ROLES OF HDACS AND MEF2 IN ADULT HIPPOCAMPAL NEUROGENESIS

APPROVED BY SUPERVISORY COMMITTEE

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Jenny Hsieh, Ph.D.

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Chun-Li Zhang, Ph.D.

_____

Eric N. Olson, Ph.D.

_____

Jane E. Johnson, Ph.D.

TO MY PARENTS

ROLES OF HDACS AND MEF2 IN ADULT HIPPOCAMPAL NEUROGENESIS

by

YINDI JIANG

DISSERTATION

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YINDI JIANG

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Supervising Professor: **JENNY HSIEH, Ph.D.**

Abstract

The maintenance of the resident adult neural stem/progenitor cell (NSPC) pool depends on the precise balance of proliferation, differentiation, and maintenance of the undifferentiated state. Identifying the mechanisms that regulate this balance in adult hippocampal NSPCs can provide insight into basic neurogenesis principles important for tissue homeostasis and preventing tumor formation. Pharmacological inhibition of histone deacetylases (HDACs), a class of histone-modifying enzymes, have promising effects in cancer cells, yet the specific roles of individual HDACs in adult NSPCs are unclear. In this dissertation, I focus on dissecting the roles of two different HDACs in adult hippocampal neurogenesis: the Class I HDAC, HDAC3 and the Class IIa HDAC, HDAC5 as well as the Class IIa HDAC binding partner, myocyte enhancer factor 2 (MEF2).

Using conditional knockout (cKO) mice and *in vitro* cell culture, I show that histone deacetylase 3 (HDAC3) is required for the proliferation of adult NSPCs. Detailed cell cycle analysis of NSPCs from Hdac3 cKO mice reveals a defect in cell cycle progression through G2/M phase, but not S phase. Moreover, HDAC3 controls G2/M phase progression mainly through post-translational stabilization of the G2/M cyclin-dependent kinase-1 (CDK1). These results demonstrate that HDAC3 plays a critical role in NSPC proliferation.

HDAC5 is the most abundant Class IIa HDAC in adult dentate gyrus. HDAC5 is only expressed in immature and mature neurons. Using Hdac5 knockout mice and *in vitro* cell culture, I show that HDAC5 is necessary and sufficient to restrict the neuronal differentiation of NSPCs. However, the detailed mechanisms are yet to be determined.

Class IIa HDACs bind to myocyte enhancer factor 2 (MEF2) in the nucleus to repress transcription of pro-neuronal genes. Thus, we also examined the function of Mef2 genes in adult hippocampal neurogenesis. In adult hippocampus, the three most highly expressed MEF2 proteins are MEF2A, 2C, and 2D, which are expressed in immature and mature neurons similar to HDAC5. We have shown that one synthetic small molecule, Isoxazole-9 (Isx-9) could trigger neuronal differentiation robustly *in vitro* and *in vivo*. Inducible knockout of all three Mef2 genes specifically in NSPCs and their progeny revealed their critical roles in mediating Isx-9 induced neurogenesis and baseline neurogenesis.

In summary, these results demonstrate that HDACs and MEF2 control different stages of adult hippocampal neurogenesis and suggest that strategies aimed at pharmacological modulation of these proteins may be beneficial for tissue regeneration and controlling tumor cell growth in mammalian brain.

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Prior Publications

1. Shanmei Cheng*, Scott Edwards*, **Yindi Jiang**, and Frauke Gräter. Glycosylation enhances peptide hydrophobic collapse by impairing solvation. 2010. *Chemphyschem*, 11(11): 2367-74. * Equal author contribution.

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3. David Petrik, **Yindi Jiang**, Shari G. Birnbaum, Craig M. Powell, Mi-sung Kim, Jenny Hsieh, and Amelia J Eisch. Functional and mechanistic exploration of an adult neurogenesis-promoting small molecule. 2012. *The FASEB Journal*, 26(8): 3148-62.

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6. **Yindi Jiang** and Jenny Hsieh. The Class IIa HDAC5 restricts adult neurogenesis by repressing MEF2. In preparation.

7. Sarah Latchney*, **Yindi Jiang***, David Petrik, Amelia Eisch, and Jenny Hsieh. Inducible knockout of Mef2A, C, and D from nestin-expressing stem/progenitor cells and their progeny unexpectedly uncouples neurogenesis and dendritogenesis *in vivo*. In revision. * Equal author contribution.

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List of Abbreviations

AC3: activated caspase-3

bHLH: basic helix-loop-helix

BMP4: bone morphogenic protein 4

BrdU: bromodeoxyuridine

CaMK: calmodulin-dependent protein kinases

CDK1: cyclin-dependent kinase-1

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

FACS: fluorescence-activated cell sorting

FF: free-floating

Fgf2: fibroblast growth factor 2

G2/M: gap 2/mitosis

GABA: γ-aminobutyric acid

GCL: granule cell layer

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

HAT: histone acetyltransferase

HDAC: histone deacetylase

HCN: hippocampal neural progenitor

HDAC3i: HDAC3-selective inhibitor

IHC: immunohistochemical

iKO: inducible knockout

i.p.: intraperitoneally

iPSC: induced pluripotent stem cell

IRES: internal ribosome entry site

MADS: MCM1-agamous-deficiens-serum response factor

MEF: mouse embryonic fibroblast

Mef2: myocyte enhancer factor 2

NAD: nicotinamide adenine dinucleotide

NCoR: nuclear receptor co-repressor

NEC: Notch-activated epicardium-derived cell

NKCC1: Na-K-Cl cotransporter

NSPC: neural stem/progenitor cell

NuRD: nucleosome remodeling deacetylase

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PI: propidium iodide

PMSF: phenylmethanesulfonyl fluoride

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

QNP: quiescent neural progenitor

qPCR: quantitative PCR

RGL: radial glial-like

RMS: rostral migratory stream

SCI: spinal cord injury

SEM: standard error of mean

SGZ: subgranular zone

shRNA: short hairpin RNA

SM: slide-mounted

SMRT: silencing mediator for retinoic acid and thyroid hormone receptors

SVZ: subventricular zone

TAM: tamoxifen

TAP: transit amplifying progenitor

TBS: tris-buffered saline

TK: thymidine kinase

tKO: triple knockout

TSA: trichostatin A

VPA: valproic acid

WT: wildtype

YFP: yellow fluorescent protein

Chapter 1

Introduction

Discovery of adult neurogenesis

Pioneering neuroanatomist Santiago Ramón y Cajal first stated a century-long belief that “In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.” (1). However, with the development of new tools and techniques, modern biologists could observe what most insightful biologists could not imagine.

Four years after the discovery of the double helix structure of DNA, Dr. J. Herbert Taylor successfully synthesized tritium-labeled thymidine to study DNA replication in plant cells in 1957 (2). This novel tool stimulated the study of cell proliferation of different tissues in mouse and rats, including the brain (3). In the 1960s, Joseph Altman published a series of papers entitled “Autoradiographic and histological studies of postnatal neurogenesis” (4-6). He injected tritium-labeled thymidine intraperitoneally into rats and discovered tritium signals in the hippocampal dentate gyrus, neocortex, and olfactory bulb, indicating that new neurons might be produced in these regions. However, his work was discounted, partially because he could not show that these new neurons were functional.

In the 1980s, intracellular potential was recorded from radioactively labeled neurons in adult songbirds injected with tritium-labeled thymidine (7), suggesting that adult-born new neurons are functional. With the development of a monoclonal antibody of a thymidine analog, 5-bromo-2-deoxyuridine (BrdU) (8), detection of proliferating cells became more convenient and accurate. Incorporation of BrdU was then detected in the

brain of a variety of species, including fish and monkey (9, 10). In 1998, Fred Gage's group first demonstrated the existence of newborn neurons in the dentate gyrus of adult humans by immunostaining of BrdU and NeuN (a marker of mature neurons) on postmortem tissues from patients treated with thymidine analogs (11). These discoveries suggest that adult neurogenesis is a universal process occurring in broad spectrum of species.

Overview of adult neurogenesis

It is now well accepted that new neurons are continuously produced in two predominant regions in the adult brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) lining the lateral ventricles (Fig. 1.1*A-B*). Although several reports showed that adult neurogenesis could also happen in other areas of the brain, such as neocortex, striatum, and amygdala (12), these findings are still not conclusive.

