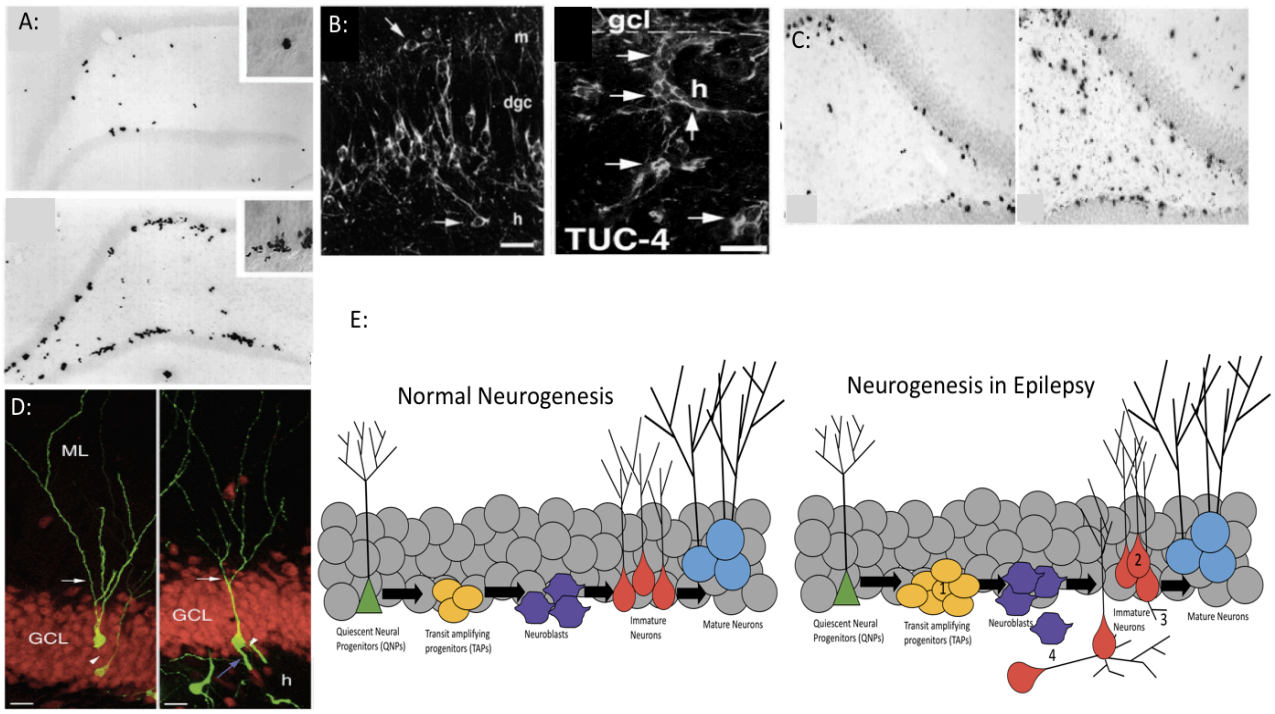

Figure 1.3: Aberrant neurogenesis in epilepsy A: Rats subjected to experimental seizures using pilocarpine showed a significant increase in the total number of BrdU labeled cells in the hippocampus at 13 days post-seizure. B: Ectopic migration of Tuc-4+ and TOAD-64+ neuroblasts in the pilocarpine epilepsy model. C: Rats treated with pilocarpine to induce seizures have a significant increase in the number of ectopically localized cells in the hilar region at 2 weeks. D: Retroviral labeling of newborn neurons showing the addition of a basal dendrite (right panel) in an animal treated with kainic acid (KA), compared to control (left panel). E: Cartoon summarizing the changes that occur to the process of neurogenesis in the hippocampus, 1) increased proliferation 2), increased maturation 3) Hilar basal dendrites and 4) Ectopic migration (90, 95, 97)

In order to try to test whether these aberrant neurons were important after seizure several groups have used ablation approaches to kill off dividing cells in the hippocampus. Continuous infusions of the anti-mitotic agent cytosine-b-D-arabinofuranoside (Ara-C) after pilocarpine induced SE led to a significant reduction in the number of ectopic granule cells and subsequently a reduction in seizure susceptibility (106). Similarly, rats exposed to radiation one day prior to the kindling model of epilepsy showed lower levels of neurogenesis and neuroblast production in the SGZ (107). However, in this study rats that underwent radiation treatment developed more severe seizures in a reduce period of time compared to control rats (107). While both of these studies present evidence for the role of neurogenesis in epilepsy, neither of their experimental approaches were specific to just the proliferating NSC population. Both Ara-C and radiation exposure are capable of killing any dividing cell in this region including microglia, and reactive astrocytes that respond to injury in the brain. Therefore, the possibility still remains that these cells are still a confounding factor within each of these studies, and suggests a need for greater specificity when examining the role of adult neurogenesis in epilepsy.

NeuroD1

Neurogenic differentiation 1 (NeuroD1) is a member of the neurogenic differentiation gene class of the bHLH transcription factors important in embryonic and adult neurogenesis. Other members in this class include NeuroD2, Atoh3, Math1, and NeuroD4 (108). First discovered and characterized in Drosophila, the mammalian bHLH proteins form heterodimers with E-proteins and subsequently bind to E boxes found in many genes throughout the genome (108).

Expression of NeuroD1 begins during embryonic development around E9.0 during the same time frame that development of the CNS is initiated (109). One of the first studies to identify a role for NeuroD1 in development of the CNS was performed in 1995 by Dr. Jacqueline E. Lee. In this study Dr. Lee and colleagues noted NeuroD1 showed transient expression is a subset of neurons as

they underwent terminal differentiation (110). By performing overexpression of NeuroD1 in Xenopus embryos she was able to show premature differentiation of neuronal precursor cells (110). Further, in this same study overexpression of NeuroD1 in cultured ectoderm was able to drive conversion of these cells, presumably destined for the epidermis, into neurons (110). Work performed in the developing mouse retina also suggested a role for NeuroD1 in neurogenic differentiation as knockout of NeuroD1 in retinal explant cultures yielded far more glial cells as compared to controls (109). Overexpression of NeuroD1 in the retina also reduced the clone size of retinal progenitor cells and suppressed glial development (109). Both of these studies provided evidence to suggest a critical role for NeuroD1 in differentiation and maturation of progenitor cells in the CNS.

One of the first major steps to understanding the role of NeuroD1 in CNS development was the creation of a NeuroD1 knockout (KO) mouse line developed by the lab of Dr. Ming-Jer Tsai (111). Surprisingly, the mice from this line were born severely ill and died not long after birth. Further investigation revealed severe defects in B cells number and pancreatic islet formation in the pancreas leading these mice to be born severely diabetic (111). As a result, this mouse line proved to be insufficient for analysis of the role of NeuroD1 in the developing CNS. In order to enable proper study of NeuroD1 in the developing CNS Dr. Lee's group designed a second NeuroD1 KO mouse, only this time they included a copy of the NeuroD1 coding region under control of the insulin promoter (112). The resulting mice were able to survive into adulthood, although they were noticeably smaller as compared to their wild-type littermates (112). Analysis of the brains of these mice showed severe defects in the granule cell populations in both the cerebellum and hippocampus (112). As a result many of these mice showed severe ataxia, a phenotype often noted with defects of the cerebellum (112). Interestingly, NeuroD1 KO mice were also reported to develop seizures of the limbic system characterized by abnormal EEG recordings in the cortical and hippocampal regions (113). With successful analysis of this mouse line it became clear that

NeuroD1 had a significant role to play in development of the cerebellum and hippocampus, and suggested a possible role for NeuroD1 in adult neurogenesis.

In the adult brain, NeuroD1 is typically expressed in the proliferating neuroblasts and immature neuronal populations suggestive a putative role for its control of differentiation and maturation of these cells. Development of a conditional knock out (cKO) mouse line for NeuroD1 was critical in our understanding of the role of NeuroD1 in adult neurogenesis (114). Breeding of this mouse line to different Cre expressing reporter mouse lines has allowed for precise interrogation of the requirement for NeuroD1 in different cell populations in the adult hippocampus and SVZ. Previous work from our lab made use of the Nestin CreERt2 reporter mouse line to drive deletion of NeuroD1 in progenitor cells of the hippocampus and SVZ. Hippocampal sections taken from the brains of these resulting mice showed a severe reduction in the number of surviving neuronal cells at 40 days post tamoxifen (TAM), but not earlier time points examined (16). Further analysis using markers specific for different populations of cells found in the process of hippocampal neurogenesis suggested a role for NeuroD1 in the differentiation and survival of newborn neurons, whereas it appeared to be dispensable in earlier progenitor cell populations (16). Additionally, loss of NeuroD1 led to a shortening of dendritic processes found on neurons in the hippocampus which may lead to altered synaptic integration of these newborn cells into the existing circuitry (16)

Reprogramming and Transdifferentiation

During development of the embryo, pluripotent stem cells organize and differentiate into the 3 germ cell layers, the mesoderm, endoderm, and ectoderm which will ultimately compose all of the tissues found in the adult body. These pluripotent stem cells encounter environmental cues, and complex transcriptional and epigenetic programs that help to mold them into their fated cell type. Conrad H. Waddington described this process in his famous “epigenetic landscape” model whereby

a stem cell could be viewed as a marble perched upon the top of a hill (115). As the marble began to move and rolled downhill it encountered different paths and grooves that represented the different transcriptional networks critical for cell fate determination so that by the time the marble reached the bottom of the hill it was a fully differentiated cell incapable of defying gravity and returning back to the top (115). This was the accepted view of cell fate determination, that it was a unidirectional process that once completed could not be reversed.

This idea of unidirectional development was first challenged by work from Dr. Joseph Gurdon and colleagues in 1958 (116). Dr. Gurdon, utilizing a protocol for transplantation of living cell nuclei into Xenopus eggs developed by Briggs and King in 1952, was able to successfully clone frogs using transplantation of nuclei from intact somatic cells of the adult intestinal epithelia into enucleated eggs (116, 117). This important experiment thus demonstrated the key finding that even cells in the adult body possess all the necessary genetic material for the development of the whole organism. Therefore, the process of development was no longer seen as a pruning of the genetic potential of pluripotent cells along their path to their somatic cell fate, but the silencing or repression of genetic programs that might confuse and confound cellular identity. Another important breakthrough in the field occurred in 1987 when Andrew Lassar's group showed that forced expression of the transcription factor MyoD in mouse fibroblasts was capable of converting these cells into myoblasts that expressed muscle specific proteins (118). This experiment was one of the first to demonstrate that expression of a single transcription factor was sufficient to induce inter-lineage conversion. This experiment would also suggest that cell fate identity could be controlled by a single transcription factor and paved the way for the many studies that would come later working to identify transcription factors key to specific cell fate identities.

Then in 2007 the scientific world was rocked by the discovery of four transcription factors, Sox2, Klf4, Oct3/4, and c-Myc, that when expressed in an adult somatic cell could lead to their

conversion back into the pluripotent state in a process called "reprogramming" (119). Further, this work demonstrated that once pluripotency was achieved, these cells were capable of differentiation into cell types found throughout the 3 germ cell layers (119). This work from Dr. Shinya Yamanaka was the first to identify factors needed for pluripotency and the induction of the "reprogrammed" cell fate, and essentially opened the door for the many publications that followed utilizing these cells. No longer was the isolation of human pluripotent stem cells with all of its ethical and legislative concerns a roadblock in the study of cell fate determination and lineage potential a concern, scientists could now easily derive stem cells from any tissue that they liked. For this work Dr. Yamanaka won the Nobel Prize in Physiology or Medicine in 2012 along with Dr. Joseph Gurdon for their pioneering work demonstrating that "mature specialized cells can be reprogrammed to become immature cells capable of developing into all tissues of the body" (Nobelprize.org).

Around the same time as the four reprogramming factors were identified, there were a number of labs experimenting with the process of transdifferentiation, or direct inter-lineage conversion. Cells that undergo transdifferentiation do so without the need for a pluripotent intermediate, leading to a 1:1 conversion. Some of the first labs to demonstrate transdifferentiation of astrocytes to neurons in the CNS made use of the bHLH family of transcription factors in their protocols. Work from Dr. Magdalena Gotz's group in 2002 showed retroviral overexpression of the bHLH transcription factor Pax6 in cultured postnatal cortical astrocytes was capable of successful neuronal conversion (120). A few years later this same group also identified the bHLH transcription factors Ngn2, and mammalian achaete schute homolog 1 (Mash1/Ascl1) as being capable of direct neuronal conversion (121). However these neurons failed to develop the ability to fire action potentials and matured much slower than their embryonic counterparts (121). Subsequent work from a number of other labs identified other key factors capable of astrocyte to neuron conversion, and improved upon previous work by demonstrating neuronal subtype identity, and electrophysiological competence of the newly converted cells (122-124). The process of

transdifferentiation has gained much attention over the last decade in part because of its direct therapeutic potential in patients. Identification of viral vectors for safe and efficient delivery of the necessary transcription factor(s) would allow in vivo targeting in patients suffering from a number of neurological disorders where replacement of neurons would be beneficial. Current work in the field is focused on better understanding the transcriptional and epigenetic changes these cells undergo during the conversion process, methods for improving conversion rates, and safer more robust targeting of the desired cell population.

Summary and conclusions

Adult neurogenesis is a complex and multi-step process that takes careful orchestration of a variety of transcription factors, environmental cues, signaling pathways, and synaptic inputs. While our current understanding of this process is quite exceptional given where we started when adult neurogenesis was first discovered there is still much that is to be done in this field. How quiescent neural stem cells are maintained and activated in the SGZ, the function of neurogenesis in the development of epilepsy, and the susceptibility of different populations of cells to transdifferentiation in the intact CNS are all still important questions in the field. The next three chapters will seek to shed light on these important questions by focusing on the role the bHLH transcription factor NeuroD1, in physiological and pathological neurogenesis.

Chapter 2

Materials and Methods

Animal Studies

All the experiments were performed in compliance with the animal care guidelines issued by the National Institutes of Health and by the Institutional Animal Use and Care Committee (IACUC) at The University of Texas Southwestern Medical Center. All mice were bred and housed in the animal facility with a 12-h light, 12-h dark cycle with no more than five mice per cage, food (2916 Global irradiated diet, Teklad Labs), and water ad libitum. NeuroD1$^{loxP/loxP}$ mice bred with Nestin-CreER^{T2}/R26R-YFP mice (114, 125) were genotyped by PCR using genomic DNA and primers for NeuroD1 (5' GTT TTT GTG AGT TGG GAG TG 3', 5' TGA CAG AGC CCA GAT GTA 3'), NestinCreER^{T2} (5' GGT CGA TGC AAC GAG TGA TGA GG 3', 5' GCT AAG TGC CTT CTC TAC ACC TGC G 3'), and R26R-YFP (5' AAA GTC GCT CTG AGT TGT TAT 3', 5' GCG AAG AGT TTG TCC TCA ACC 3', 5' GGA GCG GGA GAA ATG GAT ATG 3'). Nestin-CreER^{T2}/R26R-YFP; NeuroD1$^{loxP/lox}$ mice were backcrossed to C57BL/6NHsd mice obtained from Envigo Laboratories (Cat. no: 4403F/M) for at least four generations prior to beginning studies. Male and female mice at approximately 5 weeks of age were administered tamoxifen (TAM) intraperitoneally (i.p.) at 150 mg/kg per day for 5 days prior to status epilepticus (SE). TAM was dissolved in 10% EtOH/90% sunflower oil. All wildtype animals used in epilepsy studies were female C57BL/6NHsd (Cat no: 4403F) purchased from Envigo. To generate the mice used in the transdifferentiation studies we crossed male hGFAP-CreER mice (originally from K.D. McCarthy's lab) with female Ai14; B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J mice (obtained from Jackson Laboratories)(126). hGFAP-CreER mice were genotyped by PCR using genomic DNA and primers for Cre: (5' GGT CGA TGC AAC GAG TGA TGA GG 3') and (5' GCT AAG TGC CTT CTC TAC ACC TGC G 3')(126). Ai14 mice were genotyping by PCR using genomic DNA and the following primers: 9020 (5' AAG GGA GCT GCA GTG GAG TA 3'), 9021 (5' CCGAAAATCTGTGGGAAGTC 3'), 9103 (5' GGC ATT AAA GCA GCG TAT CC

3'), and 9105 (5' CTG TTC CTG TAC GGC ATG G 3'). Nursing mothers were administered tamoxifen (TAM) via oral gavage (o.g.) at 50 mg/kg per day for 3 days prior to AAV9 jugular vein injection (IV).