In recent years, the number of adult-born neurons has been estimated in adult human dentate gyrus and SVZ (13, 14). By measuring the concentration of nuclear bomb test-derived ^{14}C in genomic DNA from the human post-mortem hippocampus, Spalding et al. found 700 new neurons generated in each hippocampus per day (15). In SVZ, robust migration of immature neurons can be detected before 18 months after birth, but the turnover rate of neurons is extremely low in the adult olfactory bulb (< 1%/100 years) (16), suggesting very limited new neurons are generated in adult human olfactory bulb. Thus, in this dissertation, I focus my research on adult neurogenesis happened in the hippocampal dentate gyrus because these studies may provide more clues for treating diseases in human with cognitive impairment.

In SGZ and SVZ, rarely dividing radial-glia like quiescent neural progenitors (QNPs) are activated to generate transit-amplifying progenitors (TAPs), which adopt the neuronal cell fate to produce neuroblasts. Neuroblasts in SGZ will migrate into the granule cell layer (GCL) as they differentiate into immature and mature granule neurons with axons projecting into CA3 (Fig. 1.1*C*). In SVZ, neuroblasts migrate through the rostral migratory stream (RMS) to reach the olfactory bulb and differentiate into periglomerular and granular interneurons.

These stages in adult neurogenesis can be distinguished by immunohistochemical staining of stage-specific markers. QNPs are positive for glial fibrillary acidic protein (GFAP) and Sox2. Rarely activated QNPs can also be labeled by proliferation markers, such as Ki67 and BrdU. For the identification of neurons, doublecortin (DCX) is commonly used for immature neurons, whereas NeuN is used for post-mitotic mature neurons. In the next two sections, I will discuss the specific stages in adult neurogenesis.

NSPC maintenance and proliferation

Our bodies contain many differentiated cells that carry out specific functions. In contrast, stem cells are undifferentiated cells that are capable of differentiating into specialized cells. Stem cells can be categorized based on their ability to differentiate into other cell types. Somatic or adult stem cells residing in different organs are either multipotent to generate several kinds of specialized cells or unipotent to produce only one type of cells. Till now, adult stem cells have been found in many organs, including bone marrow, gut, skin, and brain (17-20).

Adult neural stem/progenitor cells (NSPCs) were first isolated from the striatum of the adult mouse brain (20). Under epidermal growth factor (EGF) condition, these

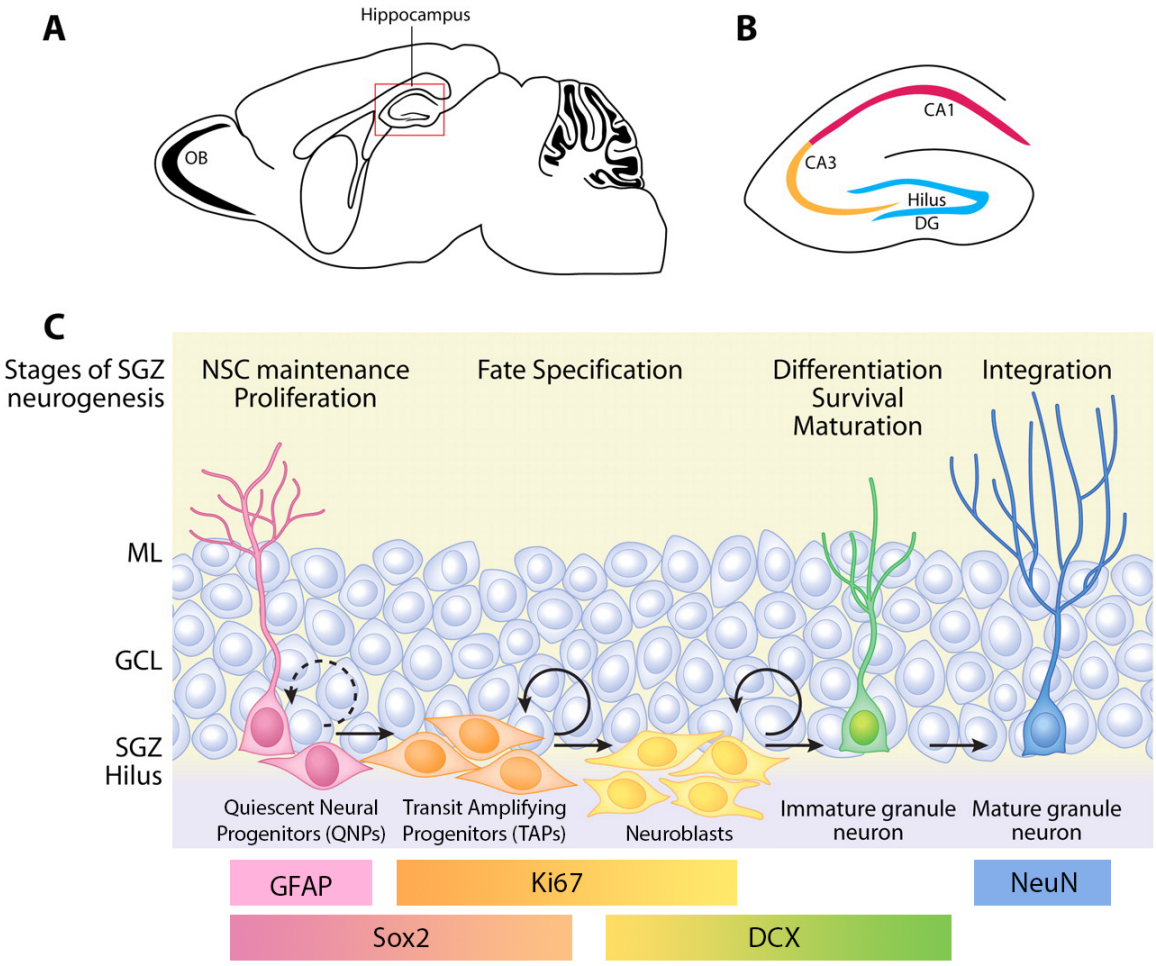


Figure 1.1. Adult hippocampal neurogenesis in the subgranular zone of the dentate gyrus. (*A*) Diagram of the location of hippocampus in the brain. (*B*) Diagram of the structure of the hippocampus. (*C*) Adult neurogenesis is a multistep process. (Modified from Hsieh (2012) Genes Dev.).

cells formed spheres of proliferating cells, which we call “neurospheres” today. After transferred to poly-L-ornithine-coated plates, they would differentiate into neurons and astrocytes. Not only from striatum, neural stem cells have also been isolated from many other regions of the adult brain, including spinal cord, hippocampal, and cerebellum (21-23).

There are two emerging models of neural stem cell (NSC) self-renewal and differentiation in the adult SGZ. In one model where there is repeated-cycling of NSCs between quiescent and activated states, QNPs divide symmetrically to generate two identical daughter stem cells or asymmetrically to produce one daughter stem cell and one restricted progenitor cell (24). In a second model where QNPs divide asymmetrically a finite number of times before terminally differentiating into an astrocyte, the NSC is considered “disposable”, which could explain why there is progressive loss of the neurogenic capacity of NSCs during aging (25). In both models, NSCs can directly differentiate into astrocytes. Meanwhile, the number of NSCs is also controlled by their location and the experience of animals (26). Once QNPs become activated, they leave the neurogenic niche and proliferate, however it remains unclear how the niche signals to nuclear epigenetic mechanisms to regulate the self-renewal of QNPs. Clearly, a more thorough understanding of the transcriptional and epigenetic regulatory mechanisms that control QNPs activation may address some of these outstanding questions.

QNPs rarely enter cell cycle from quiescence to produce TAPs, which can undergo several rounds of additional proliferation to generate neuroblasts, immature and mature neurons. Proliferation of NSPCs is tightly controlled by cell cycle regulators. Both QNPs and TAPs are capable of proliferation, however, the underlying mechanisms are likely to be different. For example, the kinase inhibitory protein 1 (Kip1) family member, cell cycle inhibitor $p27^{Kip1}$ can specifically control the proliferation of TAPs in the adult

SVZ, without affecting QNPs (27). Thus, it is necessary to separate the proliferation of QNPs and TAPs when discussing the specific functions of transcription or epigenetic regulation. These results suggest that there may be separate target genes in different cells depending on the stage of neurogenesis.

In addition, whether these cell cycle regulators merely control cell proliferation in adult NSPCs or even control cell fate decisions, such as remaining quiescent or undergoing differentiation, is not entirely clear. Recent studies reveal the roles of G1 phase regulators E2f transcription factor 3 and Cyclin D not only in cell proliferation, but also in cell fate commitment (28, 29), suggesting a possible role of cell cycle regulators in mediating self-renewal and differentiation of adult NSPCs.