Pilocarpine Model of Epilepsy

Mice were administered scopolamine methyl nitrate (intraperitoneally (i.p.); 2 mg/kg; Sigma-Aldrich S2250) and terbutaline hemisulfate salt (i.p.; 2 mg kg; Sigma- Aldrich T2528) to block peripheral effects of pilocarpine and dilate the respiratory tract, respectively. Thirty minutes later, pilocarpine hydrochloride (i.p.; Sigma-Aldrich P6503) at 220 mg/kg for males and 260 mg/kg for females (Nestin-CreER^{T2}/R26R-YFP; NeuroD1$^{loxP/lox}$ mouse line), or 185 mg/kg for female (C57BL/6NHsd mouse line) was injected, and mice were placed in an incubator maintained at 31 °C (ThermoCare). Acute seizures were behaviorally monitored using a modified Racine's scale (stage 1, mouth and facial movement; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing with forelimb clonus; stage 5, rearing and falling with forelimb clonus)(127). Once status epilepticus began (defined by continuous tonic clonic convulsive seizures), mice were placed at room temperature for 3 h and returned to the incubator after seizure activity was reduced with diazepam (10 mg/kg; Sigma- Aldrich D0899). Only mice showing SE for a total of 3 hours were included in EEG recording studies. After SE, mice were administered 5% dextrose solution (i.p.; 1 ml) and saline (i.p.; 1 ml) to facilitate their recovery. Mice were weighed each day during the recovery period and if found to have lost ≥ 2 g from the previous recorded weight were given a single i.p. dose of 1 mL 5% dextrose, and moistened chow. At 3 days after SE, mice were returned to their home cage. Wildtype animals used in this study were treated the same as above, but did not receive TAM injections. Mice included in the sham seizure group were given (TAM) intraperitoneally (i.p.) at 150 mg/kg per day for 5 days prior to sham treatment. All sham animals were administered scopolamine methyl nitrate (i.p.; 2 mg/kg; Sigma-Aldrich S2250) and terbutaline hemisulfate salt (i.p.; 2 mg/kg; Sigma- Aldrich T2528) 30 minutes prior to an i.p. vehicle (saline) injection. In some

cases Nestin-CreER^{T2}/R26R-YFP; NeuroD1$^{loxP/loxP}$ mice were given access to Metoclopramide (Henry Schein Animal Health, Cat no. 055411) treated water (1mg/kg) for 5 days prior to SE to help alleviate gastrointestinal symptoms (constipation) associated with use of pilocarpine in this mouse line.

Video/EEG Monitoring

Video/EEG recording was performed between 5–7 weeks after pilocarpine injection. For wireless EEG recording one week before EEG recording, mice were stereotaxically implanted with cortical surface electrodes connected to wireless EEG transmitters placed subcutaneously under the skin on the back (TA11ETAF10, Data Sciences International, St. Paul, MN). Mice were anesthetized using 2-3% isoflurane gas mixed in a 1L/min mixture of 70% nitrous oxide and 30% oxygen. Two cortical electrodes were placed at the coordinates from Bregma AP: +0.1 ML: +0.1 (Reference; R) and AP: -0.2 ML: +0.22 (Left parietal cortex; LPC). Mice received the analgesic Buprenorphine (subcutaneously; 0.05 mg/kg) as necessary following surgery. Animals underwent continuous monitoring by video/EEG for a total of two weeks. Video-EEG data was reviewed and quantified by a user blinded to the experimental groups using NeuroScore Software (Version 3.0.7703-0, Data Sciences International). Behavioral seizures were defined by repetitive epileptiform spiking activity (≥3Hz) that persisted for (≥10s) and was confirmed using video recordings. Seizure activity was marked at the beginning and end of each event to account for seizure duration and the number of seizures for each mouse was recorded. After EEG recording was completed, mice were sacrificed and brains harvested for IHC.

Intravascular jugular vein injection of AAV9

10 day old hGFAP-CreER; Ai14 mouse pups were anesthetized using 3-5% isoflurane in 70% nitrous oxide and 30% oxygen. Once anesthetized pups were laid on their backs underneath a dissecting microscope and a 1cm incision was made in the skin overlying the external jugular vein.

Blunt dissection was performed until the external jugular vein was visible. A 3/10 cc syringe needle was placed through the overlying muscle into the jugular vein, and a total of 130 ul of AAV9 virus was dispensed. The needle was withdrawn carefully and the vein checked for residual bleeding before the wound was closed and pups were allowed to recover on a pre-warmed heating pad. After all surgeries were complete from one litter, the surgical areas from each of the pups were cleaned with sterile water to remove residual betadine and EtOH. Pups were then rubbed with a powdered mouse chow and bedding to better facilitate acceptance back by their mother to nurse.

Immunohistochemical (IHC) staining

Mice were anesthetized and perfused transcardially with cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed and post-fixed in 4% PFA overnight, then cryoprotected in 30% sucrose in 0.1 M PBS. Brains were bisected and half-brains were coronally sectioned 30 um thick on a freezing microtome (Leica, SM 2000R). Immunohistochemistry was performed with either tissue mounted on charged slides or free-floating tissue sections.

Slides underwent antigen retrieval using 0.01 M citric acid, pH 6.0 at 100 °C for 15 min, followed by 12 min in 1x TBS at room temperature. Staining of free-floating tissue sections was the same except for the antigen retrieval step that was omitted. For Tyramide Plus signal amplification, we removed endogenous peroxidase activity by incubating sections with 0.3% H2O2 for 30 min at room temperature. Nonspecific binding was blocked with 3% normal donkey serum and 0.3% Triton-X-100 or 3% normal donkey serum and 1% Triton X-100 in 1x TBS for 1 h at room temperature.

Primary antibodies used in this study were as follows: goat anti-NeuroD1 (1:500, Santa Cruz Biotechnology sc-1084), chicken anti-GFP (1:1,000 for free floating sections, 1:8,000 for Tyramide Plus Amplification Aves Lab GFP-1020), goat anti-DCX (1:1,000, Santa Cruz Biotechnology sc-8066), guinea pig anti-DCX (1:1,000, Millipore AB2253), rabbit anti-Prox1

(1:5,000, Millipore AB5475), mouse anti-NeuN (1:1,000, Millipore MAB377), mouse anti-S100B (1:1000, Sigma Aldrich, Cat no. S2532), goat anti-Sox2 (1:1000, Santa Cruz Biotechnology, sc-17320), rabbit anti-dsRed (1:1000, Living Colors Cat no. 632496), and rabbit anti-Ki67 (1:250, ThermoScientific MA5-14520). For double or triple labeling, primary antibodies were simultaneously incubated and further processed for each antibody. For GFP, dsRed, Dcx, Prox1, Ki67, S100B, Sox2, and NeuN, a fluorescent-tagged secondary antibody was used (1:100-1:500, Jackson ImmunoResearch). For GFP and NeuroD1 (slide mounted), primary antibody incubation was followed with an appropriate biotin-tagged secondary antibody (1:200, Jackson ImmunoResearch) for 1 h at room temperature, followed by ABC (Vector Laboratories PK-6100) for 1 h, and Tyramide-Plus signal amplification (1:50, PerkinElmer NEL701001KT) for 1-3 min. Sections were counterstained with DAPI (4,6-diamidino-2-phenylindole; 1:5,000, Roche 236276).

For DCX and Prox1, after biotin-tagged secondary antibody followed by ABC labeling was completed, sections were visualized with a metal-enhanced DAB substrate kit (Thermo Scientific 34065). Sections visualized with DAB were mounted and dehydrated (70, 80, 90, 95, and 100% EtOH - 3 min each; and 50% EtOH/xylene, and 100% xylene- 3 min each) before coverslipping. Fluorescence stained sections were mounted in a 2.5% PVA-DABCO Media (PVA Sigma #D2522, DABCO: Sigma #D2522) while DAB stained images were mounted with DPX Media (VWR International).

Western Blot

Cells were washed with cold PBS before lysis in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for western blot. The buffer was supplemented with phenylmethanesulfonyl fluoride (PMSF), a cocktail of protease (Roche) and phosphatase inhibitors (Sigma). Cells were rocked in a 37 degree cold room for 1hr in the lysis buffer before centrifugation at 12,000 rpm at 4°C. After protein quantification by the

bicinchoninic acid (BCA) colorimetric assay system (Thermo Scientific), protein was denatured by boiling in 2× SDS loading buffer at 95'C for 5 min. Around 40ug of protein was loaded per well onto 7% SDS PAGE gels for Western blotting. After electrophoresis in the 7% SDS PAGE gels, the proteins were transferred to adsorbent PVDF membrane, blocked in 5% milk and incubated with primary antibody in blocking solution overnight with shaking at 4'C (1/1000 Goat-anti -NeuroD1 Santa Cruz Biotechnology sc-1084). After washing with buffer, the membranes were incubated with HRP-conjugated (Cell Signaling) secondary antibodies. Immunoblots were developed with an ECL-plus kit (GE Healthcare).

Retrovirus and Lentivirus production

The mouse cDNA for NeuroD1 was sub-cloned into the pCAG-IRES-mCherry retroviral vector (gift from Fred Gage) using BamHI and SfiI sites to generate pCAG-NeuroD1-IRES-mCherry. The control for all retrovirus experiments was the empty vector alone, pCAG-IRES-mCherry. Viral particles were packaged from 293T cells using the Profection Mammalian Transfection System (Promega, Cat # E1200), and further concentrated by ultra centrifugation at 19,000 rpm, and purified on ViraTrap RV Purification Maxi Kit columns (Biomiga Cat # V1272-02). The viral titer was between 10^7-10^8 viral particles per ul. For studies using AAV9 virus the mouse cDNA for NeuroD1 was cloned into an AAV9 vector backbone (pZac) obtained from Dr. Mauro Giacca. Expression of the protein was confirmed in 293T HEK cells via western blot analysis and the construct was sent for AAV9 custom packaging (SAB Tech) to obtain a viral titer of at least 1e13vg/ml. AAV9-GFP virus was purchased from SAB Tech to use as a control in these studies. For in vitro studies, the cDNA for NeuroD1 was cloned into the lentiviral vector (FUW; Addgene) to produce Lenti-CAG-NeuroD1-UbiC-GFP. The control lentivirus for all experiments was Lenti-CAG-UbiC-GFP. To prepare lentivirus, HEK293T cells were co-transfected with each of these constructs and lentiviral packaging vectors (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) using polyethylenimine (Polysciences). The culture supernatants were collected 48 hrs after transfection, and the virus was

introduced into primary cultured astrocytes by adding the supernatants to the culture medium. The viral titer was between 10^7-10^8 viral particles per ml.

In vitro cell culture

The rat adult hippocampal neural progenitor (HCN) cells were cultured in Dulbecco's Modified Eagle's Media/Ham's F12 (DMEM/F-12, Omega Scientific) supplemented with N2 (Invitrogen), glutamine (Omega Scientific), and PSF (Invitrogen). In all experiments with HCN cells, proliferation medium containing FGF2 (20 ng/mL) was used to maintain growth of the cells. For overexpression of NeuroD1 the retrovirus pCAG-NeuroD1-IRES-mCherry was used to infect HCN cells. Infection was performed in a minimum volume of media for 24 hrs after which the total media volume was brought up to 2 mL. Cells were allowed to grow 3 days after infection before fixation, and quantification using immunocytochemistry (ICC). Primary astrocytes were isolated and cultured as previously published (128). Briefly, primary astrocyte cultures were prepared from P1 mouse brains and cultured on uncoated dishes in 10% FBS DMEM media. After 7 days the cultures were shaken at 200 rpm for 1 hr to remove contaminating cells, and then treated with Ara-C (5uM) to kill proliferating cells for a total of 2 days. Cultures were shaken once more, and then infected with a lentivirus expressing NeuroD1 together with GFP (Lenti-CAG-NeuroD1-UbiC-GFP). Approximately 1 day later the culture media was changed to N2/B27 and the culture was allowed to continue for an additional 6 days before cells were fixed.

Electroporation of HCN Cells

Overexpression of AAV9-NeuroD1 (experimental) or pZac (control) in rat HCN cells was achieved by electroporation. Electroporation was performed with an Amaxa electroporator at a ratio of 2.5 ug AAV9-NeuroD1 DNA + 2.5ug pllU2g-GFP DNA per 5 million HCN cells. Cell culture media was changed to remove the growth factor FGF2 approximately 12 hrs after electroporation.

Cells were fixed 5 days after initial electroporation using 4% paraformaldehyde solution, and subsequently stained with antibodies for ICC.

Immunocytochemistry (ICC)

Non-specific antibody binding was blocked using blocking buffer (3% Normal donkey serum+0.3% Triton X-100 in 1x PBS) for 1 hr at room temperature. Primary antibodies used were diluted in blocking buffer (Chicken anti-GFP 1:500 Aves Lab GFP-1020, Rabbit anti-Tuj1 1:5,000 Covance) and incubated simultaneously overnight on a shaker at 4 degrees. Primary antibodies were then further processed for each antibody using a fluorescently labeled secondary antibody. All samples were counterstained using DAPI (4,6-diamidino-2-phenylindole; 1:5,000, Roche 236276).

Microscopic analysis and quantification

Quantification of cell number was performed by a user blinded to the experimental groups. In fluorescence labeled sections, quantification was performed using an upright microscope (BX60; Olympus), or a confocal microscope (LSM510/LSM700/LSM710; Carl Zeiss Microscopy). Subgranular and hilar zones were defined as the area within and beyond the diameter of one granule cell from the margin of granule cell layer, respectively. Immunoreactive cells were quantified in every twelfth 30-mm coronal section throughout the dentate gyrus. The numbers counted from each section were added and multiplied by 24 to estimate the total number of cells in one mouse brain.

Statistical Analysis

All of the data are expressed as mean ± S.E.M. Experimental groups were assigned by simple randomization. No statistical methods were used to pre- determine sample sizes. Data was collected blind. All statistical analyses were performed using GraphPad Prism 6 /7. Statistical differences were analyzed using two-tailed Student's t-test for the data with equal variances, or

Student's t-test with Satterthwaite's correction for the data with unequal variances. Samples were sometimes analyzed for statistical significance using Kruskal-Wallis nonparametric test, which included Dunn's multiple comparison test. P values of ≤ 0.05 were considered statistically significant.

Chapter 3

NeuroD1 instructs neuronal conversion in non-reactive astrocytes

This chapter adapted from:

Brulet Rebecca, Matsuda Taito, Zhang Ling, Miranda Carlos, Giacca Mauro, Kaspar Brian K., Nakashima Kinichi, and Hsieh Jenny. **NeuroD1 instructs neuronal conversion in non-reactive astrocytes.** 2016. *Stem Cell Reports*, in revision. Note that additional information has been added.

Introduction

The regenerative capacity of the mammalian central nervous system (CNS) is largely restricted to two areas of neurogenic potential found in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle (3, 13). Neurons lost outside these areas due to injury or disease cannot be replaced, and can often have devastating consequences for affected patients. Recent studies have focused on therapies involving the transdifferentiation, or direct lineage conversion, of other resident cell types into a desired neuronal population in vivo with the hopes of being able to restore or replace lost neurons (122, 123, 129-132).

During the acute phase of injury to the CNS, astrocytes become hypertrophic, resume proliferation, and up-regulate expression of the intermediate filament proteins GFAP and Vimentin in a process called reactive gliosis (133-136). Many of the cellular processes associated with this phenomenon are transitive in nature, and are complete several weeks to a month after injury (135-137). After injury to the CNS astrocytes become proliferative, and in some cases begin to express markers of neural stem/progenitor cells and neurogenic differentiation, indicating that they may be prime cellular candidates for transdifferentiation approaches (134, 138-141). Recent work has demonstrated robust

transdifferentiation of reactive cortical astrocytes into glutamatergic neurons with the overexpression of a single transcription factor, NeuroD1 (123). By direct injection of a retrovirus overexpressing NeuroD1 in the adult mouse cortex these authors were able to successfully target and convert reactive astrocytes to neurons (123). However, what is not well understood from this study and others in the field is whether astrocyte-to-neuron conversion can still occur following the initial injury phase after reactive gliosis is resolved (122, 123, 132, 135). A better understanding of the neurogenic potential of non-reactive astrocytes is therefore necessary for the future design of therapeutics administered outside the window of reactive gliosis. Here we make use of the previously reported transdifferentiation factor, NeuroD1 in order to interrogate this question.

AAV9-GFP labels neocortical and striatal astrocytes in the p10 mouse brain

Previous work suggests that intravascular delivery of AAV9 is an ideal tool for targeting astrocytes in certain brain regions (142). In order to further extend these findings and validate our experimental model we wanted to confirm the identity of AAV9 infected cells in the cortex and striatum. To investigate the identity of the newly infected cells we used an AAV9 vector that expressed green fluorescent protein (GFP) under the control of the chicken ß-actin hybrid promoter (AAV9-GFP) (142). We used a Cre inducible hGFAP reporter mouse to label astrocytes in the cortex and striatum irreversibly with tdTomato (Ai14) to determine the level of overlap between AAV9-GFP infected cells and GFAP-expressing astrocytes. In order to accomplish this, we bred hGFAP-CreER male mice to Ai14 mice, and administered tamoxifen (TAM) to nursing females starting on day 7 after delivery of the pups (Fig 3.1 A). TAM administration was repeated for 3 consecutive days in order to label GFAP-expressing astrocytes with tdTomato, and on the 4th day when the pups were 10 days old AAV9-GFP was injected into the jugular vein (Fig 3.1 A). Mice were sacrificed at 5 days post injection to determine the overlap of AAV9-GFP and tdTomato. We confirmed

overlap between the two markers indicating a strong preference of AAV9 for targeting astrocytes in the cortex and striatum (Fig 3.1 B,C).

Next, to provide additional confirmation that AAV9 predominantly labels astrocytes and to rule out the possibility that AAV9 infects cells that are already neuronal in identity, we repeated intravascular injections with AAV9-GFP and stained these sections with the marker doublecortin (Dcx) for immature neurons, and neuronal nuclei (NeuN) for mature neurons (Fig 3.1 D). Out of all the labeled cells counted throughout the cortex and striatum only 1 cell, or <0.003% of GFP+ cells were identified as double positive for Dcx (Fig 3.1 E,F). Likewise, only 0.79% of GFP+ cells in the cortex, and 1.07% of GFP+ cells in the striatum were positive for NeuN, indicating that AAV9 does not target neuronal cells (Fig 3.1 E,F). Moreover, approximately 42.46% of GFP+ cells in the cortex and 44.77% of GFP+ cells in the striatum stained with the astrocyte marker S100ß consistent with intravascular AAV9 predominantly targeting astrocytes in this region (Fig 3.1 E,F). These data confirm that intravascular delivery of AAV9-GFP efficiently labels astrocytes and not neurons in the cortex and striatum, reinforcing this approach to evaluate NeuroD1's effects in neuronal transdifferentiation. Of interest, AAV9-GFP when expressed under a ubiquitous promoter and introduced into the vascular system also showed the capability of infecting cells in every organ system examined at 2 weeks post injection (Fig 3.1G). Future work will be necessary to identify the specific cell types infected in each organ. This result underscores the need for precise and non-pathogenic means of introducing therapeutic factors.

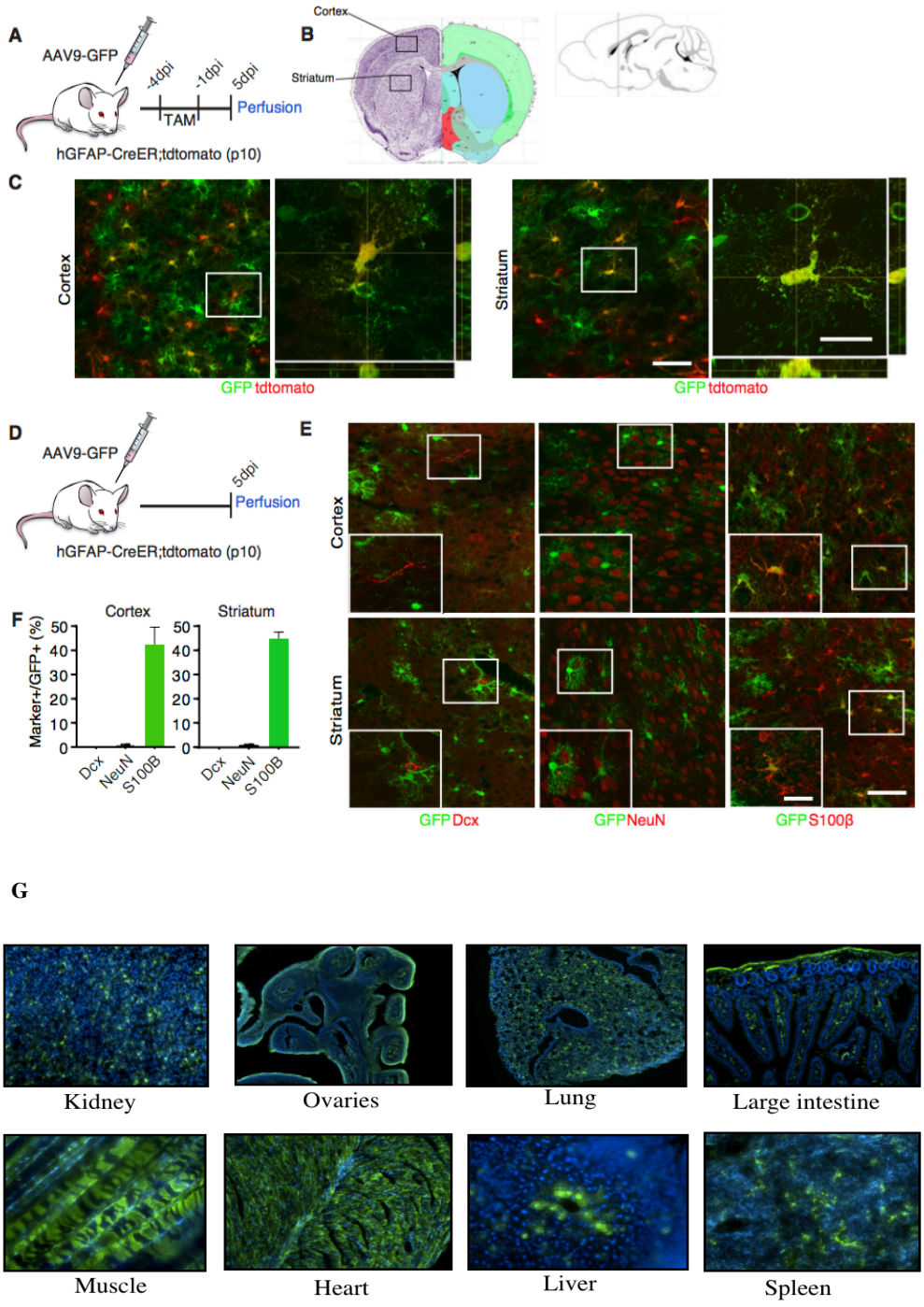

Figure 3.1 AAV9-GFP labels neocortical and striatal astrocytes in P10 mouse brain:
A: Timeline showing the experimental design. B: Schematic of the brain showing the areas in which representative images were taken. C: Representative images of AAV9-GFP infection in the cortex and striatum at 5 days post injection. D: Timeline showing experimental design for validating identity of GFP+ cells in Fig 1E. E: Representative images showing co-labeling of GFP+ cells with the neuronal markers Dcx and NeuN, and the astrocyte marker S100ß. F: Quantification of overlap of GFP+ cells for respective markers in the cortex and striatum. G: Representative images showing AAV9-GFP infection in various organ systems 2 weeks post injection. F; N=3. Scale bar in C,E; 50um, inset in C,E; 25um. All data are shown as mean +/- S.E.M.

Confirmation of NeuroD1 overexpression in neocortical and striatal astrocytes

Previous work indicates that it takes approximately 3 days to 1 week after infection of reactive astrocytes with retrovirus expressing NeuroD1 in order to see neuronal transdifferentiation (123). Since our strategy for overexpressing NeuroD1 involved injection of AAV9 virus into the vascular system, we wanted to perform a time course to determine the kinetics of virus transduction based on expression of GFP in astrocytes. In order to test this we injected AAV9-GFP virus into 10-day old hGFAP-CreER; Ai14 mouse pups via the jugular vein and sacrificed the mice at different time points to check GFP expression in the brain (Fig 3.2 A). Our results showed robust GFP+ infected cells in cortex and striatum beginning around 3 days post injection (Fig 3.2 B,C). Therefore, in all subsequent in vivo experiments using AAV9 we decided to sacrifice the mice at 10 days post infection in order to accommodate the time necessary for the virus to infect cells of the brain and express NeuroD1, as well as allow for successful transdifferentiation based on previous published work (123).

To overexpress NeuroD1 in astrocytes of the cortex and striatum we cloned the mouse cDNA for NeuroD1 into an AAV9 vector and confirmed NeuroD1 protein expression using western blot analysis (Fig 3.2 D). Because NeuroD1 has been previously shown to instruct differentiation of the rat hippocampal neural stem cell line (HCN) into neurons we decided to further confirm our construct by electroporating it into this same cell line and stained for Tuj1, a marker of neuronal cells 6 days later (Fig 3.2 E)(143). HCN cells electroporated with AAV9-NeuroD1 showed significant numbers of Tuj1+ neurons as compared to control electroporated cells (Fig 3.2 F,G). After viral packaging we wished to confirm NeuroD1 expression in infected astrocytes by immunohistochemistry in the brain so we mixed AAV9-NeuroD1 and AAV9-GFP and co-injected both viruses into 10-day old mice and sacrificed them 5 days post injection (Fig 3.2 H). Growth curves performed between 10 and 20 days post injection confirmed that both AAV9-GFP and AAV9-NeuroD1 injected groups

were healthy with no significant differences in weight between groups (data not shown). As expected cortical and striatal sections stained for NeuroD1 showed high expression in GFP+ astrocytes in animals that received a mixture of AAV9-NeuroD1 and AAV9-GFP, whereas no NeuroD1 expression was noted in control animals that received only AAV9-GFP injection (Fig 3.2 I). Taken together, these results indicate intravascular AAV9 delivery is a suitable approach for widespread overexpression of NeuroD1 in the brain.

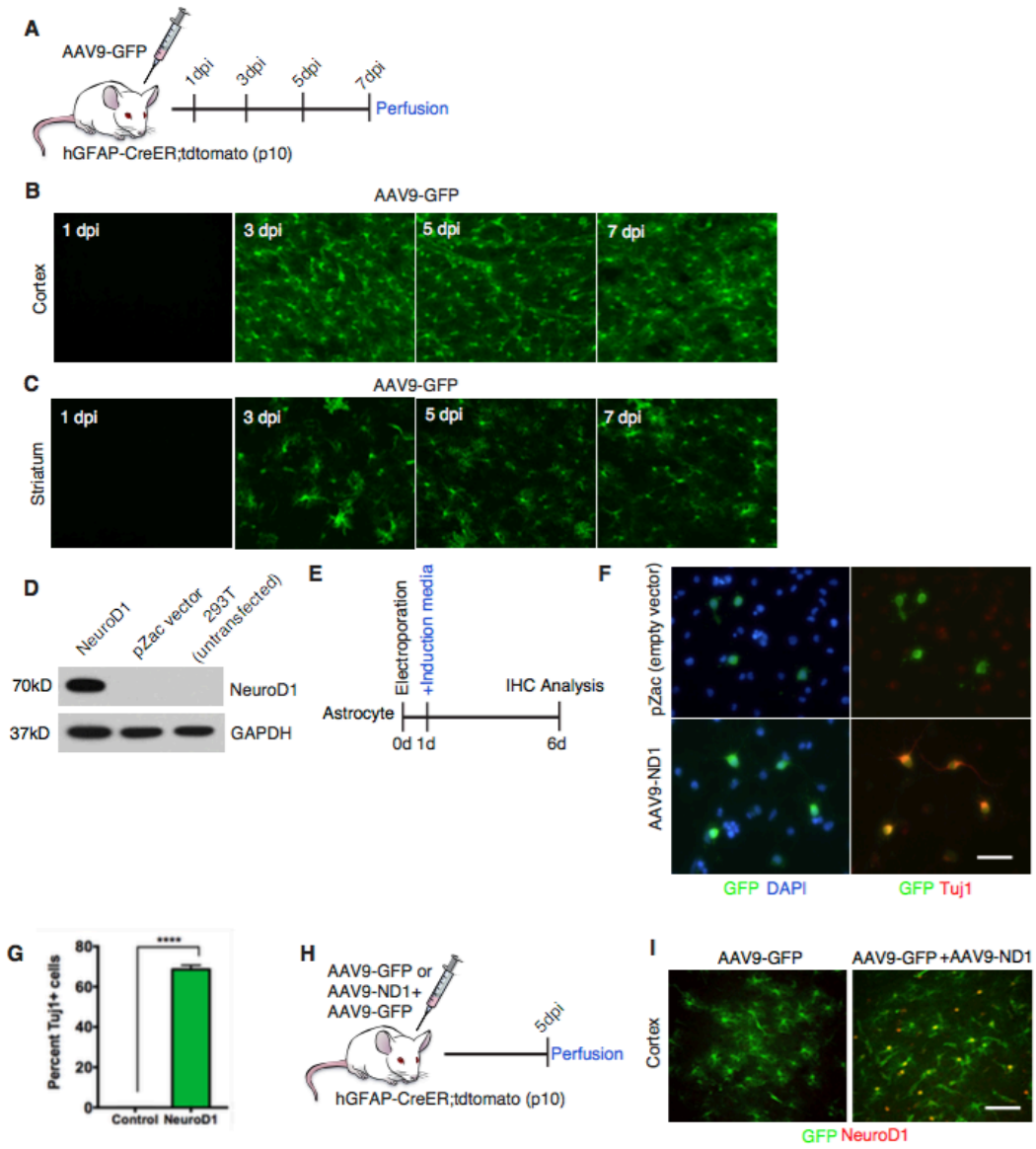

Figure 3.2 Confirmation of NeuroD1 overexpression in neocortical and striatal astrocytes: A: Timeline showing experimental design. B, C: Representative images taken from the cortex and striatum showing AAV9-GFP infection from 1-7 days post injection. D: Western blot showing successful overexpression of NeuroD1 after cloning into the AAV9 backbone. E: Timeline showing experimental design. F,G: Quantification and representative images from HCN cells electroporated with AAV9-NeuroD1 or pZac (AAV9 control vector). H: Timeline showing experimental design. I: Representative images from the cortex showing significant overlap of infected astrocytes and NeuroD1 in mice injected with AAV9-NeuroD1, but not AAV9-GFP control at five days post injection. Scale bar in B,C,F; 50um. Scale bar in I: 25 um.

NeuroD1 mediates a small but significant astrocyte-to-neuron conversion in the striatum

In order to determine whether NeuroD1 is able to convert astrocytes to neurons without direct brain injury we injected 10-day old mice with a mixture of AAV9-NeuroD1 and AAV9-GFP or AAV9-GFP as a control intravascularly and then sacrificed the animals 10 days later (Fig 3.3 A). Brain sections were stained for Dcx and NeuN to determine the total number of GFP+ converted immature and mature neurons, respectively. Overexpression of NeuroD1 did not lead to a significant number of transdifferentiated cells expressing DCX or NeuN neuronal markers in the cortex (Fig 3.3 B,D). Interestingly, a small number of GFP+/NeuN+ cells in the striatum were higher in the AAV9-NeuroD1 transduced group (2.42% of GFP+ cells) compared to the AAV9-GFP transduced group (0.34%), although there was no difference in GFP+/DCX+ cells (Fig 3.3 C,E). Moreover, the GFP+/NeuN+ striatal cells observed appeared to exhibit neuron-like morphology, with longer, more elaborate processes (Fig 3.3 C). These data suggest NeuroD1 expression alone can mediate astrocyte-to-neuron conversion even under physiological conditions, although this was a relatively rare event.

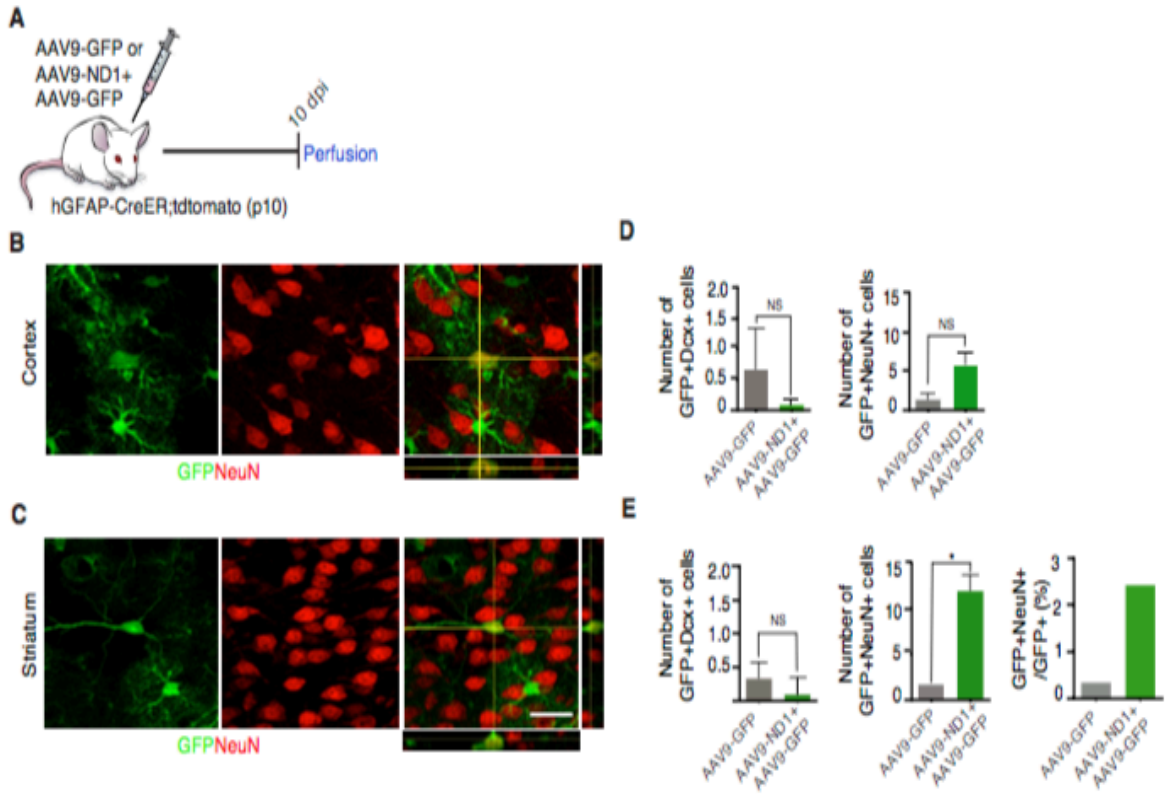

Figure 3.3 NeuroD1 expression in striatal astrocytes led to limited neuronal conversion: A: Timeline showing experimental design. Mice at the age of 10 days old were injected with either AAV9-NeuroD1+AAV9-GFP (experimental) or AAV9-GFP (control) and sacrificed 10 days post injection to determine whether neuronal conversion had occurred. B: Representative images showing one of the few cells expressing NeuN in the cortex. C: Representative images showing one of the few cells expressing NeuN in the striatum. D: Quantification showing the total number of GFP+ cells that co-labeled for the neuronal markers Dcx and NeuN in the cortex E: Quantification showing the total number and percent of GFP+ cells that co-labeled with the neuronal markers Dcx and NeuN in the striatum. n=3 AAV9-GFP, n=11 AAV9-NeuroD1+AAV9-GFP. Scale bar B,C: 50um. All data shown as mean +/- S.E.M. $p<0.05$ statistically significant.