Fate specification, survival, migration, maturation, and function of adult-generated neurons

Besides preserving their multipotency in one daughter cell, adult NSCs can adopt two outcomes for another daughter cell, neural progenitors or glial progenitors. Studies of cortical development reveal that sequential neurogenesis (E12 to E17) and gliogenesis (E17 to P1) depends on the levels of a basic helix-loop-helix (bHLH) transcription factor, neurogenin-1 (30). Unlike developmental stages, it is unclear whether cell fate determination in adult stages occurs in a strictly spatial or temporal manner, rather it would appear that neurons and glia are generated on an as-needed basis.

In the transition from TAPs to neuroblasts, most of the newborn cells in the hippocampal dentate gyrus will be eliminated by microglia through apoptosis-coupled phagocytosis (31). Surviving neuroblasts will migrate a short distance to the GCL to mature and integrate into current neuronal circuits. In pilocarpine-induced status

epilepticus, newborn granule cells are found ectopically in the hilus and believed to cause epilepsy (32). Neuroblasts from SVZ will migrate along RMS to the olfactory bulb. In some pathological conditions, such as stroke, neuroblasts could migrate to the injured sites to compensate for the local cell death (33). However, the effect of this endogenous response after stroke is limited for regeneration.

In the GCL, maturation of immature neurons is highly regulated by neurotransmitters. Newborn immature neurons express high levels of Na-K-Cl cotransporter 1 (NKCC1), which brings Cl^- into the cells to reach higher internal chloride concentration. Local γ-Aminobutyric acid (GABA) activates GABA receptor on immature neurons to allow outflux of Cl^-, thereby depolarizing the cells. Block this depolarization in newborn neurons by knocking down NKCC1 leads to dendritic arborization defects. This excitatory effect of GABA will finally become inhibitory when these newborn neurons mature and express less NKCC1 and more K-coupled Cl transporter 2 (KCC2), which exports chloride ions (34).

During the third week of the adult hippocampal neurogenesis, newborn cells receive glutamergic inputs from the entorhinal cortex via perforant path, and extend their axons to dendritic shafts of CA3 pyramidal neurons to integrate into existing neural circuitry. Synapses were observed between newborn neurons and existing neurons through electron microscope (35). Between 1 month and 1.5 months of the neurogenesis, newborn neurons exhibit enhanced synaptic plasticity, which distinguished them from mature neurons (36). However, increased synaptic plasticity would not be observed after two months.

The hippocampus is required for learning and memory. To study the function of these newly integrated neurons, adult hippocampal neurogenesis is blocked to examine defects in hippocampus-dependent learning and memory tasks, such as Morris water

maze. Ionizing radiation (IR) is often used to block neurogenesis in these studies because of its non-invasion and effectiveness. Under a dose of 1-15 Gy, IR only kills the proliferating progenitor cells but leave mature neurons intact (37). However, this radial approach is not specific. To eliminate newborn neurons specifically, transgenic mice with thymidine kinase (TK) expression driven by neural stem cell specific promoter were generated (38). After four-week treatment of ganciclovir (GCV), which is toxic to cells expressing TK, over 90% of DCX+ cells will be depleted.

However, the results of these behavior studies after radial or genetic ablation of adult neurogenesis are confusing. Some results have been summarized in Supplementary Information S1 in Deng et al. 2010 Nature Review Neuroscience paper (39). These discrepancies may originate from the experimental designs or the ways to perform these behavior tests. Thus, whether adult neurogenesis is required for learning and memory is still not conclusive. Recently, newborn neurons were shown to be necessary and sufficient for another hippocampus-dependent task, pattern separation (40, 41).

Transcriptional and epigenetic regulation of adult hippocampal neurogenesis

It is now well accepted that adult hippocampal neurogenesis does exist and function in the murine and human brain, however, some fundamental information is still lacking, such as the regulatory mechanisms controlling the maintenance of the NSPC pool and differentiation of adult-generated neurons. Most recent studies have focused on cell-extrinsic factors, such as receptor-mediated signaling pathways (42-44). Less is known about cell-intrinsic mechanisms, including transcription and epigenetic factors, particularly the crosstalk between extrinsic and intrinsic regulation to control adult neurogenesis. Disruptions in transcriptional or epigenetic regulation within the brain have

been associated with neurodevelopmental and neuropsychiatric disorders (e.g., Angelman, Prader-Willi, and Rett syndrome) (45-47). Thus, there is intense interest to understand how the transcription and epigenetic factors integrate extrinsic and intrinsic signals in NSPCs.

The role of transcription factors has been well described in neurogenesis (48). They receive extrinsic and intrinsic signals and execute their function through binding to the promoter of target genes in a sequence-specific manner. A well-characterized example is the previously mentioned bHLH transcription factor, neurogenin-1. During early neurogenesis stages of cortical development, neurogenin-1 can activate neuronal genes through preferentially binding with the coactivator CBP/p300 and SMAD1, which can also form a complex with signal transducers and activators of transcription 3 (STAT3) to activate astrocyte genes. By sequestering away STAT3, neurogenin-1 can activate the neuronal program and simultaneously repress the astrocytic program. Downregulation of neurogenin-1 after neurogenesis allows STAT3 to associate with the CBP complex to promote the neurogenic to gliogenic switch.

In addition to transcription effects on gene expression, epigenetics refers to potentially heritable changes in gene expression without a change at the DNA sequence level (49, 50). In recent years, epigenetic regulation has emerged as an equally important mechanism for gene regulation compared to regulation by transcription factors. For example, epigenetic processes are critical throughout early embryonic development – where pluripotent stem cells differentiate into hundreds of distinct cell types throughout the body by selectively activating or repressing lineage-specific genes (51).

There are four major types of epigenetic mechanisms: DNA methylation, histone modification marks, chromatin remodeling, and non-coding RNAs. DNA methylation can occur at the fifth position at the cytosine residue of CpG dinucleotides (5'MeC) to form a

stable carbon-carbon single bond (49, 52, 53). X-chromosome inactivation and genetic imprinting are classic examples by which DNA methylation controls gene expression through the actions of DNA methyltransferases (DNMTs) (54-56). DNA methylation of CpG nucleotides is mainly catalyzed by three different DNMTs, DNMT1, DNMT3a, and DNMT3b (57).

During development, deletion of DNMT1 in nestin-positive neural progenitor cells resulted in an increase of mature astrocytes (58). Loss of DNMT1 mediated passive demethylation of CpG islands in the astrocyte gene GFAP promoter as well as in the promoter of STAT3, which leads to an upregulation of astrocyte genes by binding to their promoters (58). The role of DNMT1 in adult NSCs remains elusive. Nevertheless, in adult neurons, loss of DNMT1 alone did not yield an observable phenotype. Since DNMT3b is not expressed in adult stages (59), it is thus speculated that DNMT3a and DNMT1 may play redundant roles in adult neurons (60). Using a postmitotic neuron specific calcium/calmodulin-dependent protein kinase IIα (Camk2α) Cre recombinase, deletion of DNMT1 and 3a impaired synaptic plasticity, learning and memory through de-repression of immune genes, such as the major histocompatibility complex 1 (MHC1) (61).

Another well-studied epigenetic mechanism, histone modification is based on chromatin structure, whose nucleosome core particles are composed of octameric core histones, H2A, H2B, H3, and H4, wrapped by 147 base pairs of DNA (48). When chromatin is in its compacted conformation, the transcription machinery has limited access to DNA to regulate gene expression. Lysine and arginine residues of N-terminal domain of histone tails protruding from the nucleosome core can be modified covalently by acetylation, methylation, phosphorylation, ribosylation, and SUMOylation (62).

Acetylation is the one of the most studied histone modifications. It was first discovered on histones in 1964 (63). Acetylation of histone tails can reduce the electrostatic interaction between negatively charged DNA and positively charged histone tails, so the chromatin becomes less compacted. This process is catalyzed by histone acetyltransferases (HATs), such as GCN5, CREB-binding protein (CBP), p300, and p300/CBP-associated factor (PCAF). Some HATs have been studied in the brain, such as CBP. CBP haploinsufficient mice have impaired differentiation of stem cells in embryonic neuroepithelium, thus leading to early cognitive dysfunction. This phenotype in mice is similar to Rubinstein-Taybi syndrome (RTS) in humans, who carry a mutated or deleted CBP gene (64).