NeuroD1 expression in cultured astrocytes induces neuronal differentiation

Our in vivo NeuroD1 expression results indicating limited astrocyte-to-neuron conversion suggest that reactive astrocytes induced by injury may be a vital component for robust neuronal transdifferentiation. To extend our findings, we established mouse astrocytes in vitro, treated them with AraC and allowed them to grow without the addition of growth factors, which more closely resembles the physiologic state of non-proliferating astrocytes (Fig. 3.4 A)(144-146). We infected cultured mouse astrocytes with Lenti-CAG-NeuroD1-UbiC-GFP or Lenti-CAG-UbiC-GFP as a control, switched the cultures to serum-free induction medium the following day, and stained with markers of astrocytes (GFAP) or neurons (βIII-Tubulin) at 7 days post infection (Fig. 3.4 B). Interestingly, we observed minimal βIII-Tubulin+ neuronal differentiation in NeuroD1-expressing cultured mouse astrocytes compared to control (Fig. 3.4 C-E). These results further corroborate the idea that NeuroD1 expression alone can convert astrocytes into neurons under physiological conditions although the efficiency is very limited.

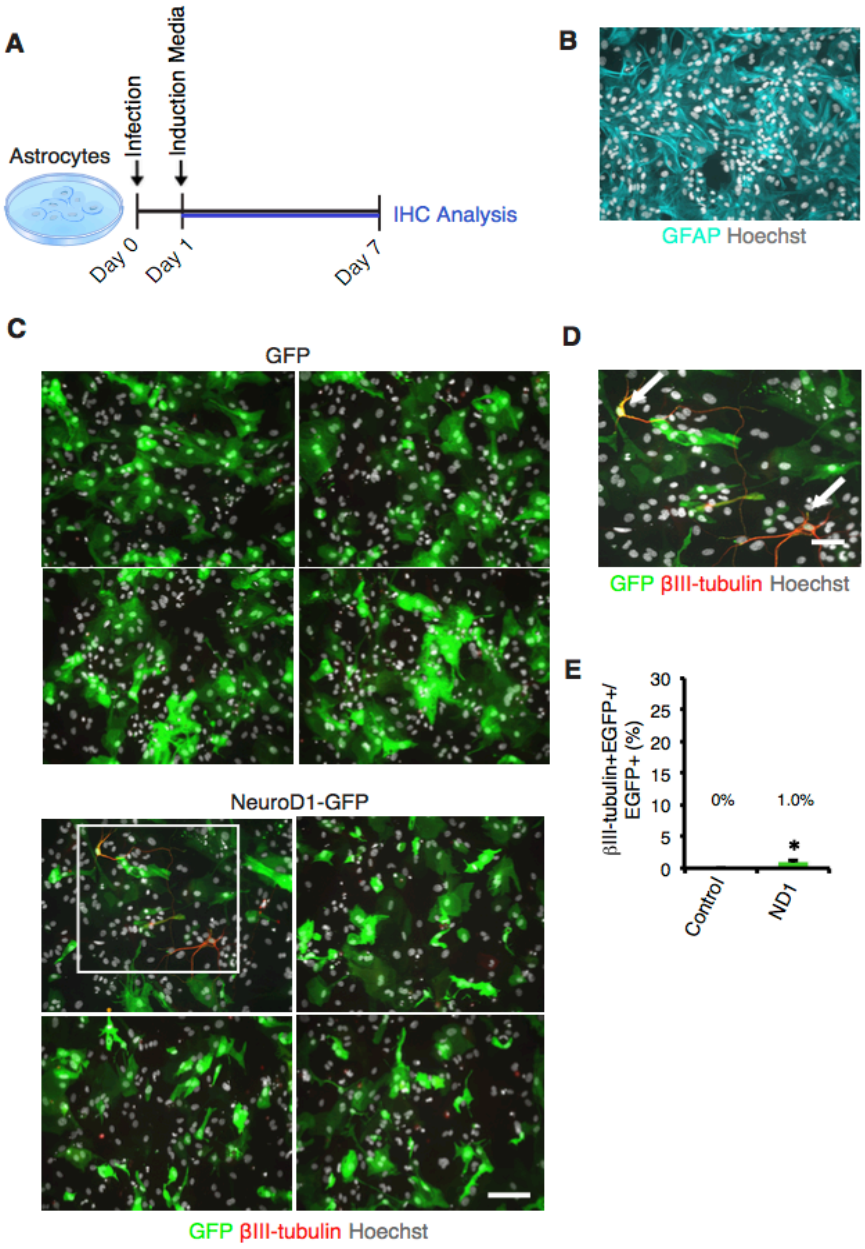

Figure 3.4 Overexpression of NeuroD1 resulted in minimal neuronal differentiation in vitro: A: Timeline showing experimental design. B: Representative image of cultured astrocytes showing expression of the astrocyte marker GFAP. C: Representative images taken in vitro showing astrocytes infected with Lenti-CAG-NeuroD1-UbiC-GFP or Lenti-CAG-UbiC-GFP at 7 days after infection co-labeled with the immature neuronal marker βIII-tubulin. Arrows denote GFP+/βIII-tubulin+ cells. D: Inset image from C showing a magnified view of GFP/ βIII-tubulin+ cells E: Graph showing the percentage of all astrocytes expressing βIII-tubulin 7 days after infection. n=4 control, n=4 NeuroD1. Scale bar in C; 50um. Scale bar in D; 25um. All data expressed as mean +/- S.E.M.

Conclusions

In this work we demonstrate a method for indirect, non-invasive, and widespread targeting of astrocytes in the cortex and striatum. By utilizing overexpression of the transcription factor NeuroD1, previously reported to yield both rapid and efficient conversion of astrocytes to neurons, we present data in support of the idea that expression of this transdifferentiation factor via a non-invasive vascular route can yield newly converted neurons. While we did observe newly converted mature NeuN+ neurons in the striatum, the overall number of newly generated cells remained low compared to those reported in the previously published study (123). Future work will be necessary to not only explain why intravascular AAV9 delivery of NeuroD1 yields low numbers of newborn neurons, but also identify, and characterize these newly converted cells, as well as their ability to integrate and synapse with pre-existing neurons already present in the adult brain.

Chapter 4

Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline

This chapter adapted from:

Cho Kyung-Ok, Lybrand Zane R, Ito Naoki, Brulet Rebecca, Tafacory Farrah, Zhang Ling, Good Levi, Ure Kerstin, Birnbaum Shari G, Scharfman Helen E, Eisch Amelia J, Hsieh Jenny. **Aberrant Hippocampal neurogenesis contributes to epilepsy and associated cognitive decline.** 2015. *Nature Communications*, **26**(6). The data presented in this chapter are a portion of a larger body of work. Here I provide a smmary of the background and relevant data I directly contributed to. Due to copyright restrictions, readers are encouraged to refer to the published work (147).

Introduction

Previous work has shown an increase in adult neurogenesis in the hippocampus of several animal models of epilepsy (95, 96, 148). In contrast to steady-state neurogenesis, neurons born after epileptic seizures have been demonstrated to become "aberrant", often mis-migrating and forming inappropriate connections in the surrounding hippocampal network (95, 149). This has led to the hypothesis that these aberrantly integrated adult-generated neurons contribute to the spontaneous recurring seizures (SRS) seen in mTLE (91, 147, 150). However, the role of aberrant neurogenesis in epilepsy still remains a controversial subject. Previous work using anti-mitotic agents and radiation to kill off dividing cells in the hippocampus have proven inconclusive at providing a link between aberrant neurogenesis and SRS (106, 107, 151). In each of these studies ablation of the neural stem cell pool was accomplished through use of non-specific techniques. Therefore, the question still remains whether the aberrant neurogenesis seen in epilepsy is causative in the progression of SRS and associated memory decline.

Genetic ablation of adult-born granule neurons

In this work we utilized the Nestin-δ-HSV-thymidine kinase-EGFP (Nestin-TK) transgenic mouse to specifically target adult hippocampal neurogenesis. In order to achieve near-complete ablation of neurogenesis we implanted these mice with mini osmotic pumps that administered gancyclovir (GCV) (experimental) or saline (control) over a period of 4 weeks prior to inducing status epilepticus (SE). Animals that received GCV treatment showed a ~98% reduction in the number of Dcx+ immature neurons in the SGZ as compared to controls indicating successful ablation of neurogenesis. Additionally, we were able to confirm the absence of NeuroD/doublecortin (DCX)-positive late-stage progenitors and neuroblasts at this same time point. In order to better understand the affect that GCV treatment had on certain aspects of aberrant neurogenesis we quantified the total number of Dcx+ immature neurons in both the GCL and hilus, as well as the total number of Prox1+ mature neurons in the hilus of animals 6 weeks after treatment. GCV treated animals showed a significant reduction not only in the total number of Dcx+ cells in the GCL, but also in the total number of Dcx+ and Prox1+ ectopically localized to the hilus indicating successful ablation of aberrant neurogenesis in our mouse model.

After we confirmed ablation of neurogenesis in our mouse model we wanted to determine whether this would have any effect on seizure formation or frequency. In order to test this, we treated animals with pilocarpine, an M1 muscarinic receptor agonist, to induce chronic epilepsy. Mice were implanted with EEG recording devices at approximately 4 weeks after SE to monitor the total number and duration of each seizure experienced during the chronic phase of the disease. Ablation of neurogenesis resulted in an approximately 40% reduction in the average number of seizures each mouse experienced, however there was no noted change in the duration of each seizure event. This data shows that newborn neurons

present prior to SE contribute to the development of spontaneous reoccurring seizures (SRS). Interestingly, animals in which ablation of neurogenesis was performed in both the pre and post SE period did not show a change in the SRS frequency experienced. Taken together this data would suggest that while aberrant neurogenesis does play a critical role in the development of epilepsy, there are likely other contributing factors that help to produce the seizures characteristic of the disease.

Ablation of Adult Neurogenesis rescues deficits in cognitive function

Many epileptic patients report deficits in memory and cognitive function as comorbidities associated with the disease (152). Since hippocampal neurogenesis is known to be important in learning and memory, we next wanted to determine the role aberrant neurogenesis plays in cognitive deficits seen in the disease. In order to test this we subjected vehicle or GCV treated animals to a hippocampal-dependent memory task, the novel location recognition test (NL)(153). Interestingly GCV treated animals showed significant improvement in hippocampus-dependent memory function indicating that ablation of neurogenesis in epilepsy rescues hippocampal spatial memory impairment associated with chronic seizures.

NeuroD1 is required for seizure-induced neurogenesis

From a practical standpoint near complete ablation of neurogenesis is not feasible in a clinical setting, because hippocampus neurogenesis also plays a critical role in learning and memory. Therefore, in order for more targeted therapeutics to be developed we must identify specific genetic and molecular regulators important in the process of aberrant neurogenesis. In an effort to identify these molecular regulators we induced acute seizures using the glutamatergic receptor agonist Kainic Acid (KA) in wildtype animals and stained

hippocampal sections with the neuroblasts marker NeuroD1. Interestingly, we showed that NeuroD1 expressing cells were significantly increased in both the GCL and hilus of animals that had undergone acute seizures compared with sham treated controls. In order to determine whether expression of NeuroD1 is required for the formation of aberrant neurons seen after an acute seizure event we performed tamoxifen administration in Nestin-CreER^{T2}; NeuroD1$^{loxP/loxP}$;Rosa(R26R)-YFP (cKO) mice and Nestin-CreER^{T2};NeuroD1$^{+/+}$;R26R-YFP (wild-type, WT) mice in order to delete NeuroD1 in nestin-expressing stem cells (16). Interestingly, proliferating neuroblasts identified by triple labelling with YFP/DCX/Ki67 were significantly decreased in NeuroD1 cKO mice, compared with WT littermates three weeks after treatment with KA. Additionally, NeuroD1 deletion led to a significant reduction in the number of Prox1-positive mature neurons in the granule cell layer and the hilus. Therefore, targeting NeuroD1 could potentially be an alternative approach to eliminating aberrant adult neurogenesis.

Conclusions

Our work suggests that aberrant neurogenesis plays a significant role in the development of epilepsy and associated memory decline. Additionally, through this work we have identified a candidate transcription factor, NeuroD1, that is required for seizure-induced neurogenesis. In this next chapter I will examine the role of this factor further in the context of epilepsy, and present work showing the functional consequences of NeuroD1 deletion in the pilocarpine model of epilepsy in an effort to test its therapeutic relevance as an anti-epileptic target.

Chapter 5

Mice with conditional NeuroD1 knockout display reduced aberrant hippocampal neurogenesis but no change in epileptic seizures

This chapter adapted from:

Brulet Rebecca, Zhu Jingfei, Aktar Mahafuza, Hsieh Jenny, and Cho Kyung-Ok. **Mice with conditional NeuroD knockout display reduced aberrant hippocampal neurogenesis but no change in epileptic seizures.** 2016. *Experimental Neurology*, in preparation. Note that additional information has been added.

Introduction

As discussed in the previous chapter our work shows a significant link between adult neurogenesis and epilepsy (147). In addition, we identified the bHLH transcription factor, NeuroD1, as a potential target for modulation of aberrant neurogenesis and seizure frequency (147). Here, we made use of the NeuroD1 conditional knockout transgenic mouse to investigate the role of NeuroD1 in aberrant neurogenesis in the pilocarpine mouse model.

Pilocarpine-induced SE increases the number of NeuroD1+ cells in the hippocampus

To determine the role of NeuroD1+ cells in chronic epilepsy, we used the cholinergic agonist pilocarpine to induce status epilepticus (SE). This model has been shown to faithfully recapitulate much of the pathology seen in human patients with temporal lobe epilepsy (TLE) and has the benefit of leading to spontaneous recurring seizures (SRS) in mice with only a single intraperitoneal (IP) injection of pilocarpine (154-157). Wild-type mice at approximately 6 weeks of age were given a single injection of pilocarpine (185mg/kg), to induce status epilepticus (SE), which was allowed to continue for 3 hours before seizures were terminated with diazepam. Mice were euthanized 3 weeks later to determine the total number NeuroD1 cells found in both the GCL and hilar regions of the hippocampus (Fig 5.1 A).

Epileptic animals that had been treated with pilocarpine compared to saline control animals showed a significant increase in the total number of NeuroD1+ cells in both the GCL and hilus (Fig 5.1 B,C). In addition, the vast majority (>99%) of NeuroD1+ cells in both the GCL and hilus were found to co-express doublecortin (Dcx) (Fig 5.1 D,E), a marker of immature neuroblasts. Based on these results and previous work from our lab showing the important role of neurogenesis in epilepsy we decided to further investigate what the role of NeuroD1 might play in the cellular and functional outcomes of epileptogenesis (147).

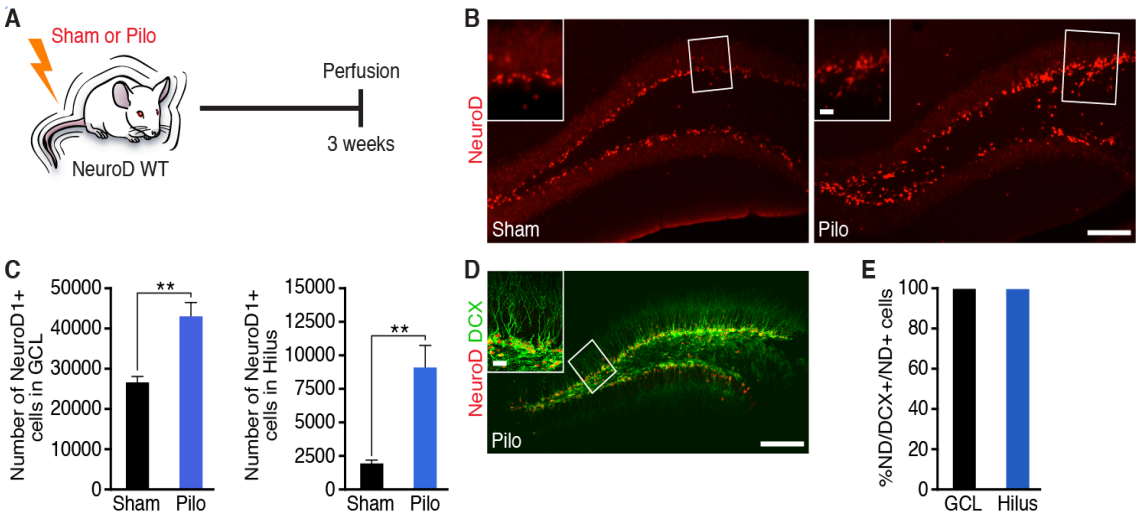

Figure 5.1 Acute seizures increase the number of NeuroD1-positive cells in adult dentate gyrus: A: Experimental Timeline. Wild-type animals were subjected to pilocarpine induced status epilepticus (SE) and then euthanized 3 weeks post-pilocarpine. B: Brain sections were stained with NeuroD1 to determine the total cell number in the granule cell layer (GCL) and hilus. (n=6 sham, n=6 pilo) C: There was a significant increase in the number of NeuroD1+ cells in both the GCL (p=0.0013) and hilus (p=0.0016) in pilocarpine treated animals compared to sham treated controls. D,E: The majority of pilocarpine induced NeuroD1+ cells were co-localized with DCX. (n=8 sham, n=8 pilo). Inset bar in B,D: 25 um.

Deletion of NeuroD1 reduces the number of adult-generated neurons in the hippocampus

In order to delete NeuroD1 from the adult neural progenitor cell population found in the hippocampus we decided to take advantage of a conditional knock-out (cKO) mouse model for NeuroD1. NeuroD1$^{loxP/loxP}$ mice were crossed with Nestin-CreER^{T2}/R26R-YFP mice to conditionally delete NeuroD1 in the adult nestin stem/progenitor cell population. Mice were given 5 consecutive days of tamoxifen injections to induce cre-mediated recombination followed by pilocarpine induced SE (220 mg/kg for males and 260 mg/kg for females), and then sacrificed 3 weeks later to confirm NeuroD1 deletion in YFP+ recombined cells (Fig 5.2 A,B,E). NeuroD1 cKO mice showed a strong trend in the reduction of the total number of YFP+/NeuroD1+ recombined cells in the GCL and hilus compared to Nestin-CreER^{T2}/R26R-YFP littermate controls (NeuroD1 WT) (Fig 5.2 E). Moreover, NeuroD1 cKO mice showed a strong trend in reduction in the number of YFP+/DCX+ immature neuroblasts and YFP+/Prox1+ mature neurons in the GCL, consistent with NeuroD1 being required for aberrant neurogenesis in the pilocarpine model (Fig 5.2 C,D,F,G). Additionally, total number of NeuroD1+ cells was almost significant in the GCL, but not hilus (Fig 5.2 H,I) Taken together, these data confirm the effectiveness of NeuroD1 cKO mice to address the role of adult-generated NeuroD1+ cells in chronic seizures.

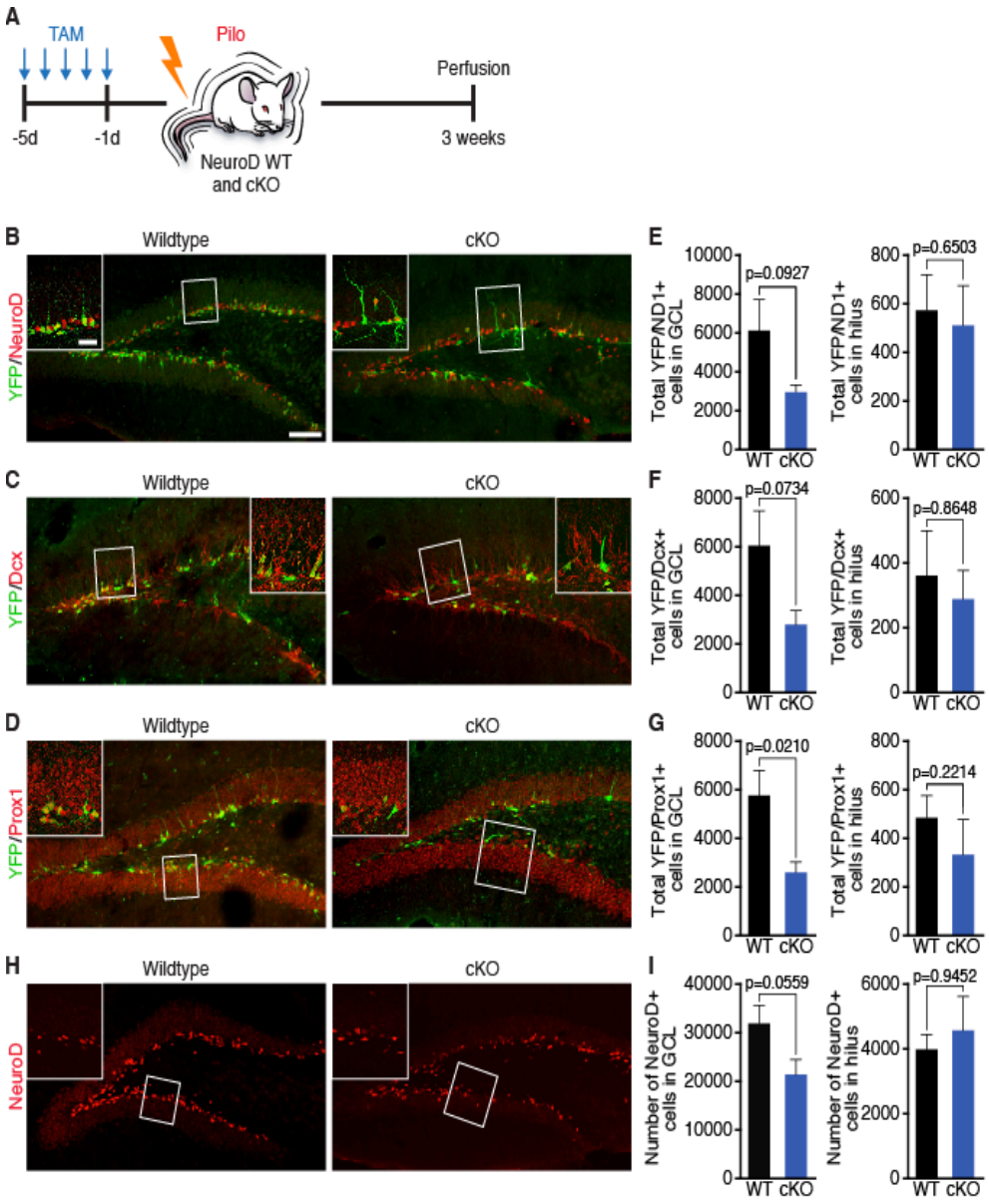

Figure 5.2 NeuroD1 is required for pilocarpine-induced adult-generated neurons

A: Experimental Timeline. Animals were given 5 days of tamoxifen and then subjected to pilocarpine induce status epilepticus (SE). Mice were euthanized 3 weeks post pilocarpine. B: Sections were stained for YFP and NeuroD to confirm reduction of NeuroD+ adult-born neurons in the GCL and hilus. C: Sections were stained with YFP/Dcx to assess reduction of immature neuronal cells in GCL and hilus. D: Sections were stained with YFP/Prox1 to assess reduction of mature neuronal cells in the recombined population in the GCL and hilus. E: Quantification of total number of YFP/ND1+ cells in GCL and hilus F: Quantification of total number of YFP/Dcx+ cells in GCL and hilus G: Quantification of total number of YFP/Prox1+ cells in GCL and hilus H: Sections were stained with NeuroD1 to quantify total number of NeuroD1+ cells in the GCL and hilus. I: Quantification of total number of ND1+ cells in GCL and hilus. (n=6 WT n=6 cKO) All data shown as mean +/- SEM.

Deletion of NeuroD1 in adult-generated neurons does not reduce SRS

To examine the role of NeuroD1 in epilepsy we treated NeuroD1$^{loxP/loxP}$; Nestin-CreER^{T2}/R26R-YFP mice with 5 days of tamoxifen, at which point they were given pilocarpine (220 mg/kg for males and 260 mg/kg for females) to induce epilepsy. These animals were then implanted with wireless EEG recording devices at 4 weeks post-pilo (Fig 5.3 A,B). Two cortical epidural screws were placed, one located over the hippocampus (Left parietal cortex; LPC) for recording generalized seizure activity, and the other over the olfactory bulb (Reference; R) to serve as reference activity. Starting at five weeks post-pilocarpine freely moving animals were video/EEG recorded for a period of two continuous weeks (Fig 5.3 A). The total number of generalized seizures was quantified and confirmed using video. Removal of NeuroD1 from adult-generated neurons prior to SE did not significantly alter either the total number of seizures or the total duration of each seizure event (Fig 5.3 C,D). Together these results suggest that adult neural stem/progenitor specific knockout of NeuroD1 does not significantly impact SRS.

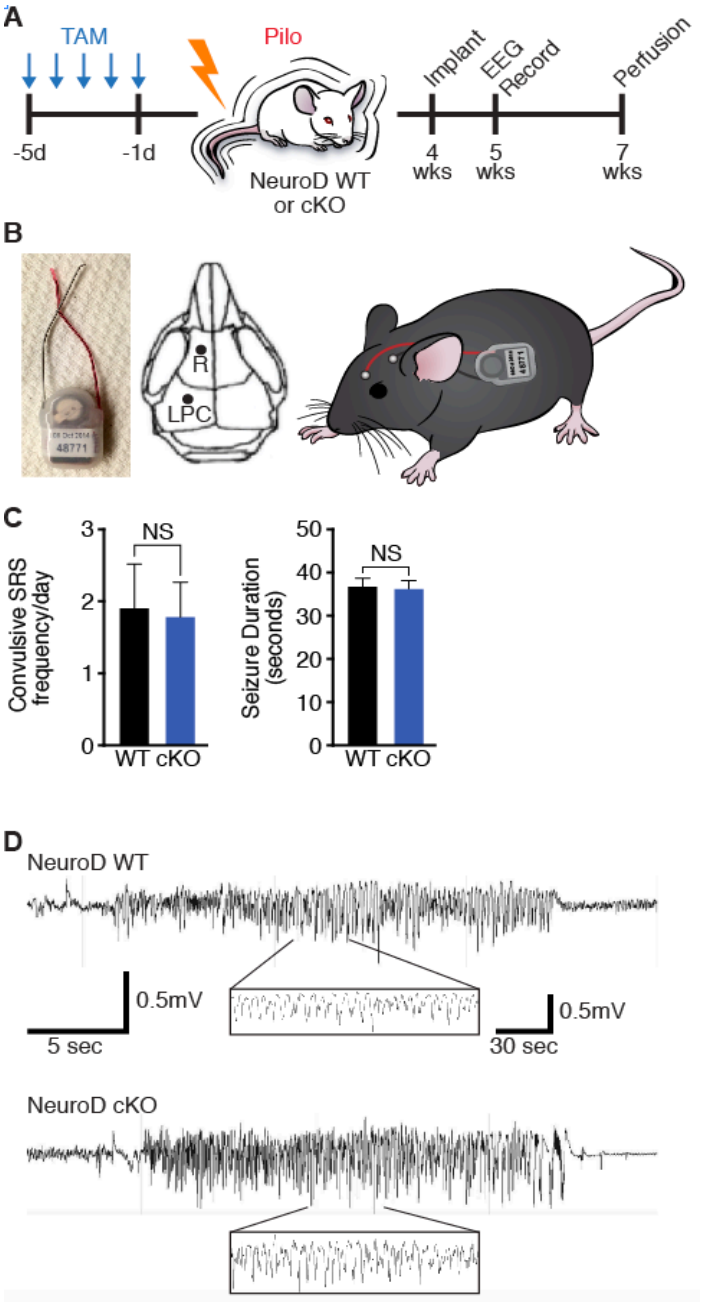

Figure 5.3 No change in SRS in animals with NeuroD1-deficient adult-born neurons: A: Experimental Timeline. Animals were implanted with wireless EEG recording devices at 4 weeks post pilocarpine (pilo). EEG recording began at 5 weeks post-pilo and continued for a total of 2 weeks. B: Representative images showing where the wireless EEG screws were implanted and how the electrodes were attached. R=Reference, LPC=Left parietal cortex C: Deletion of NeuroD1 did not have a significant effect on spontaneous recurrent seizure (SRS) frequency or duration. D: Representative EEG seizure traces from WT and cKO animals. (n=11 wt, n=10 cKO, NS=not significant).

NeuroD1 deletion does not alter the number of aberrant adult-generated neurons

To reconcile our data that NeuroD1 deletion reduces the number YFP+NeuroD1+ adult-generated neurons in both the GCL and the surprising finding that NeuroD1 cKO mice had similar levels of SRS compared to WT, we hypothesized that the level of aberrant neurogenesis was still above the threshold to reduce SRS. Thus, we performed histological analysis on mice seven weeks after pilocarpine treatment to determine the total number of immature and mature granule neurons present in the hippocampus during the chronic period of epilepsy (Fig 5.4 D). First, sections were stained with the immature neuroblast marker Dcx. There was no difference in the total Dcx+ cells within the GCL in NeuroD1 cKO mice compared to WT (Fig 5.4 E-F). This result is in contrast to sham treated animals sacrificed at the same time point (Fig 5.4 A-C). Deletion of NeuroD1 in sham treated animals produced a significant reduction of Dcx+ cells in the GCL (Fig 5.4 B-C). Deletion of NeuroD1 did however reduce the total number of Dcx+ immature neuroblasts ectopically localized to the hilus (Fig 5.4 G), but showed no significant reduction in the total number of Prox1+ mature granule neurons ectopically localized to the hilus (Fig 5.4 H). These results suggest that while NeuroD1 deletion can remove a population of YFP+ adult-generated neurons, it does not alter the total number of aberrant adult-generated neurons in the pilocarpine model. Moreover, these results also support that NeuroD1 cKO still have SRS similar to WT mice due to persistent levels of aberrant neurogenesis.

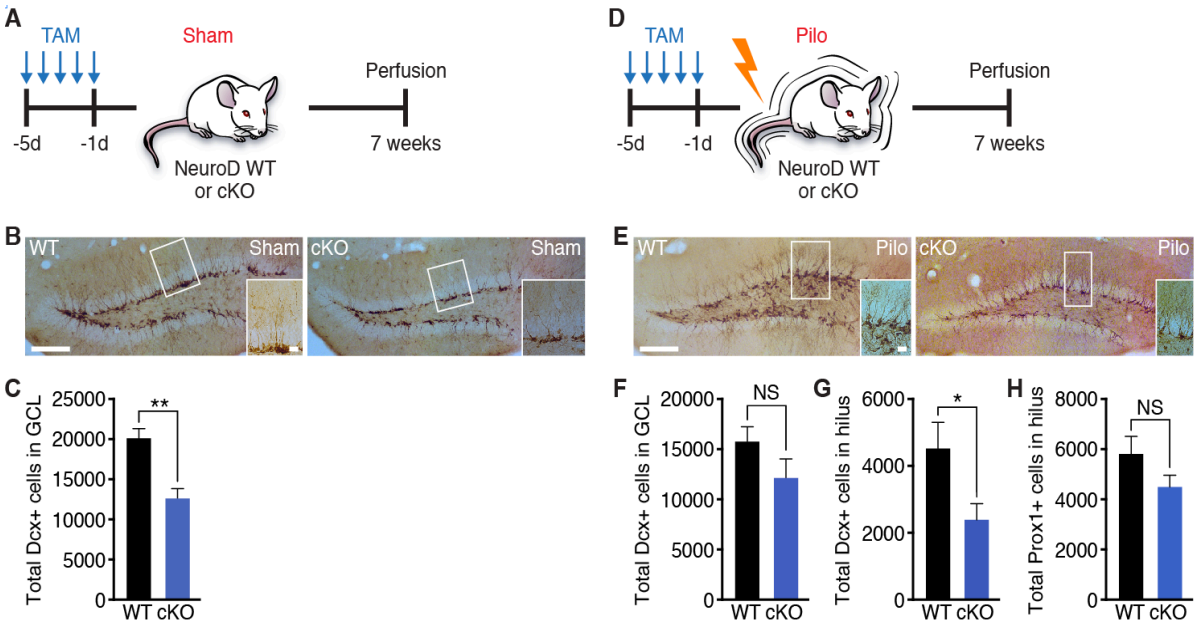

Figure 5.4 NeuroD1 is not required for Prox1-positive ectopic granule neurons: A, D: Experimental Timeline. Animals were given 5 days of tamoxifen injections and then subjected to sham- or pilocarpine treatment. Mice were euthanized 7 weeks post-sham or post-pilocarpine. B, E: Representative images from sham-treated or pilocarpine-treated WT and NeuroD1 cKO animals stained with DCX. C, F: Deletion of NeuroD1 significantly reduced the number of DCX+ cells in the GCL (p=0.0011) in sham-treated mice (n=6 WT, n=8 cKO) but not in pilocarpine-treated mice (n=11 WT, n=10 cKO). G: Deletion of NeuroD1 significantly reduced the total number of DCX+ cells ectopically localized to the hilus (p=0.0362)(n=11 WT, n=10 cKO). H: Deletion of NeuroD1 did not change the total number of Prox1+ ectopic granule neurons (n=9 WT, n=10 cKO). Inset bar in B, E: 25 um. NS=Not significant

Conclusions

Aberrant adult neurogenesis is one of the key hallmarks of mTLE and has been demonstrated by our lab and others to contribute significantly to the development of SRS (95-97, 147, 158-160). Therefore, it is critical to our understanding of the disease and the development of future therapeutics to understand the role of key molecular players involved in controlling aberrant neurogenesis. NeuroD1 is a bHLH transcription factor that is transiently expressed in late-stage adult neural progenitors and neuroblasts and is essential for the differentiation and survival of newborn neurons in the hippocampus (16). Previously implicated in the control of epileptic aberrant neurogenesis, this study examined the role of NeuroD1 in a chronic epilepsy model. Here, we have shown that the number of NeuroD1+ immature neuroblasts increases in the hippocampus after SE, and interestingly can be found in a large population of ectopic adult-generated neurons. Surprisingly, deletion of NeuroD1 from adult-generated neurons did not have any functional outcome on SRS frequency or duration. These results seem in contrast to previous work from our laboratory discussed in Chapter 4 using a genetic ablation model where we showed that ablation of >98% immature neuroblasts prior to pilocarpine administration led to a significant reduction in SRS frequency (147). In Chapter 6 I will discuss the possibilities that may have led to the different results seen in each of these studies.