Acetylation of histone tails will be reversed by histone deacetylases (HDACs). HDACs can be categorized into four classes based on their sequence similarities (65). Class I HDACs (HDAC1, 2, 3, and 8) are homologous to yeast transcriptional regulator Rpd3 (66). They include an HDAC domain, which harbor the deacetylase activity. Class IIa HDACs consist of one adapter domain at their N-terminus and one deacetylase domain at their C-terminus (67, 68). Distinct from Class IIa, Class IIb HDAC6 contains two deacetylase domains at its N-terminus and a zinc-finger domain at its C-terminus. HDAC6 is well known for its role in tubulin deacetylation (69). Class III HDACs are nicotinamide adenine dinucleotide (NAD)-dependent instead of zinc-dependent like the other HDACs, and involved in metabolism and aging (65). HDAC11 is the unique member in Class IV (70) (Fig.1.2).

Because acetylation is one of the most fundamental modifications in biological systems, it is not surprising that HDACs are involved in many processes beyond gene transcription. This enables HDACs to become a hot target for therapeutics. In recent years, small molecule inhibitors for HDACs have been developed to treat many

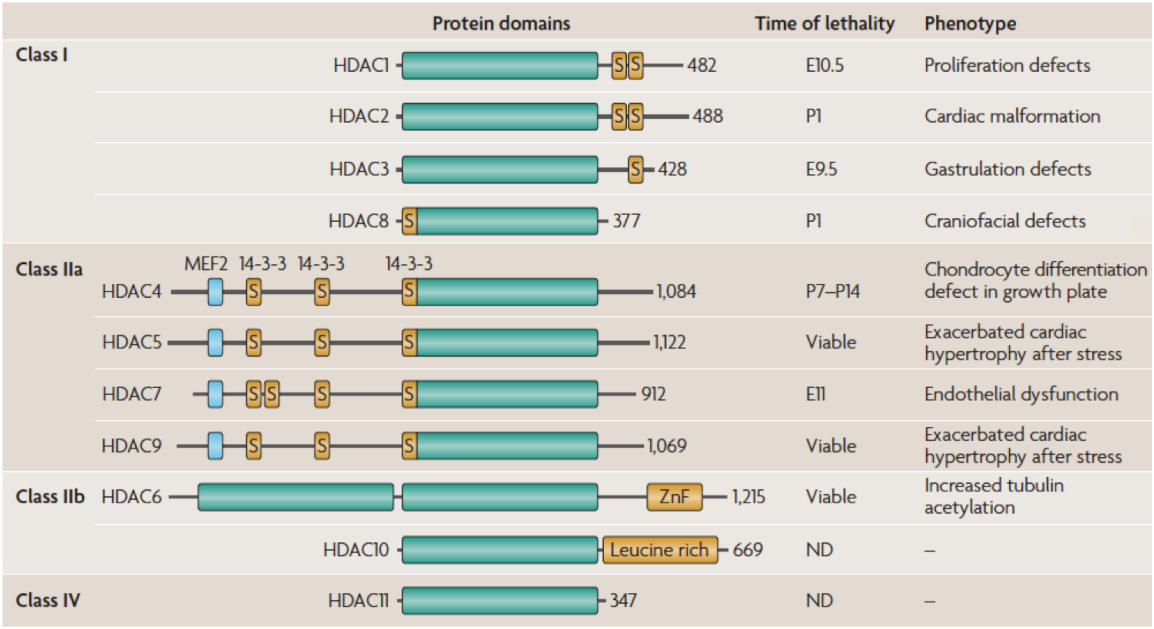


Figure 1.2. Protein structure and loss-of-function phenotypes of zinc-dependent HDACs. Green boxes indicate deacetylase domains; blue boxes indicate Mef2 binding domain. S: serine phosphorylation site; ZnF: zinc finger domain. (Adapted from Haberland et al. (2009) Nat. Rev. Genetics)

different kinds of diseases, including cancers. For example, Zolinza (Merck & Co. Inc.) and Istodax (Celgene Corp.) have been approved by Food and Drug Administration (FDA) to treat cutaneous T-cell lymphoma. Our previous work demonstrated that treatment of broad HDAC inhibitors, such as valproic acid (VPA), decreased adult NSPC proliferation and promoted their neuronal differentiation *in vitro* and *in vivo* (71).

This result demonstrated that HDACi are powerful chemical biological tools to determine neuronal cell fate in NSCs, they also raised the question of the role of specific HDACs involved in adult NSPCs maintenance and neurogenesis. In the next two sections, I will introduce Class I and Class IIa HDACs in detail.

Class I HDACs

HDACs 1 and 2 in Class I HDACs share very high similarity, with 83% amino acids identity. They are usually found in a complex with Sin3, nucleosome remodeling deacetylase (NuRD), and corepressor of RE-1 silencing transcription factor (CoREST). Thus, HDACs 1 and 2 appear to function redundantly in many different systems, such as adipogenesis, oligodendrocyte differentiation, and progression of neural precursors to neurons during brain development (72-74). In these studies, a dramatic phenotype with single deletion of HDAC1 or HDAC2 was not observed, but HDAC1/HDAC2 double knockout mice showed severe defects.

Nevertheless, in the nervous system, HDAC1 and HDAC2 are broadly expressed during embryonic and adult stages, with HDAC1 enriched in neural stem/progenitor cells and glial cells, whereas HDAC2 is mainly found in neuroblasts and immature/mature neurons (75). Overexpression of HDAC2, but not HDAC1 in neurons, resulted in defects in hippocampus-dependent memory formation by Pavlovian fear conditioning and Morris water maze tests (76). Another study demonstrated that HDAC2 deletion, both globally

as well as conditionally using a GLAST-CreERT2, had a severe impact on the maturation and survival of adult-generated neurons in the SGZ and SVZ (77). However, these studies do not exclude a role for HDAC1 in adult neurogenesis.

HDAC3 is best known for its actions to repress gene expression through interacting with SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor) (78). HDAC3 can also function in a deacetylase-independent way, because deacetylase-dead HDAC3 can rescue the severe hepatosteatosis caused by conditional deletion of Hdac3 in mouse liver (79).

Recently, a role of HDAC3 in cell cycle progression has also been uncovered. In human colon cancer cells, HDAC3 levels are elevated, which have been suggested to control cells in both S and G2/M phase (80). Loss of HDAC3 in hematopoietic progenitor cells results in only S phase progression defects (81), while in Hela cells, a G1/S transition defect was observed after knockdown of HDAC3 (82). However, it is still unclear which stage(s) of cell cycle HDAC3 controls in NSPCs and the underlying mechanisms.

While HDAC3 is highly expressed in the brain (83) and has documented roles in learning and memory associated with cocaine-seeking behavior (84), its role in adult NSPCs is largely unknown. In Chapter 3, I will describe the function of HDAC3 in adult hippocampal neurogenesis in detail.

Class IIa HDACs

Class IIa HDACs (HDAC4, 5, 7, and 9) show high sequence similarity with Hda1 protein in *Saccharomyces cerevisiae* (85, 86). They contain a highly conserved deacetylase domain at carboxyl-terminus, but with very weak catalytic activity. This is due to a replacement of tyrosine with histidine in their catalytic pockets (87). They exert

their deacetylase function through bridging an enzymatically active Class I HDAC (88). In the amino-terminus of Class IIa HDACs, they contain a myocyte enhancer factor 2 (MEF2)-binding motif and a 14-3-3 chaperone protein binding site required for shuttling between the nucleus and cytoplasm to control gene expression (89).

Unlike ubiquitous expression of Class I HDACs, Class IIa HDACs are expressed in a tissue-specific manner. They were found highly expressed in heart, skeletal muscle, thymus, and brain. To reach target specificity, they interact with sequence-specific DNA-binding transcription factors at their N terminal regions, such as NFATc3 (nuclear factor activated T cells c3; (90)), RUNX2 (Runt-related transcription factor 2; (91)), NRSF (neuron-restrictive silencer factor; (92)), and MEF2. Class IIa HDAC knockout mice also exhibit tissue-specific phenotypes. Loss of HDAC4 shows hypertrophy of chondrocytes, resulting in premature ossification of cartilage (91). Mice lacking either HDAC5 or HDAC9 are viable, but hypersensitive to cardiac stress signals (93, 94). Deletion of HDAC7 resulted in upregulation of an endoprotease MMP10 (matrix metalloproteinase 10) and destroyed vascular integrity (95). Although Class IIa HDACs are abundant in the brain, their functions are largely unknown.

Cytoplasmic HDAC4 is known to control survival of cerebellar granule neurons and retinal neurons (96-98). Either deletion of HDAC4 or overexpression of HDAC4 in the nucleus would result in apoptosis of these neuronal cells. Several mechanisms have been put forward to explain the cell survival role of HDAC4. In post-mitotic cerebellar granule neurons, HDAC4 inhibits the activity of the cell cycle protein CDK1 to block cell cycle progression (97). In retinal neurons, HDAC4 may control the activity of hypoxia-inducible factor 1 α (HIF1 α), a protein critical for preventing light-induced retinal degeneration (98, 99).