Chapter 6

Conclusions and Future Directions

In the previous three chapters I have presented data from several connected projects focusing on either the increase of adult neurogenesis in non-neurogenic brain regions as a therapeutic strategy for patients with damage to the CNS, or the removal of aberrant neurogenesis as a treatment for epilepsy (Fig 6.1). In this chapter I will discuss how these results advance our understanding of the field and provide questions that still remain or are raised as a result of this work. I will also provide suggestions for future work that could be done in order to answer some of these critical questions.

NeuroD1 regulation of astrocyte-to-neuron transdifferentiation is dependent on reactive gliosis

One of the more promising therapies currently being studied for the treatment of traumatic brain injury and neurodegenerative disorders is the transdifferentiation of resident glial cells into neurons. A number of studies have been published recently showing that a variety of transcription factors, many of them bHLH transcription factors known to be important in the developing CNS, are capable of mediating this process. Interestingly however, is that in all of these studies the starting population of targeted cells are not quiescent, but reactive astrocytes. Reactive astrocytes have several unique properties that might "prime" them and make them more susceptible to undergo cell fate conversion. Interestingly, work from others support the idea that injury to the brain can lead to the recruitment of resident astrocytes to form neuroblast-like cells, whereas astrocytes isolated from non-injured brain fail to realize this potential (139, 140). Previous work also shows that after injury reactive astrocytes in the brain up-regulate expression of EGF, FGF2, and VEGF receptors allowing them to respond to signaling pathways critical to neuroblast cell fate (135, 137, 161). In Chapter 3, I utilize expression of the bHLH transcription factor NeuroD1, previously reported to

mediate reactive astrocyte conversion into neurons in the cortex, in non-reactive astrocytes to try to shed light on whether the reactive state is necessary in order for cell fate conversion to occur in the intact brain.

Reactive gliosis is a transient process in the brain, and is usually concluded approximately 1 month after injury. For patients that seek therapy outside this window of reactive gliosis it will be critical to understand whether non-reactive astrocytes may also be induced to functional neurons, or whether the identification of additional factors are needed. In my work I demonstrate that while widespread overexpression of NeuroD1 in non-reactive astrocytes of the cortex and striatum is capable of astrocyte-to-neuron conversion, this number is much lower than what was previously reported in the Guo et al. study targeting reactive astrocytes in the cortex. This would suggest that there are fundamental genetic differences between non-reactive astrocytes and their reactive counterparts. Alternatively, the local environment around the site of injury may be more conducive to transdifferentiation approaches due to the presence of reactive astrocytes. In support of this idea, it has been demonstrated that a higher yield of newly converted neurons can be achieved when overexpression of a neurogenic transcription factor is combined with the addition of growth factors to the lesion site, supporting the idea that fundamental environmental differences exist between the injured and non-injured brain (162). Consistent with this idea, astrocytes in our work were cultured in the absence of the growth factors EGF and FGF2 in contrast to previous work (123). Additionally, injury to the brain has been shown to cause disruption of the Notch1 signaling pathway in astrocytes leading to the activation of a latent neurogenic program in these cells which might make them more primed to adopt the neuronal lineage if the correct factors are present (140). Future work will therefore need to focus on the identification of genetic and epigenetic roadblocks present in these cells, as well as environmental differences unique to the un-injured brain that prevent efficient astrocyte-to-neuron conversion.

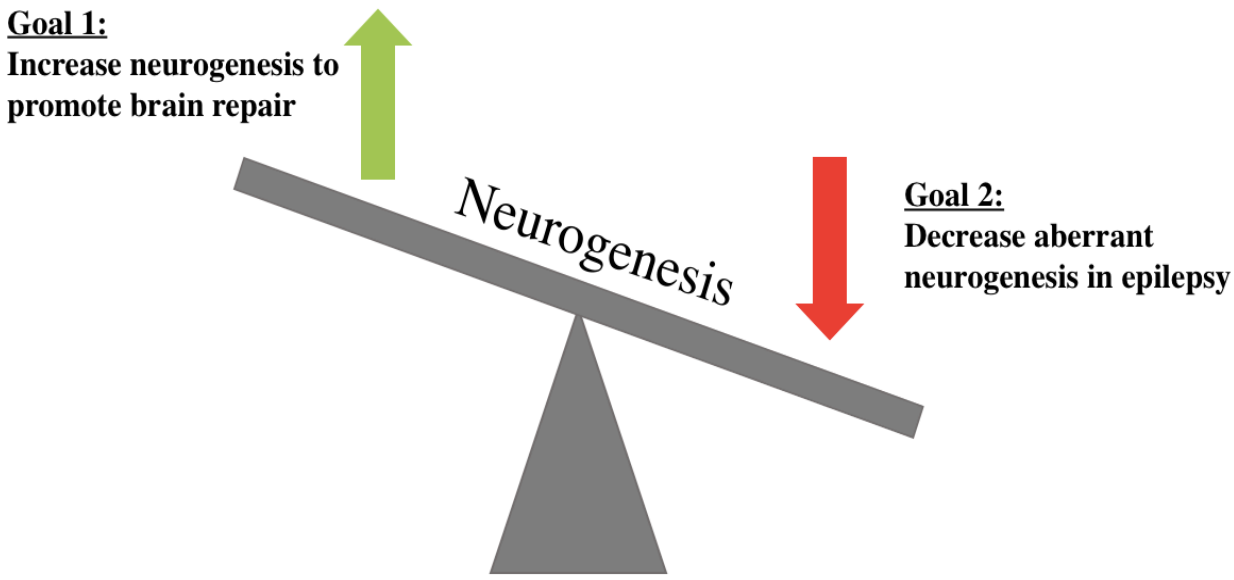

Figure 6.1 Thesis overview In chapter 3 I discuss the strategy of using expression of NeuroD1 in non-neurogenic regions of the brain to promote astrocyte to neuron transdifferentiation with the goal of increasing neurogenesis to promote brain repair (Goal 1). In chapters 4 and 5 I discuss modulating the levels of NeuroD1 expression in order to achieve less aberrant neurogenesis in epilepsy and fewer associated seizures (Goal 2).

Another potential explanation for the lack of conversion efficiency in our current work could be explained by the preferential targeting of astrocytes by AAV9. While in Chapter 3 we confirm AAV9 labels a large number of astrocytes while selectively bypassing resident neuronal cells we have not yet excluded the possibility that AAV9 might be targeting other cells types found in the cortex and striatum such as NG2+ oligodendrocyte precursor cells, which have been previously reported to undergo conversion into neurons with overexpression of NeuroD1 (123). Targeting of additional cell types might be necessary to increase the number of newly converted neurons if the initially infected cell is amenable to conversion using NeuroD1.

Additionally, all work performed in this study was done in the context of the transcription factor NeuroD1, while other transcription factors or pools of transcription factors previously reported to yield efficient astrocyte-to-neuron conversion were not tested. Therefore, it is possible that some of these transcription factors or pools already carry the genes necessary for efficient non-reactive astrocyte conversion. For example, in many transdifferentiation studies the transcription factor Sox2 is included as part of the experimental pool. As previously mentioned, Sox2 is one of the four key transcription factors that has been shown to be necessary for the induction of pluripotency in adult somatic cells. Therefore, it is not improbable that addition of one of these four reprogramming "stem-ness" factors might be necessary for successful astrocyte-to-neuron conversion by predisposing the astrocytes to a more stem-like state facilitating easier transdifferentiation.

Lastly, the identification of the subtypes of neurons generated through this approach will be critical when designing therapeutic strategies for patients as introduction of non-native neuronal subtypes may lead to worsening of disease or development of other disruptions to the CNS (163-166). For instance, grafting of fetal hippocampal tissue into the intact adult hippocampus led to the development of seizures in 30% of rats compared to rats

in which the grafts failed to survive (164). Similarly, transplantation of serotonin-rich neuroblast grafts resulted in worsening of behavioral dyskinesias in a rat model of Parkinson's disease (165). These studies highlight the need for a better understanding of the type of neurons produced when NeuroD1 is introduced under physiological conditions.

Neurogenesis plays a critical role in the progression of epilepsy

While several earlier studies hinted at the idea that neurogenesis could play a role in the development and progression of epilepsy there has yet to be a specific targeted approach for the ablation of newborn neurons that could isolate adult neurogenesis as a causative factor. However, in my co-authored paper with Dr. Cho (Chapter 4) we present evidence that suggests near complete ablation of neurogenesis using the Nestin-TK mouse line prior to SE results in an approximately 40% reduction in the overall number of seizures experienced. Interestingly, near complete ablation of neurogenesis did not abolish the existence of SRS in these animals suggesting that epilepsy is a complex and multi-faceted disease that will require much future effort to determine what other cell populations or circuitry could be contributing to the development of SRS as well. Several of the other changes that have been noted to occur in the hippocampus of epileptic patients is death of granule cell layer neuron, dispersion of the granule cell layer, and reactive gliosis. Ablation of neurogenesis prior to SE would not affect any of these cell populations, and therefore future work should focus on each of these processes as well to determine whether any can contribute to that pathology of epilepsy.

In our work we demonstrated that through use of the Nestin-TK mouse approach we could achieve ~98% ablation of neurogenesis prior to SE in these animals. However, in a practical sense complete ablation of neurogenesis is not ideal from a therapeutic standpoint as hippocampal neurogenesis has been shown to play a significant role in learning and memory. Therefore, future work will need to focus on the identification of which population(s) of cells in the neurogenesis

pathway are most critical or susceptible to becoming aberrant leading to the progression of the disease. Are proliferating neuroblasts the cell population most susceptible to becoming aberrant? Or are the immature neurons just on the verge of proper integration the cells that are most affected? And if we can identify a specific population of cells, how do we go about targeting it to eliminate aberrant neurogenesis whilst maintaining functional neurogenesis? Each of these questions will require the identification/development of specific tools that allow for precise targeting of these transient cell stages while maintaining the delicate balance of neurogenesis that occurs in this brain region.

Aberrant neurogenesis in the hippocampus of epileptic patients can take many forms. Neurons may succumb to death when exposed to SE, begin to aberrantly migrate, form incomplete or inappropriate connections with the surrounding circuitry, or fail to be selected for death allowing the survival and integration of ill-equipped neurons. In our work using near-complete ablation of neurogenesis we were unable to show exactly which one of these processes is critical to the development of SRS. Currently, no in vivo tools exist that allow for the distinction between "normal" and "aberrant" neural precursors, and therefore it is impossible to answer this question. Future work in our lab and others using single-cell RNA sequencing and other transcriptome approaches will be dedicated to identifying specific genetic regulators in the hopes of targeting each of these aberrant populations to interrogate their function in the development of epilepsy.

Additionally, in our study we chose to ablate neurogenesis prior to SE, but from a clinical standpoint it is difficult if not impossible to identify patients that will develop epilepsy prior to their first seizure event. Therefore, future work examining the role of neurogenesis in epilepsy will need to identify whether post-seizure ablation alone can yield the same level of SRS reduction as pre-seizure ablation. Unpublished work from another member of our lab, Dr. Parul Varma, suggests that there might be a trend for reduction in SRS if neurogenesis is ablated over two separate rounds of treatment after SE, but this data is still inconclusive. Even more critical will be the identification of a

therapeutic "window" of treatment as previous work suggests that neurons present during the seizure event are those most susceptible to becoming aberrant. Therefore, we need a better understanding of how long after a seizure event intervention may be effective, and whether treatment of aberrant neurons once integrated can have any effect on SRS outcome in patients.

NeuroD1 as a tool for targeting aberrant neurogenesis

As discussed in the previous section, the treatment of aberrant neurogenesis in epilepsy will require a more precise approach to target specific stages or classes of cells involved in the process of neurogenesis. In Chapter 5 I present evidence that suggests modulation of the bHLH transcription factor NeuroD1 could be an important therapeutic target. In both an acute seizure model (kainic acid), and chronic seizure model (pilocarpine) I have demonstrated a significant increase in the number of NeuroD1+ cells in the hippocampus, and more notably ectopically localized to the hilar cell region. In an effort to test whether deletion of NeuroD1 could affect SRS I used the Nestin-CreER^{T2} driver line to delete NeuroD1 in a subset of progenitor cells prior to SE. While deletion of NeuroD1 using this approach resulted in a promising trend towards the reduction of YFP/NeuroD1+ cells in the GCL, it did not significantly reduce the total number of YFP/NeuroD1+ cells in the hilus. Therefore, using this approach we were unable to successfully target many of the ectopically localized NeuroD1+ cells that could be contributing to the formation of SRS. While we were able to see a significant trend in reduction of YFP/Dcx+ cells in the GCL, this unfortunately was not enough to result in an overall decrease in the total number of Dcx+ cells in the GCL layer at 7 weeks post-SE. Interestingly, while YFP/Dcx counts conducted at 3 weeks post pilo showed no significant change in the number of cells found in the hilus, the total number of Dcx+ cells at 7 weeks ectopically localized to the hilus was shown to be significantly different. This would suggest that NeuroD1 may be dispensable for the formation of immature EGCs, as there was no significant change in the total number of Prox1+ mature EGCs at 7 weeks post pilo.

Ultimately, deletion of NeuroD1 did not lead to a significant reduction in SRS, in direct contrast to what was shown with near complete ablation of neurogenesis in Chapter 3. This would suggest one of three things: 1. NeuroD1 is not necessary for the formation of aberrant neurogenesis, 2. Experimental NeuroD1 deletion approaches were insufficient to show the effect of NeuroD1 deletion, or 3. NeuroD1 cooperates with other transcription factors and pathways to induce aberrant neurogenesis that were not targeted in this work. In regards to possibility #1; since NeuroD1 expression is predominantly found in immature neuroblasts (e.g., Dcx+ cells) in both physiological conditions and after acute seizures (16), conditional ablation of NeuroD1+ cells may not affect maturing adult-generated neurons that no longer require NeuroD1, leaving a population of aberrant adult-generated neurons that contribute to SRS. Our results raise the idea that targeting multiple molecules that govern aberrant neurogenesis might be necessary to reduce it below a threshold to see a functional impact on SRS.

In regards to possibility #2; in Chapter 5, I deleted NeuroD1 in a subset of Nestin+ precursor cells prior to SE using 5 days of tamoxifen treatment, but this subset of cells is nowhere near comprehensive. Because only a subset of cells undergoes cre-mediated recombination, there is still a significant population of NeuroD1+ cells that persist and could be confounding results shown here in this study. Previous work from our lab has shown that near complete ablation (>98%) of adult-generated neurons prior to seizure can reduce overall seizure frequency by approximately 40% (147). In contrast, in this work there was no significant change in the total number of adult-generated neurons in NeuroD1 cKO mice compared to control mice after pilocarpine treatment. Since there was no change in the total number of adult-generated neurons in NeuroD1 cKO mice, we presume there are still extensive aberrant adult-generated neurons present to contribute to SRS. It would be interesting to determine whether chronic treatment of tamoxifen longer than 5 days in

NeuroD1 cKO mice might lead to more efficient labeling of the adult nestin-expressing stem/progenitor pool, leading to enhanced removal of NeuroD1+ immature neuroblasts. Also, there is emerging work that suggests functional heterogeneity within adult neural stem/progenitor subpopulations, as exemplified by a recent study of adult neural stem cells labeled using different cre recombinase drivers that show distinct proliferative responses to running and antimitotic drug treatment (167). The use of alternative inducible neural stem/progenitor cre transgenic mice (e.g., Gli1-CreERT2) may allow for more comprehensive labeling and deletion of NeuroD1+ immature neuroblasts within the stem/progenitor population (46). Future work will be necessary to determine if more comprehensive ablation of NeuroD1, perhaps through multiple rounds or more chronic treatment with tamoxifen can result in a change in SRS.