Behavior of mice lacking Class IIa HDACs has also been examined in recent years. HDAC4 could regulate synaptic plasticity and hippocampal-dependent learning and memory (100). However, mice lacking HDAC5 only show impaired hippocampus-dependent spatial memory, but not context-dependent fear conditioning (100, 101). Also, HDAC5 is involved in reducing chronic, but not acute, cocaine reward in the nucleus accumbens (102). This process is controlled by cAMP induced dephosphorylation and nuclear accumulation of HDAC5 (103).

The above results reveal that Class IIa HDACs play important roles in mature, postmitotic neurons. However, their function in the development of adult-born neurons is still unknown. In Chapter 4, I will describe their contributions in adult hippocampal neurogenesis.

In recent years, the complex interplay between transcription factors and nuclear epigenetic mechanisms such as histone modification marks in control of adult NSPC proliferation has been revealed. For example, the orphan nuclear receptor TLX was shown to recruit a histone demethylase, lysine specific demethylase 1 (LSD1) and a histone deacetylase, HDAC5 to the promoter regions of the cell cycle inhibitor p21 gene and tumor suppressor phosphatase and tensin homolog (Pten) gene to repress their expression and maintain the proliferative state (104) (105). However, how Class IIa HDACs work in concert with transcription factors to control adult hippocampal neurogenesis remains elusive. In the next section, I will introduce Mef2, a transcription factor that interacts with Class IIa HDACs.

MEF2

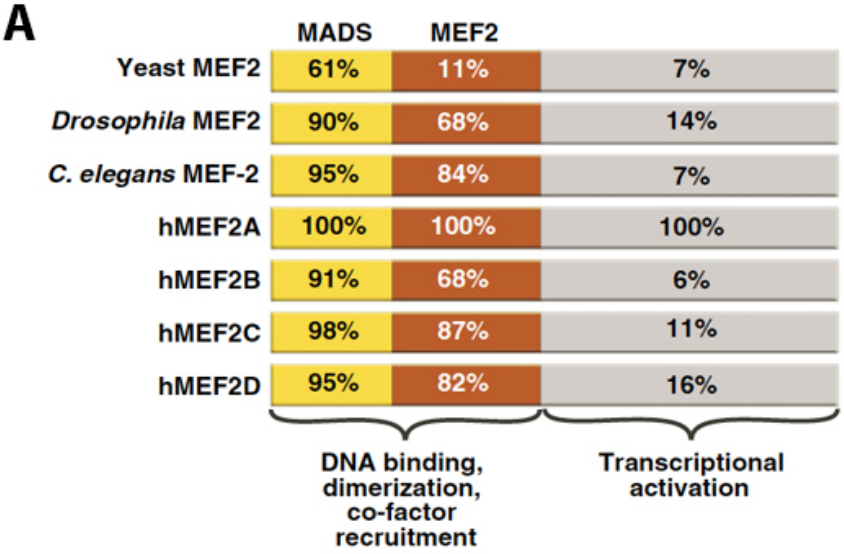
MEF2 proteins belong to MADS (MCM1-agamous-deficiens-serum response factor) family of transcription factors (106, 107). The MEF2 protein is encoded by one

single Mef2 gene in yeast, *C. elegans*, and Drosophila. In the vertebrate, there exist four Mef2 genes, Mef2A, 2B, 2C, and 2D. The protein structure of MEF2 includes a highly conserved N terminal MADS domain adjacent to a MEF2 domain. These two domains are highly conserved and responsible for DNA binding, homo- or heterodimerization, and recruitment of cofactors, such as Class IIa HDACs. In the C-terminal, they possess two highly variable transcriptional activation domains subject to covalent modifications, such as phosphorylation, acetylation, and sumoylation (Figure 1.3*A*).

MEF2 was first identified as a myocyte-specific enhancer-binding factor to activate muscle-specific genes in 1989 (108). These proteins could bind to the consensus DNA sequence (C/T)TA(A/T)$_4$TA(A/G) in the enhancers or promoters of MEF2 target genes. They could also be found in brain, heart, spleen, and bone (107). Knockout mice for all four Mef2 genes have been generated. Mice homozygous for the null mutation of Mef2A die of mitochondrial deficiency in postnatal cardiac myocytes by the first week after birth (109). Mef2b-null mice are viable, with no significant defects at birth (110). A knockout of Mef2C resulted in embryonic lethality by E10.5 due to defects in cardiac myogenesis (111). Mice lacking Mef2D are also viable, but resistant to stress-dependent cardiac hypertrophy and fibrosis (112). These studies suggested that each Mef2 gene plays a different role in heart development.

MEF2 activity could be modulated through its interaction with Class IIa HDACs. In the cell nucleus, Class IIa HDACs associate with the MADS domain of MEF2 to repress MEF2-dependent gene expression. Calmodulin-dependent protein kinases (CaMKs) activated by the calcium signaling will phosphorylate Class IIa HDACs, thereby exporting these repressors to the cytoplasm and de-repressing MEF2 (113). MEF2 activity could also be regulated via its phosphorylation states. Mitogen-activated protein kinase (MAPK) p38 and extracellular-signal-regulated kinase 5 (ERK5) phosphorylate

A



B

		Days postcoitum					
		12.5	14.5	16.5	Nn	2 week	Adult
Cerebral cortex	2A	–	–	+	+	+	+
	2B	+	++	++	+++	++	+++
	2C	++	+++	+++	+++	+++	+++
	2D	+	+	++	++	++	++
Hippocampus	2A	nd	–	–	+	+++	+++
	2B	nd	+	+	+	+	+/–
	2C	nd	+++	+++	++	++	++
	2D	nd	+	+	+	+++	+++

Figure 1.3. Expression of four Mef2 genes in developing mouse brain. (*A*) Sequence conservation of four Mef2 genes (Adapted from Potthoff et al. (2007) Development). (*B*) Expression levels of Mef2 genes in cerebral cortex and hippocampus (Adapted from Lyons et al. (1995) J Neurosci.).

transcriptional activation domains of MEF2 to promote its transcriptional activity (114, 115).

The levels of Mef2 genes have been determined in the developing central nervous system by in situ hybridization with gene specific probes (116). Mef2C is the first detected Mef2 gene in the CNS and remains as a predominant form in the cerebral cortex from the embryonic stage to the adult stage (Fig. 1.3*B*). In the hippocampus, Mef2C is highly expressed in the embryonic stage, whereas its levels are decreased in the adult stage. Mef2B cannot be detected in the hippocampus. Levels of Mef2A, and 2D are low in the prenatal and neonatal stages, but upregulated in the adult stage. However, the function of this upregulation in adult hippocampus is still unknown.

MEF2 is known for its role in neuronal survival. Activity induced calcium influx into cerebellar granule neurons *in vitro* will activate MAPK p38 to phosphorylate MEF2 at serine 387. Phosphorylated MEF2 stimulates MEF2-dependent genes for neuronal survival (117). After DNA damage, ataxia telangiectasia mutated (ATM) kinase becomes activated to phosphorylate MEF2D at serine 259, 275, 294, and 314. Phosphorylation of MEF2D promotes neuronal survival through transcriptional activation of survival genes (118). Recently, massive apoptosis in the brain was observed in Mef2A/2C/2D triple knockout, but not Mef2A/D double knockout mice, suggesting that MEF2 redundantly regulates neuronal survival *in vivo* (119).

MEF2 could also promote neuronal excitatory synapse elimination. Activity-dependent calcium influx into cultured hippocampal neurons will activate calcium/calmodulin regulated phosphatase calcineurin to dephosphorylate MEF2A and 2D at serine 221, 255, and 408. Dephosphorylated MEF2 promotes the expression of genes required for synapse elimination, including Arc (activity-regulated cytoskeletal-

associated protein) and synGAP (synaptic RAS GTPase-activating protein) (120). Further studies identified Fragile X Mental Retardation Protein (FMRP) as a downstream target of MEF2 for excitatory synapse elimination (121). These studies indicated that MEF2 could regulate synapse number, but which specific Mef2 gene is involved is still controversial.