In regards to possibility #3; if comprehensive deletion of NeuroD1 in the nestin+ progenitor cells was indeed achieved but there was still no change in SRS, it is possible that NeuroD1 cooperates with a larger transcriptional pathway that requires targeting of multiple members in order for an effect to be seen. Compensation from another transcription factor or arm of the pathway may explain the lack of phenotype seen in our current work. Therefore, future work should focus on identifying differentially regulated genes in the aberrant neuronal population that could be potential therapeutic targets.

Final Words

As described throughout this work adult neurogenesis is a complex and multi-faceted process that is governed by a number of environmental stimuli, transcription factors, epigenetic changes, signaling pathways, and disease contexts. In this work I have attempted to shed some light on the role of the transcription factor NeuroD1 in the regulation of neurogenesis in both the physiological and pathological state. NeuroD1 is a critical transcription factor in the regulation of

adult neurogenesis, and upon deletion leads to accelerated differentiation and maturation of the neural progenitor cell pool. Here, I have presented work interrogating the role of NeuroD1 in the context of epilepsy, as well as the ability of NeuroD1 to mediate the conversion of non-reactive astrocytes to neurons. As more precise tools are developed it will be interesting to see progress in the field of adult neurogenesis, particularly in the context of epilepsy.

Acknowledgements

I am particularly grateful to my mentor, Dr. Jenny Hsieh for accepting me as an ambitious yet inexperienced first year graduate student. She has spent much time and effort training me on a number of important skills from presentation to experimental design, scientific writing, and critical thinking. I appreciate her support in my endeavors to gain experience working with undergraduate students, and for encouraging me in my career choice in teaching the next generation of scientists. I appreciate her understanding and support during the difficult times in my graduate career when the journey seemed dark and I didn't believe I could make it. She believed that I could, and that is part of the reason you have this work before you today.

I would like to thank all past and current Hsieh lab members for their discussion and assistance: Kyung-Ok Cho, Zane Lybrand, Shradha Mukherjee, Yindi Jiang, Ling Zhang, Jing Ma, Jingfei Zhu, Farrah Tafacory, Peiguo Ding, Zhengling Gao, Kerstin Ure, Parul Varma, Takeshi Matsui, and Drew Thodeson. I would also like to thank my thesis committee members, Drs. Jay Schneider, Amelia Eisch, and Woo-ping Ge for their helpful suggestions, support, and time.

I would like to thank all the participants in NeuroGlee Club, SCIN (Stem Cells In Neuroscience), and Stem Cell WIPs for sharing their scientific stories and feedback.

I would like to thank Dr. Brian Kaspar, Dr. Carlos Miranda, Dr. Kinichi Nakashima, and Dr. Mauro Giacca, and Dr. Robert Hamer for their collaboration in the transdifferentiation study. I would also like to thank Dr. Amelia Eisch for providing Nestin-CreER^{T2}/Rosa-YFP mice, Dr. Woo-ping Ge for providing hGFAP CreER mice, Dr. Chun-Li Zhang for providing Ai14 Rosa;Tddtomato mice, Dr. Klaus Nave for providing NeuroD1 cKO mice, and Dr. Fred Gage for the HCN cell line, and retroviral backbones used in this work.

Support for the transdifferentiation studies, in part, was provided through the Genetics Training Program grant **T32GM0823831,** John Abrams, Ph.D. (July 1 2012-December 31 2014)

Finally, my deepest gratitude goes to my parents and sister for all their love and support during these years.

BIBLIOGRAPHY

1. Palmer TDT JGFH (1997) The adult rat hippocampus contains primodial neural stem cells. *Molecular and Cellular Neuroscience*.
2. Ming GL SH (2011) Adult Neurogenesis in the mammalian brain: significant answers, and significant questions. *Neuron* 70(4):687-702.
3. Ming GL SH (2005) Adult Neurogenesis in the Mammalian Central Nervous System. *Annual Reviews Neuroscience* 28:223-250.
4. Taylor JH WP, Hughes WL. (1957) The organization and duplication of chromosome as revealed by autoradiographic studies using tritium-labeled thymidine. *PNAS* 43(1):122-128.
5. GD. AJaD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *THe Journal of Comparative Neurology* 124(3):319-331.
6. Paton JA NF (1984) Neurons generated in the adult brain are recruited into functional circuits. *Science* 225(4666):1046-1048.
7. Gould E RA, Graziano M, Gross CG. (1999) Neurogenesis in the neocortex of adult primates. *Science* 286:548-552.
8. P R (1985) Limits of neurogenesis in primates. *Science* 227:1054-1056.
9. Zupanc GK HI (1995) Proliferation zones in the brain of adult gymnotiform fish: a quantitative mapping study. *THe Journal of Comparative Neurology* 353(2):213-233.
10. Eriksson PS PE, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. *Nature Medicine* 4(11):1313-1317.
11. Reynolds BA WS (1992) Generation of Neurons and Astrocytes from Isolated cells of the adult mammalian central nervous system. *Science* 255(5052):1707-1710.
12. Doetsch F CI, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. (1999) Subventricular Zone Astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703-716.
13. Kempermann Gerd HS, Fred H. Gage (2015) Neurogenesis in the Adult Hippocampus. *Cold Spring Harbor Perspectives in Biology*.
14. Zhao C DW, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. *Cell* 132(4):645-660.
15. Imayoshi I SM, Kageyama R (2011) Genetics methods to identify and manipulate newly born neurons in the adult brain. *Frontiers of Neuroscience* 5(64):1-11.
16. Gao Z UK, Ables JL, Lagace DC, Nave K, Goebbels S, Eisch AJ, Hsieh J. (2009) NeuroD1 is essential for the survival and maturation of adult-born neurons. *Nature Neuroscience Brief Comm* 12(9).
17. Kronenberg G RK, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G. (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *Journal of Computational Neurology* 467(4):455-463.

18. Dayer AG FA, Cleaver KM, Yassaee M, Cameron HA. (2003) Short-term and long-term survival of new neurons in the rat dentate gyrus. *Journal of Computational Neurology* 460(4):563-572.
19. Ma DK KW, Ming GL, Song H (2009) Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. *Annals of the New York Academy of Science* 1170(1):664-673.
20. Tashiro A MH, Gage F. (2007) Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. *Journal of Neuroscience* 27:3252-3259.
21. Van Praag H SA, Christie BR, Toni N, Palmer TD, Gage FH. (2002) Functional neurogenesis in the adult hippocampus. *Nature* 415(6875):1030-1040.
22. Laplange DA EM, Piatti VC, Morgenstern NA, Zhao C, Van Praag H, Gage FH, Schinder AF. (2006) Functional convergence of neurons generated in the developing and adult hippocampus. *PLoS Biology* 4(12):e409.
23. Seki T AY (1995) Age-related production of new granule cells in the adult dentate gyrus. *Neuroreport* 6(18):2479-2482.
24. Kuhn HG D-AH, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *Journal of Neuroscience* 16(6):2027-2033.
25. G. K (2015) Activity Dependency and Aging in the regulation of adult neurogenesis. *Cold Spring Harbor Perspectives in Biology* 7(11).
26. van Praag H ST, Zhao C, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. *Journal of Neuroscience* 25(38):8680-8685.
27. Kempermann G. KH, Gage FH (1998) Experience-induced neurogenesis in the senescent dentate gyrus. *Journal of Neuroscience* 18(9):3206-3212.
28. Nusse R VH (2012) Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J* 31(12):2670-2684.
29. Wang HY LT, Malbon CC. (2006) Structure-function analysis of Frizzleds. *Cell Signaling* 18(7):934-941.
30. Varela-Nallar L IN (2013) Wnt signaling in the regulation of adult hippocampal neurogenesis. *Frontiers in Cellular Neuroscience* 7(100):1-11.
31. Machon O BM, Machonova O, Kozmik Z, Vacik T, Andersen L, Krauss S. (2007) A dynamic gradient of Wnt signaling controls intitiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus. *Developmental Biology* 311(1):223-237.
32. Lie D CS, Song H, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signaling regulates adult hippocampal neurogenesis. *Nature Letters* 437(27):1370-1375.
33. Jessberger S CR, Broadbent NJ, Clemenson GD Jr, Consiglio A, Lie DC (2009) Dentate gyrus-specific knockdown of adult neurogenesis impair spatial and object recognition memory in adult rats. *Learning and Memory* 16:147-154.
34. Shi Y CL, Taupin P, Nakashima K, Ray J, Yu RT (2004) Expression and function of the orphan nuclear receptor TLX in adult neural stem cells. *Nature* 427:78-83.
35. Goodrich LV ML, Higgins KM, Scott MP. (1997) Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 277:1109-1113.

36. Marigo V DR, Zuo Y, Cunningham JM, Tabin CJ. (1996) Biochemical evidence that patched is the Hedgehog receptor. *Nature* 384:176-179.
37. Ingham PW MA (2001) Hedgehog signaling in animal development: paradigms and principles. *Genes and Development* 15:3059-3087.
38. Stone DM HM, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H, Noll M, Hooper JE, De Sauvage F, Rosenthal A (1996) The tumour-suppressor gene patched encodes a candidate receptor for Sonic Hedgehog. *Nature* 384:129-134.
39. Dahmane N R-i-AA (1999) Sonic hedgehod regulates the growth and patterning of the cerebellum. *Development* 126:3089-3100.
40. Corrales JD BS, Mahoney EM, Joyner AL (2006) The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. *Development* 133:1811-1821.
41. Fuccillo M RM, McMahon AP, Fishell G. (2004) Temporal requirement for hedgehog signaling in ventral telencephalic patterning. *Development* 131:5031-5040.
42. Gunhaga L JT, Edlund T (2000) Sonic Hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo. *Development* 127:3283-3293.
43. Dakubo GD WY, Mazerolle C, Campsall K, McMahon AP, Wallace VA (2003) Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. *Development* 130:2967-2980.
44. Huh S HV, Marcus RC, Li SC, Lai E (1999) Dorsal-ventral patterning defects in the eye of BF-1-deficient mice associated with a restricted loss of shh expression. *Developmental Biology* 211:53-63.
45. Lai K KB, Gage FH (2002) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. *Nature Neuroscience* 6(1):21-27.
46. Ahn S JA (2005) In vivo analysis of quiescent adult neural stem cells responding to sonic hedgehog. *Nature* 437(7060):894-897.
47. Faigle R SH (2013) Signaling mechanisms regulat adult neural stem cells and neurogenesis. *Biochemica et Biophysica Acta* 1830(2):2435-2448.
48. Stump G DA, Klein AL, Lutolf S, Suter U, Taylor V (2002) Notch 1 and its ligands Deltalike and Jagged are expressed and active in distinct cell populatino in the postnatal mouse brain. *Mechanisms of Development* 114:153-159.
49. Breunig JJ SJ, Vaccarino FM, Sestan N, Rakic P (2007) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. *PNAS* 104(51):20558-20563.
50. Ables JL DN, Johnson MA, Rivera PD, Gao Z, Cooper DC, Radtke F, Hsieh J, Eisch AJ. (2010) Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. *Journal of Neuroscience* 30(31):10484-10492.
51. Bonaguidi MA PC, McGuire T, Falciglia G, Gobeske KT, Czeisler C, Kessler JA (2008) Noggin expands neural stem cells in the adult hippocampus. *Journal of Neuroscience* 28:9194-9204.
52. DM K (1994) The TGF-beta superfamily: new members, new receptors, and new genetic test of function in different organisms. *Genes and Development* 8:133-146.
53. Liu A NL (2005) Bone morphogenetic protein signaling and vertebrate nervous system development. *Nature Reviews Neuroscience* 6:945-954.

54. Lim DA TA, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A (2000) Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* 28:713-726.
55. Tozuka Y FS, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. *Neuron* 47(6):803-815.
56. Ge S GE, Sailor KA, Kitabatake Y, Ming G, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439(7076):589-593.
57. R B (2000) NMDA and AMPA/Kainate glutamate receptors modulate dentate neurogenesis and CA3 synapsin-1 in normal and ischemic hippocampus. *JCBFM* 20(12):1669-1680.
58. Makeyev EV ZJ, Carrasco MA, Maniatis (2007) The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Molecular Cell* 27(3):435-448.
59. Zhao C SG, Li S, Shi Y (2009) A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nature Structural Molecular Biology* 16(4):365-371.
60. Gao Z ea (2011) The master negative regulator REST/NRSF controls adult neurogenesis by restaining the neurogenic program in quiescent stem cells. *Journal of Neuroscience* 31:9772-9786.
61. Szulwach KE LX, Smrt RD, Li Y, Luo Y, Lin L, Santistevan NJ, Li W, Zhao X, Jin P (2010) Cross talk between microRNA and epigenetic regulation in adult neurogenesis. *Journal of Cell Biology* 189(1):127-141.
62. Liu C TZ, McQuate AL, Jobe EM, Christ CC, von Hoyningen-Huene SJ, Reyes MD, Polich ED, Xing Y, Li Y, Guo W, Zhao X (2013) An epigenetic feedback regulatory loop involving microRNA-195 and MBD1 governs neural stem cell differentiation. *PLOS One* 8(1):1-12.
63. Liu C TZ, Santistevan NJ, Szulwach KE, Guo W, Jin P, Zhao X (2010) Epigenetic regulatino of miR-124 by MBD1 governs neural stem cell proliferation and differentiation. *Cell Stem Cell* 6(5):433-444.
64. Broccoli V CG, Sessa A, Rubio A (2015) Histone modifications controlling native and induced neural stem cell identity. *Current Opinions in Genetics and Development* 34:95-101.
65. Hsieh J NK, Kuwabara T, Mejia E, Gage FH (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *PNAS* 101(47):16659-16664.
66. Sun G YR, Evans RM, Shi Y (2007) Orphan nuclear receptor TLX recruits histone deacetylases to repress transcrition and regulat neural stem cell proliferation. *PNAS* 104(39):15282-15287.
67. Jiang Y HJ (2014) HDAC3 controls gap2/mitosis progression in adult neural stem/progenitor cells by regulating CDK1 levels. *PNAS* 111(37):13542-13546.
68. Ajamian F ST, Salminen A, Reeben M (2003) Upregulation of class II histone deaceytlase mRNA during neural differentiation of cultured rat hippocampal progenitor cells. *Neuroscience Letters* 346:57-60.

69. Kirby ED KA, Messer RL, Wyss-Coray T (2015) Adult hippocampal neural stem and progenitor cells regulate the neurogenic niche by secreting VEGF. *PNAS* 112(13):4128-4133.
70. Yancopoulos GD DS, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407(242-248).
71. Fournier NM LB, Banasr M, Elsayed M, Duman RS (2012) Vascular endothelial growth factor regulates adult hippocampal cell proliferation through MEK/ERK- and PI3K/Akt-dependent signaling. *Neuropharmacology* 63(4):642-652.
72. Greene J BM, Lee B, Warner-Schmidt J, Duman RS (2009) Vascular endothelial growth factor signaling is required for the behavioral actions of antidepressant treatment: pharmcological and cellular characterization. *Neuropsyhopharmacology* 34(2459-2468).
73. Lee JS JD, Lee N, Ko HG, Kim YS, Kim B, Son J, Kim SH, Chung H, Lee MY, Kim WR, Sun W, Zhuo M, Abel T, Kaang BK, Son H (2009) Induction of neuronal vascular endothelial growth factor expression by cAMP is the dentate gyrus of the hippocampus is required for antidepressant-like behaviors. *Journal of Neuroscience* 29:8493-8505.
74. Warner-Schmidt JL DR (2007) VEGF is an essential mediator of the neurogenic and behavioral action of antidepressants. *PNAS* 104:4647-4652.
75. Fabel K TB, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *European Journal of Neuroscience* 18(2803-2812).
76. Lledo PM AM, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. *Nature Reviews Neuroscience* 7:179-193.
77. Petreanu L A-BA (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. *Journal of Neuroscience* 22:6106-6113.
78. Rochefort C GG, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *Journal of Neuroscience* 22:2679-2689.
79. Sakamoto M II, Ohtsuka T, Yamaguchi M, Mori K, Kageyama R (2011) Continuous neurogenesis in the adult forebrain is required for innate olfactory responses. *PNAS* 108:8479-8484.
80. Sultan S MN, Kermen F, Garcia S, Sacquet J, Didier A (2010) Learning-dependant neurogenesis in the olfactory bulb determines long-term olfactory memory. *FASEB J* 24:2355-2363.
81. Sahay A SK, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* 472(7344):466-470.
82. van Praag H CB, Sejnowski TJ, Gage FH (1999) Running enhances neurogenesis, learning, and long-term potentiation in mice. *PNAS* 96(23):13427-13431.
83. Snyder JS HN, McDonald RJ, Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. *Neuroscience* 130(4):843-852.
84. Nilsson M PE, Johansson U, Orwar O, Eriksson PS (1999) Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *Journal of Neurobiology* 39(4):569-578.