Since Mef2C was the most highly expressed Mef2 gene in hippocampus in the embryonic stage, two groups have studied the function of MEF2C in neurogenesis during embryonic and prenatal development. Stuart Lipton's group crossed Nestin-Cre mice to Mef2C floxed mice to delete Mef2C as early as E9.5 in NSPCs. They observed abnormal cortical organization, smaller soma size, and cognitive function (122). Meanwhile, Eric Olson's group utilized GFAP-Cre to remove Mef2C in GFAP positive neural progenitor cells and their progeny in development. They found more excitatory synapses and deficits in hippocampal-dependent learning in Mef2C cKO mice (123).

These studies revealed a significant role of MEF2 at embryonic stages. It is still unknown 1) if Mef2 genes also control the number of excitatory synapses in adult hippocampus; 2) if MEF2 controls other steps of adult neurogenesis besides synapse formation; and 3) which specific Mef2 gene(s) play a major role in adult hippocampal neurogenesis. These questions will be discussed in Chapter 5.

Translational potential of adult neurogenesis

Adult-generated hippocampal granule cell neurons incorporate into hippocampal circuitry and are implicated in many hippocampal functions, including learning and memory. The connection between adult-generated hippocampal neurons and the pathophysiology of neurological disorders, such as depression, addiction, schizophrenia, epilepsy, and Alzheimer's disease has also been highlighted (124-127). Therefore, it is

of great interest to identify approaches, such as stem cell transplantation and small molecule treatments that preserve or enhance neurogenesis and hippocampal function. Such manipulations could be useful in combating the diminished hippocampal neurogenesis and function that occur with aging and in certain pathological situations (128).

Stem cells have the potential to treat a variety of human neurological conditions, including acute spinal cord injury, neurodegeneration, stroke, and epilepsy (129). One strategy currently employed for stem cell therapy is exogenous stem cell transplantation(130). Currently, there are two types of stem cells used for transplantation. One is pluripotent human embryonic stem (ES) cells, but they have their own limitations, including heterogeneity and ethical issues (131). A complementary source for stem cell transplantation is multipotent adult NSCs, which can differentiate into the three major neural cell types. It has become a promising way for treating neurological disease.

As discussed above, epigenetics can control multiple stages of adult neurogenesis, thus, targeting epigenetic regulators by small molecules, or “Epi-drugs”, may provide a powerful method to modulate transplanted NSCs and enhance neurogenesis after spinal cord injury (SCI). This neurological injury is characterized by massive neuronal cell loss (132, 133). Neuronal replacement is one anticipated strategy to restore function after damage or injury in the nervous system. However, under pathological contexts, transplanted NSCs predominantly adopt glial fates, instead of generating neurons (134). Combining HDACi that regulate cell fate specification with stem cell therapy has become a promising strategy for neuronal replacement. In a recent study involving spinal cord injury, VPA was successfully used to coax transplanted NSCs to undergo a neuronal fate, resulting in restoration of hindlimb function in mice SCI (135).

Given the variety of situations where neurogenesis-increasing compounds might be employed, a large arsenal of novel candidate compounds will be needed besides HDACis. A promising route for identification of novel neurogenesis-increasing compounds is high-throughput screening of chemical libraries in stem cell-based assays. Via this route, several small molecules have been reported to increase the number or differentiation of neurons (136-138). Isoxazole 9 [Isx-9; N-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide] was particularly intriguing. Isx-9 was discovered from a NeuroD/GluR2-luciferase *in vitro* screening of a small molecule library in UT Southwestern. We have shown that Isx-9 can robustly trigger NSCs to differentiate into neuronal cells *in vitro* via a neurotransmitter-like evoked Ca^{2+} signal to activate CaMK, which leads to HDAC5 export, and MEF2 de-repression, and ultimately, activation of pro-neuronal genes critical for neurogenesis (136).

This seminal *in vitro* work with Isx-9 raised three questions that are the partial focus of Chapter 5 in this dissertation. First, does Isx-9 drive adult neurogenesis *in vivo* as the *in vitro* data suggest it might? *In vivo*, cells exist in many stages of adult hippocampal neurogenesis as described before. Correct progression through these stages of adult hippocampal neurogenesis depends on the complex environment of the neurogenic niche, which consists of embryonic-generated neurons, astrocytes, and vasculature, among other components. As this niche is largely absent from *in vitro* preparations, it is critical to assess whether the promising *in vitro* work with Isx-9 translates to increasing neurogenesis *in vivo*. Second, does Isx-9 improve hippocampal function? Prior work has shown that pharmacologically induced increases in hippocampal neurogenesis are linked to improvement in hippocampal function (137). We will address whether Isx-9 improves learning and memory using two hippocampal-dependent spatial tasks, the Morris water maze and fear conditioning. Third, does Isx-9's

ability to increase neurogenesis *in vivo* rely on intrinsic MEF2 signaling in adult-generated neurons as the *in vitro* data suggest it might? We will assess whether MEF2 is required to mediate Isx-9's *in vivo* effects and use inducible transgenic approaches to inducibly delete three brain-enriched Mef2 isoforms specifically from NSCs and their progeny.

In addition to neural stem cells, Isx-9 also shows strong differentiation effects on malignant astrocytes (139), pancreatic β cells (140), and Notch-activated epicardium-derived cells (NECs) (141). Through a screen of G_q protein-coupled receptor (G_qPCR) in NECs, the extracellular proton/pH-sensing GPCR GPR68 was identified as a potential target of Isx-9 (142). Further studies of GPR68 in hippocampus revealed its localization in the process of QNPs (143), indicating that Isx-9 may also target GPR68 in the brain.

Besides *in vitro* screening, chemical compounds were also infused directly into mouse brains for screening (137). One aminopropyl carbazole compound, named P7C3 was identified to increase neurogenesis through immunohistochemical staining of BrdU as readout of the number of neural progenitor cells. P7C3 is a neuroprotective molecule as it can block apoptosis of neural progenitor cells as well as mature neurons. This compound has great efficacy to recover the loss of neurogenesis in *npas3* (neuronal PAS domain protein 3) knockout mice. It has also been successfully applied to treat mouse models of neurological disease featuring loss of neurons, such as Parkinson disease and amytrophic lateral sclerosis (ALS) (144, 145). However, its targets and mechanisms were still unclear (146).

Summary and conclusions

In summary, ongoing neurogenesis in the adult brain is governed by three general mechanisms: (a) cell-extrinsic factors from the niche that signal in an autocrine

and/or paracrine fashion, (b) integration of niche signals by transcription factors to control NSC fate, and (c) fine-tuning of gene expression by epigenetic regulators that form a regulatory circuit with transcription factors. Each of these mechanisms and their associated networks are used to varying degrees during different stages of adult NSC self-renewal and differentiation. Despite the ability of the brain to activate selected pools of adult NSCs, these collective endogenous mechanisms are still inadequate to fully restore neuronal differentiation and function to the adult brain or spinal cord following damage or injury. We envision that more restoration of function to the nervous system after injury or during disease will benefit from a full understanding of the epigenetic and transcriptional control mechanisms. A set of therapeutic targets will involve strategies for preserving the NSC pool, directing NSCs and transit-amplifying cells to adopt a neuronal fate, suppressing glial fates, promoting differentiation and survival of immature neurons, or preventing the death of terminally differentiated neurons.

Chapter 2

Materials and Methods

Animal studies

All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (UTSW). Mice were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care at UTSW on a 12 hr light-dark cycle.

Hdac3 inducible conditional knockout (cKO) mice were generated by crossing Nestin-CreER^{T2}, Rosa-YFP line (From Dr. Amelia Eisch's lab) with Hdac3 floxed mice (From Dr. Eric Olson's lab, (147)). Mef2 inducible conditional triple knockout (iKO) mice were generated by crossing Nestin-CreERT2, Rosa-YFP line with mice carrying transgenes for Mef2A$^{F/F}$, Mef2C$^{F/F}$, and Mef2D$^{F/F}$ (From Dr. Eric Olson's lab (112, 119, 148)). As the breeding strategy did not allow production of offspring carrying wild-type (WT) alleles for Mef2a/c/d, mice with the genotype *Nestin-CreER*$^{T2+/-}$, *R26R-YFP*$^{+/-}$ were used as controls and referred to as Mef2-WT. Hdac5 knockout mice were generated by self-crossing heterozygous Hdac5 LacZ knockin mice (From Dr. Eric Olson's lab (94)). Genomic DNA extracted from tails of these mice was used for genotyping. *Nestin-CreER*^{T2}, *R26R-YFP*, *Hdac4, Hdac5* mice and *Hdac3*, *Mef2A*, *Mef2C*, and *Mef2D* floxed mice, and their genotyping have been described before (91, 149, 150).

Hdac3 cKO mice and *Mef2* iKO mice were given tamoxifen (TAM) intraperitoneally (i.p.) daily for 6 days (150 mg/kg dissolved in 10% ethanol (vol/vol) and 90% sunflower oil (vol/vol)) and sacrificed for analysis at indicated timepoints after the last injection. When the mice were given TAM, CreER^{T2} in nestin-expressing NSCs and their progeny translocates to the nucleus, allowing excision of coding exons of Hdac3 and Mef2.

Isoxazole 9 (Isx-9) was synthesized by Omm Scientific (Dallas, TX, USA). For *in vivo* administration, Isx-9 was prepared as 2 mg/ml of 30% Veh ((2-hydroxypropyl)-β-cyclodextrin, Sigma) in sterile milliQ-purified water. Veh (vol/vol) or Isx-9 (20mg/kg) was injected i.p. daily for 7 days after tamoxifen injection in Nestin-GFP, Mef2 WT, a/d KO, and a/c/d KO mice.

To label proliferating cells, mice received i.p. 5-bromo-2-deoxyuridine (BrdU, 150ml/kg, 10mg/ml of 0.9% NaCl, pH 7.4). To assess the effect of Isx-9 on survival of proliferating cells, mice received 2 injections of BrdU (6 hrs apart) 1 day before Veh or Isx-9 and were killed 30 days later. To assess the ability of Isx-9 to influence proliferation and differentiation, other mice received 1 injection of BrdU 1 day after receiving 7 days of daily Veh or Isx-9 and were killed 2 hrs or 30 days later.

Immunohistochemical staining

Immunohistochemical (IHC) staining was performed on either free-floating (FF) sections or slide-mounted (SM) sections. For FF staining, sections were washed twice with 1XTBS and then blocked with 3% normal donkey serum and 0.3% Triton X-100 in 1XTBS for 1 hour. For SM staining, sections were incubated in 0.01M citric acid (pH 6.0, 100 °C) for 15 min for antigen retrieval followed by 10 min in 1XPBS at room temperature. Treatment of 1% H_2O_2 for 30 min to block endogenous peroxidase was followed by 30 min blocking with 3% normal donkey serum and 0.3% Triton X-100. Following primary antibodies were used in this study: chicken-anti-GFP (1:15,000 SM, Aves Labs, Cat# GFP-1020), mouse-anti-GFAP (1:4000 FF, Millipore, Cat# MAB360), chicken-anti-GFAP (1:4000 FF, Millipore, Cat# AB5541), goat-anti-Sox2 (1:750 FF, Santa Cruz Biotechnology, Cat# sc-17320), rabbit-anti-Sox2 (1:750 FF, Millipore, Cat# AB5603), rabbit-anti-MEF2A (H-300) (1:500 SM, 1:1000 FF, Santa Cruz Biotechnology,

Cat# sc-10794), goat-anti-MEF2C (E-17) (1:500 SM, 1:1000 FF, Santa Cruz Biotechnology, Cat# sc-13266), mouse-anti-MEF2D (1:500 SM, 1:1000 FF, BD Biosciences, Cat# 610774), rabbit-anti-Ki67 (1:500 SM, Thermo Scientific, Cat# RM-9106), mouse-anti-Ki67 (1:500 SM, BD Pharmingen, Cat# 550609), guinea pig-anti-DCX (1:2000 FF, Millipore, Cat# AB2253), rabbit-anti-prox1 (1:1000 FF, Millipore, Cat# AB475), mouse-anti-NeuN (1:1000 FF, Millipore, Cat# MAB377), rabbit-anti-HDAC3 (1:1000, Santa Cruz Biotechnology, Cat# sc-11417), goat-anti-MCM2 (1:500, Santa Cruz Biotechnology, Cat# sc-9839), rabbit-anti-cleaved caspase-3 (1:500, Cell Signaling, Cat# 9661), rat-anti-BrdU (1:500, Accurate Chemical & Scientific, Cat# OBT0030G), goat-anti-DCX (1:4000, Santa Cruz Biotechnology, Cat# sc-8066). For double or triple labeling, primary antibodies were simultaneously incubated
(e.g., MEF2A/DCX, MEF2A/GFAP/Sox2, etc) and further processed for each antibody.

For FF staining, sections were incubated in primary antibodies for 2 days at 4°C, and washed with 1XTBS for three times before incubation in fluorescence-conjugated secondary antibodies (1:1000, Jackson ImmunoResearch) overnight at room temperature. Sections were then washed with 1XTBS for three times, mounted onto slides, and coverslipped with PVA/DABCO.

For SM staining MEF2A, MEF2C, and MEF2D, primary antibody incubation overnight at room temperature was followed with a fluorescent-tagged secondary antibody (1:200, Jackson ImmunoResearch) for 2 hours at room temperature. For YFP, and Ki67, an appropriate biotin-tagged secondary antibody (1:200, Jackson ImmunoResearch) was used for 1 hour at room temperature followed by ABC (Vector Laboratories, Cat# PK-6100) for 1 hour and Tyramide-Plus signal amplification (1:50, PerkinElmer Life Sciences, Cat# NEL701). Sections were washed with 1XTBS for three times, conterstained with DAPI (4,6-diamidino-2-phenylindole; 1:5,000, Roche, Cat#

236276), and coverslipped with PVA/DABCO.

Cell culture and immunocytochemical staining

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. Mouse primary NSPCs (neurospheres) from the hippocampus and lateral ventricle of P15 Hdac5 KO mice, 4-6 week old $Hdac3^{F/F}$, $Mef2A^{F/F}$, $Mef2C^{F/F}$, $Mef2D^{F/F}$, and $Mef2A^{F/F}2C^{F/F}2D^{F/F}$ mice were isolated as previously described (151) and were grown in DMEM/F-12 supplemented with N2 (Invitrogen), B27 (Invitrogen), glutamine (Omega Scientific), and PSF (Invitrogen) in the presence of EGF (Peprotech Inc., 20 ng/mL), FGF2 (Peprotech Inc., 20 ng/mL), and heparin (Sigma, 5 μg/mL). To induce neuronal differentiation in HCN cells or mouse neurospheres, RA (1 μM) and FSK (5 μM) were added for 4 days. In some HCN experiments, HDAC3 inhibitor (10 μM) or VPA (1 mM) was used. For BrdU studies in HCN cells or mouse neurospheres, 10 μM BrdU was added to the medium 1 hr before 4% paraformaldehyde (PFA) fixation to assess proliferation. Immunocytochemical staining of BrdU requires pretreatment of 2N HCl at 37°C for 30 min and 0.1 M borate buffer for 10 min at room temperature. The following primary antibodies were used for staining overnight at 4°C: rat-anti-BrdU (1:500, Accurate Chemical & Scientific), mouse-anti-Tuj1 (1:1000, Sigma), and chicken-anti-GFP (1:2000, Aves Labs). Cells were washed with 1XTBS for three times, and incubated in secondary antibodies (1:200, Jackson ImmunoResearch). Cells were washed with 1XTBS for three times, counterstained with DAPI (4,6-diamidino-2-phenylindole, 1:5000, Roche), and coverslipped with PVA/DABCO. To delete HDAC3 or Mef2 *in vitro*, *Hdac3* or *Mef2* floxed neurospheres were trypsinized with 0.05% trypsin

(Corning Cellgro), infected with GFP (control) or Cre-GFP adenovirus (1:10,000 of 1 X 10^{10} pfu/mL stock, University of Iowa), and plated on coated plates for 2 days. To determine cell survival rate, the MTS assay was performed with HCN cells according to manufacturer's protocol (Promega).

Cell cycle analysis

HCN cells were synchronized in G1/G0 phase by adding BMP4 (50 ng/mL, R&D Systems) to proliferation medium for 1 day and in M phase by addition of nocodazole (2 μg/mL, Sigma) to proliferation medium for 16 hrs, before releasing in either HDAC3i or DMSO containing proliferation medium. Cells were then collected, centrifuged, washed, and resuspended in 70% ethanol for fixation. For DNA content analysis, cells were stained with propidium iodide (20 μg/mL, Sigma), and RNAse A (200 μg/mL, Invitrogen). Cell cycle was monitored on the FACScalibur flow cytometer (BD Biosciences) with 10,000 events/determination and analyzed with Flowjo software (Tree Star Inc.).

FACS and microarray

One day after 7 days of Veh or Isx-9 injections, the hippocampi of adult Nestin-GFP mice (4 mice/group) were prepared for cell sorting **(29)**. Briefly, the hippocampi were microdissected and enzymatically digested into a single-cell suspension. Cell sorting was performed using a MoFlo machine (Dako,Carpinteria, CA, USA). GFP-expressing, live cells were separated from GFP-negative, dead cells using standard parameters of forward- and side-scattering. Approximately 8000 live, GFP-expressing cells per group were sorted directly into TriZol (Invitrogen, Carlsbad, CA, USA), with multiple, separate groups of mice being run via FACS.