85. Jessberger S CR, Broadbent NJ, Clemenson GD, Consiglio A, Lie DG, Squire LR, Gage FH. (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learning and Memory* 16:147-154.
86. Scott BW WJ, Burnham WM (2000) Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. *Experimental Neurology* 165:231-236.
87. Malberg JE EA, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *Journal of Neuroscience* 20(24):9104-9110.
88. Mak GK EE, Gregg C, Pakarainen T, Poutanen M, Huhtaniemi I, Weiss S (2007) Male phermon-stimulated neurogenesis in the adult female brain: possible role in mating behavior. *Nature Neuroscience* 10(8):1003-1011.
89. PO S (2014).
90. Sebastian Jessberger JP (2015) Epilepsy and Adult Neurogenesis. *Cold Spring Harbor Perspectives in Biology* 7:1-10.
91. Noebels JL AM, Rogawski MA, Olsen RW, Delgado-Escueta AV (2012) Neurogenesis and Epilepsy. *Jasper's Basic Mechanisms of the Epilepsies*, (National Center for Biotechnology Information), 4th Ed.
92. Sebastian Jessberger BR, Harish Babu, Gerd Kempermann (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippcampal progenitor cells. *Experimental Neurology* 196(2):342-351.
93. L.A. Shapiro MJK, C.E. Ribak (2005) Newly generated dentate granule cells from epileptic rats exhibit elongated hilar basal dendrites that align along GFAP-immunolabeled processes. *Neuroscience* 136(3):823-831.
94. Devin K. Binder CS (2006) Functional Changes in Astroglial Cells in Epilepsy. *Glia* 54:358-368.
95. Jack M. Parent TWY, Rebecca T. Leibowitz, Daniel H. Geschwind, Robert S. Sloviter, and Daniel H. Lowenstein (1997) Dentate Granule Cell Neurogenesis is Increase by Seizures and Contributes to Aberrant Network Reorganization in the Adult Rat Hippocampus. *The Journal of Neuroscience* 17(10):3727-3738.
96. Jack M. Parent SJ, James O. McNamara, Daniel H. Lowenstein (1998) Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. *Neurosience Letters* 247:9-12.
97. Parent JM ER, Pleasure SJ, Barbaro NM, Lowenstein DH. (2006) Aberrant seizure-induced neurognesis is temporal lobe epilepsy. *Annals of neurology* 59 (1):81-91.
98. Linda S. Overstreet-Wadiche DAB, AeSoon L. Bensen, and Gary L. Westbook (2006) Seizures accelerate functional integration of adult-generated granule cells. *Journal of Neuroscience* 26(15):4095-4103.
99. Ribak CE TP, Spigelman I, Okazaki MM, Nadler JV (2000) Status epilepticus induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. *Journal of Computational Neurology* 428:240-253.
100. Jessberger Sea (2007) Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. *Journal of Neuroscience* 27:9400-9407.

101. Kron MM, Zhang, H. & Parent, J. M. (2010) The developmental stage of dentate granule cells dictates their contribution to seizure-induced plasticity. *Journal of Neuroscience* 30:2051-2059.
102. Walter C MB, Pun RY, Spieles-Engemann AL, Danzer SC (2007) Pilocarpine-induced seizures cause selective time-depedant changes to adult-generated hippocampal dentate granule cells. *Journal of Neuroscience* 11(27):7541-7552.
103. Ozen I GC, Watts C, Parras C, Guillemot F, Raineteau (2007) Proliferating neuronal progenitors in the postnatal hippocampus transiently express the proneural gene Ngn2. *European Journal of Neuroscience* 25:2591-2603.
104. Elliott RC KS, Pleasure SJ, Parent JM, Lowenstein DH (2001) Differential regulation of basic helix-loop-helix mRNAs in the dentate gyrus following status epilepticus. *Neuroscience* 106(1):79-88.
105. Konishi Y M-uT, Mikoshiba, Tamura T (2001) Stimulation of gene expression of NeuroD-related factor in the mouse brain following pentylenetetrazol-induced seizures. *Brain Research Molecular Brain Research* 97(2):129-136.
106. Jung KH CK, Kim J, Jeong SW, Song YM, Lee ST, Kim JY, Lee SK, Roh JK. (2004) Continuous cytosine-b-D-arabinofuranoside infusion reduces ectopic granule cells in adult rat hippocampus with attenutation of spontaneous recurrent seizures following pilocarpine-induced status epilepticus. *European Journal of Neuroscience* 19(12):3219-3226.
107. Raedt R BP, Persson A, Alborn A-M, Boterberg T, Van Dycke A, Linder B, De Smedt T, Wadman WJ, Ben-Menachem E, Eriksson PS. (2007) Raditation of the rat brain suppresses seizure-induced neurogenesis and transiently enhances excitability during kindling acquisition. *Epilepsia* 48(10):1952-1963.
108. Huang C CJ, Schuurmans C (2014) Proneural bHLH Genes in Development and Disease. *Current Topics in Developmental Biology* 110:75-127.
109. Morrow EM FT, Lee JE, Cepko CL (1999) NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* 126:23-36.
110. Lee JE HS, Snider L, Turner DL, Lipnick N, Weintraub H (1995) Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268:836-844.
111. Naya FJ HH, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/NeuroD-deficient mice. *Genes and Development* 11(18):2323-2334.
112. Miyata T MT, Lee J (1999) NeuroD is require for differentiation of the granule cells in the cerebellum and hippocampus. *Development* 13:1647-1652.
113. Liu M PS, Collins AE, Noebels JL, Naya FJ, Tsai MJ, Lowenstein DH (2000) Loss of BETA2/NeuroD leads to malformation of the dentate gyrus and epilepsy. *PNAS* 97(2):865-870.
114. Goebbels S BU, Pieper A, Funfschilling U, Schwab MH, Nave KA. (2005) Cre/loxP-mediated inactivation of the bHLH transcription factor gene NeuroD/BETA2. *Genesis* 42(4).
115. CH W (1957) The strategy of the genes; a discussion of some aspects of theoretical biology. *Allen and Unwin*.
116. Gurdon JB ET, Fischberg M (1958) Sexually mature individuals of Xenopus laevis from the Transplantation of single somatic nuclei. *Nature* 182:64-65.

117. Briggs R KT (1952) Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *PNAS* 38:455-463.
118. Davis RL WH, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51(6):987-1000.
119. Takahashi K TK, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors.
120. Heins N MP, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M (2002) Glial cells generate neurons: the role of the transcription factor Pax6
. *Nature Neuroscience* 5(4):308-315.
121. Berninger B CM, Koch U, Schroeder T, Sutor B, Grothe B, Gotz M (2007) Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. *Journal of Neuroscience* 27(32):8654-8664.
122. Niu Wenze ZT, Zou Yuhua, Fang Sanhua, Smith Derek K, Bachoo Robert, and Zhang Chun-Li (2013) In vivo reprogramming of astrocytes to neuroblasts in the adult brain *Nature Cell Biology* 15(10):1164-1176.
123. Guo Ziyuan ZL, Wu Zheng, Chen Yuchen, Wang Fan, Chen Gong (2013) In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury in an alzheimer's disease model. *Cell Stem Cell*.
124. Addis RC HF, Wright RL, Dichter MA, Coulter DA, Gearhart J (2011) Efficient conversion of astrocytes to functional midbrain dopaminergic neurons using a single polycistronic vector. *PLoS One* 6(12).
125. Lagace DC WM, Noonan MA, Ables JL, DeCarolis NA, Arguello AA, Donovan MH, Fischer SJ, Farnbauch LA, Beech RD, DiLeone RJ, Greer CA, Mandyam CD, Eisch AJ. (2007) Dynamic Contribution of Nestin-Expressing Stem Cells to Adult Neurogenesis. *Journal of Neuroscience* 27(46).
126. Casper KB MK (2005) GFAP-positive progenitor cells produce neurons and oligodendrocytes throughout the CNS. *Molecular and Cellular Neuroscience* 31(4):676-684.
127. Racine R (1972) Modification of seizure activity by electrical stimulation II. Motor seizure. . *Electroencephalography* 32
.
128. Matsuda T. MN, Katano Y., Juliandi B., Kohyama J., Akira S., Kawai T., Nakashima K. (2015) TLR9 signaling in microglia attentuates seizure-induced aberrant neurogenesis in the adult hippocampus. *Nature Communications* 6(6514).
129. Aravantinou-Fatorou K OF, Chroni-Tzartou D, Antoniou N, Poulopoulou C, Politis PK, Berninger B, Matsas R, and Thamidou D (2015) CEND1 and NEUROGENIN 2 reprogram mouse astrocytes and embryonic fibroblasts to induced neural precursors and differentiated neurons. *Stem Cell Reports*.
130. Corti Stefania NM, Simone Chiara, Falcone Marianna, Donadoni Chiara, Salani Sabrina, Rizzo Federica, Nardini Martina, Riboldi Giulietta, Magri Fancesca, Zanetta Chiara, Faravelli Irene, Bresolin Nereo, and Comi Giacomo P. (2012) Direct reprogramming of human astrocytes into neural stem cells and neurons. *Experimental Cell Research* 318:1528-1541.
131. Liu Yueguang MQ, Yuan Jiacheng, Han Su'e, Zhang Panpan, Li Sanlan, Rao Zhiping, Zhao Wenlong, Ye Qian, Geng Junlan, Zhang Xiaohui, and Cheng Leping (2015) Ascl1

converts dorsal midbrain astrocytes into functional neurons in vivo. *The Journal of Neuroscience* 35(25):9336-9355.
132. Torper O. PU, Wolf D.A., Pereira M., Lau S., Jakobsson, Bjorklund A., Grealish S., Parmar M. (2013) Generation of induced neurons via direct conversion in vivo. *PNAS* 110(17):7038-7043.
133. al. AJe (2014) A transcriptional mechanism integrating inputs from extracellular signals to activate hippocampal stem cells. *Neuron* 83:1085-1097.
134. Bayraktar Omer Ali F, Alvarez-Buylla Arturo, and Rowitch David H. (2015) Astrocyte Development and Heterogeneity. *Cold Spring Harbor Perspectives in Biology* 7:1-16.
135. Burda Joshua E aSMV (2014) Reactive Gliosis and the Multicellular Response to CNS damage and disease. *Neuron* 81.
136. MV S (2014) Astrogliosis. *Cold Spring Harbor Perspectives in Biology* 7(2).
137. Robel S. BB, Gotz M. (2011) The stem cell potential of glia: lesson from reactive gliosis. *Nature Reviews Neuro* 12:88-104.
138. Anderson MA AY, Sofroniew MV (2014) Heterogeneity of reactive astrocytes. *Neuroscience Letters* 565:23-29.
139. Duan Chun-Ling LC-W, Shen Shu-Wen, Yu Zhang, Mo Jia-Lin, Chen Xian-Hua, Sun Feng-Yan (2015) Striatal astrocytes transdifferentiate into functional mature neurons following ischemic brain injury. *Glia* 63:1660-1670.
140. Magnusson JP GC, Tatarishvili J, Dias DO, Smith E, Lindvall O, Kokaia Z, and Frisen J. (2014) A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science* 346:237-241.
141. Nato G CA, Trova S, Avataneo V, Rolando C, Taylor V, Buffo A, Peretoo P, and Luzzati F. (2015) Striatal astrocytes produce neuroblasts in an excitotoxic model of huntinton's disease. *Development* 142:840-845.
142. Foust K.D.*, et al.* (2009) Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology* 27(1):59-65.
143. Hsieh Jenny NK, Kuwabara Tomoko, Mejia Eunice, Gage Fred H. (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *PNAS* 101(47):16659-16664.
144. Laywell ED RP, Kukekov VG, Holland EC. (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *PNAS* 97(25):13883-13888.
145. Leutz A SM (1981) Epidermal growth factor stimulates DNA-synthesis of astrocytes in primary cerebellar cultures. *Cell and Tissue Research* 220(2):393-404.
146. White RE RM, Gensel JC, McTigue DM, Kaspar BK, Jakeman LB (2011) Transforming growth factor alpha transforms astrocytes to a growth-supportive phenotype after spinal cord injury. . *Journal of Neuroscience* 31(42):15173-15187.
147. Cho KO LZ, Ito N, Brulet R, Tafacory F, Zhang L, Good L, Ure K, Kernie SG, Birnbaum SG, Scharfman HE, Eisch AJ, Hsieh J. (2015) Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. *Nature Communications* 26(6).
148. Wiliam Peter Gray LES (1998) Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Research* 790:52-59.
149. HE S (2002) Epilepsy as an example of neural plasticity. *Neuroscientist* 8(2):154-173.

150. Pascal Bielefeld EAVV, Jan A. Gorter, Paul J. Lucassen, and Carlos P. Fitzsimmons (2013) Different subsets of newborn granule cells: a possible role in epileptogenesis? *European Journal of Neuroscience* 39(1):1-11.
151. Jung KH CK, Lee ST, Kim J, Sinn DI, Kim JM, Park DK, Lee JJ, Kim SU, Kim M, Lee SK, Roh JK. (2006) Cyclooxygenase-2 inhibitor, celecoxib, inhibits the altered hippocampal neurogenesis with attenuation of spotaneous recurrent seizures following pilocarpine-induced status epilepticus. *Neurobiology of Disease* 23(2):237-246.
152. Hermann BPea (2006) Cognitive prognosis in chronic temporal lobe epilepsy. *Annals of neurology* 60:80-87.
153. Barker G. R. & Warburton EC (2011) When is the hippocampus involved in recognition memory? *Journal of Neuroscience* 31:10721-10731.
154. Sarkisian MR (2001) Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. *Epilepsy & Behavior* 2:201-216.
155. Giulia Curia DL, Giuseppe Biagini, Roland S.G. Jones, Massimo Avoli (2008) The pilocarpine model of temporal lobe epilepsy. *Journal of Neuroscience Methods* 172(2):143-157.
156. E.A. Cavalheiro JPL, Z.A. Bortolotto, W.A. Turski, C. Ikonomidou, L. Turski (1991) Long-term effects of pilocarpine in rats: structural damage of the brain triggers kindling and spontaneous recurrent seizures. *Epilepsia* 32:778-782.
157. L. Covolan LEM (2000) Temporal profile of neuronal injury following pilocarpine or kainic acid-induced status epilepticus. *Epilepsy Research* 39:133-152.
158. Myers CE B-HK, Scharfman HE (2013) The influence of ectopic migration of granule cells into the hilus on dentate gyrus-CA3 function. *PLoS One* 28(8).
159. Scharfman HE MD (2009) Postnatal neurognesis as a therapeutic target in temporal lobe epilepsy. *Epilepsy Research* 85:150-161.
160. Walter C MB, Pun RY, Speiles-Engemann AL, Danzer SC. (2007) Pilocarpine-induced seizures cause selective time-dependant changes to adult-generated hippocampal dentate granule cells. *Journal of Neuroscience* 11(27):75.
161. Buffo A. IR, Tripathi P., Lepier A., Colak D., Horn A., Mori T., and Gotz M. (2008) Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *PNAS* 105(9):3581-3586.
162. Grande A SK, Lopez-Juarez A, Howard J, Sakthivel B, Aronow B, Campbell K, Nakafuku M (2013) Environmental impact on direct neuronal reprogramming in vivo in the adult brain. *Nature Communications* 4.
163. Buzsaki G PG, Bayardo F, Shaw T, Gage FH (1988) Suppression and induction of epileptic activity by neuronal grafts. *PNAS* 85:9327-9330.
164. Buzsaki G ME, Chen LS, Horvath Z, Terry R, Gage FH (1991) Hippocampal grafts into the intact brain induce epileptic patterns. *Brain Research* 554(1-2):30-37.
165. Carlsson T CM, Winkler C, Bjorklund A, Kirik D (2007) Serotonin Neuron Transplants Exacerbate L-DOPA Induced Dyskinesisas in a Rat Model of Parkinson's Disease. *Neurobiology of Disease* 27(30):8011-8022.
166. Olanow CW GC, Kordower JH, Stoessl AJ, Sossi V, Brin MF, Shannon KM, Nauert GM, Perl DP, Godbold J, Freeman TB. (2003) A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Annals of neurology* 54(3):403-414.

167. DeCarolis NA MM, Petrik D, Carlton A, Ables JL, Malhotra S, Bachoo R, Gotz M, Lagace DC, Eisch AJ (2013) In vivo contribution of nestin- and GLAST lineage cells to adult neurogenesis. *Hippocampus* 23(8):708-719.