RNA was isolated using a standard phenol-chloroform extraction. The isolated RNA was amplified in a linear fashion to cDNA using 2 rounds of amplification of the

MessageAmp II aRNA kit (Ambion, Austin, TX, USA). Amplified cDNA was used for a single microarray analysis (Affymetrix GeneChip Mouse Genome 430 2.0 array; Affymetrix, Santa Clara, CA, USA) using standardized protocols of the US National Institutes of Health Neuroscience Microarray Consortium (Duke University Medical Center, Durham, NC, USA), which included loading via nanodrop and use of GAPDH as an internal control. Data analysis was performed with Partek Genomics Suite (Partek, St. Louis, MO, USA) and Ingenuity software (Ingenuity Systems, Redwood City, CA, USA). When compared with Veh-treated mice, only genes with 2-fold higher or 0.5-fold lower expression were considered significantly up- or down-regulated. Comparative analyses on biofunctions were performed using Ingenuity Pathway Analysis (Ingenuity Systems) using Fisher's exact test to calculate the *P* values.

Quantitative RT-PCR

Total RNAs were extracted using TRIzol (Invitrogen) according to manufacturer's protocol. cDNAs were synthesized from 1 μg of total RNA with a SuperScript III kit (Invitrogen). Quantitative PCR was performed with an ABI 7000 thermocycler (Applied Biosystems). Normalization was based on the expression of *GAPDH* and relative gene expression was determined by the ΔΔCT method. Primer sequences are available upon request.

Plasmids, lentivirus production, transfection, and electroporation.

A shRNA sequence targeting mouse HDAC3 was cloned into lentiviral pllu2g vector: 5'-tgatgatcgtcttcaagcctttcaagagaaggcttgaagacgatcatctttttt-3'. Lentivirus was made as previously described (152). HCN cells were infected at a multiplicity of infection (MOI) of 10 particles per cell in N2, glutamine, and PSF containing DMEM/F-12 medium with FGF2 for 3 days. Mouse *Hdac3* cDNA, *Hdac5* cDNA, HA-tagged *Cdk1* cDNAs, and

Flag-tagged *ubiquitin* were cloned into a retroviral pCAG-IRES-GFP vector. Mouse *Hdac5* and *Hdac5* (S259A and S498A) cDNAs were cloned into a retrovirus pCAG-mcherry vector. All the plasmids were confirmed by DNA sequencing. HEK293T cells were transfected using Lipofectamin 2000 (Invitrogen) according to manufacturer's protocol. Electroporation was performed with an Amaxa electroporator at a ratio of 5 μg DNA per 5 million HCN cells. Cells were maintained in proliferation condition and collected for western blot or immunoprecipitation 3 days post electroporation. Proteasome inhibitor MG132 (10 μM) was added 12 hrs before harvesting cells.

Immunoprecipitation and 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 and cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) for immunoprecipitation. These buffers were supplemented with phenylmethanesulfonyl fluoride (PMSF), a cocktail of protease (Roche) and phosphatase inhibitors (Sigma). To detect ubiquitination, 10 mM ubiquitination inhibitor N-ethylmaleimide (NEM) was added.

Cells were rocked in cold room for 45 min 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), 500 μg protein samples were used for co-immuoprecipitation. Five μg HDAC3 or CDK1 antibodies were added to the protein samples and incubated at 4°C overnight, followed by 1 hr incubation with 50 μL protein G or A sepharose (GE healthcare) beads. The beads were rinsed by cell lysis buffer for 3 times and boiled in 2 X SDS loading buffer for 5 min at 95°C.

After a brief centrifugation, supernatants were loaded onto 4-12% SDS PAGE gels for western blotting. Western blotting was performed using standard protocols with these primary antibodies: mouse-anti-CDK1 (1:1000, Santa Cruz Biotechnology), rabbit-anti-phospho-CDK1 (Tyr15) (1:1000, Cell Signaling), rabbit-anti-cyclin B1 (1:1000, Cell Signaling), mouse-anti-cyclin A (1:1000, Cell Signaling), rabbit-anti-CDK4 (1:1000, Cell Signaling), mouse-anti-cyclin D1 (1:1000, Cell Signaling), rabbit-anti-phospho-Histone H3 Ser10 (1:1000, Cell Signaling), rabbit-anti-acetyl-histone H4 (1:10,000, Millipore), rabbit-anti-acetyl-H4K12 (1:1000, Millipore), rabbit-anti-acetyl-H4K12 (1:1000, Millipore), rabbit-anti-Histone H4 (1,10,000 Millipore), rabbit-anti-HDAC3 (1:1000, Santa Cruz Biotechnology), rabbit-anti-Mef2A (H-300) (1:1000, Santa Cruz Biotechnology), goat-anti-Mef2C (E-17) (1:1000, Santa Cruz Biotechnology), mouse-anti-Mef2D (1:1000, BD Biosciences), mouse-anti-GAPDH (1:10,000, Millipore), mouse-anti-beta-actin (1:1000, Santa Cruz Biotechnology), rabbit-anti-acetyl-lysine (1:1000, Cell Signaling), mouse-anti-HA (1:10,000, Sigma), mouse-anti-Flag (1:10,000, Sigma), and rabbit-anti-ubiquitin (1:500, Santa Cruz Biotechnology). HRP-conjugated (Cell Signaling) or AP-conjugated (Santa Cruz Biotechnology) secondary antibodies were used. Immunoblots were developed by an ECL-plus kit (GE Healthcare) or detected by a BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laborotories).

Morris water maze

To assess the effects of Isx-9 on learning and memory, Morris water maze was used. For MWM, at 3 weeks after the last injection of Veh or Isx-9, mice (15/group) were trained to find a submerged platform in a pool of opaque water (diameter 150cm). Mice received 4 training trials/day (intertrial interval 30-45 min) for 10 days. In all trials, mice were allowed to swim until they landed on the platform or 60 seconds had elapsed. On day 11, a probe test was conducted in the morning with the platform removed from the

pool. The swim path, time to platform, time in each quadrant, and total distance were recorded during the training days. Results are presented as mean ± SE. For the training days, repeated-measure 2-way ANOVA (GraphPad Prism, GraphPad Software, San Diego, CA, USA) with Bonferroni *post hoc* test was used with treatment as a within-subject factor and time as a between-subject factor. For the probe test, an unpaired *t* test was used.

Statistical analysis

Data are represented as mean ± SEM. Statistical comparisons were assessed with unpaired, two-tailed Student's *t*-test. A p value < 0.05 was considered significant. All statistical analyses were performed on Graphpad Prism 5 software.

Chapter 3

HDAC3 controls G2/M progression in adult neural/stem progenitor cells by regulating CDK1 levels

As described in Chapter 1, HDAC3 is highly expressed in the brain (83) and has documented roles in learning and memory associated with cocaine-seeking behavior (84), its role in adult NSPCs is largely unknown. Here, we investigate the function and mechanism of HDAC3 in adult NSPCs. From HDAC3 loss of function experiments *in vitro* and *in vivo*, we identified that HDAC3 is required for the proliferation of adult NSPCs. We further determined that HDAC3 controls G2/M phase progression in adult NSPCs by blocking ubiquitination and degradation of CDK1. These data reveal novel insights regarding the key role of HDAC3 in maintaining the adult NSPC pool. The data presented in this chapter has been accepted in manuscript form to *PNAS*.

HDAC3 is required to promote the proliferation of adult NSPCs *in vitro*.

To rapidly determine whether HDAC3 is required for adult NSPCs fate decision *in vitro*, we first designed a short hairpin RNA (shRNA) plasmid to knockdown HDAC3 (Fig. 3.1*A*). The HDAC3 shRNA was packaged into a lentivirus vector expressing internal ribosome entry site (IRES)-EGFP and used to infect a line of previously characterized adult rat hippocampal neural progenitor cells (HCN cell line) (153). Expression of HDAC3-shRNA-IRES-EGFP resulted in fewer GFP+ HCN cells incorporating bromodeoxyuridine (BrdU), compared to control IRES-EGFP infected cells (Fig. 3.1*B*).

As a complementary approach, we obtained an HDAC3-selective inhibitor (HDAC3i) to test in HCN cells (154). HDAC3i shows ten times higher selectivity against HDAC3 relative to HDAC1 and HDAC2 (154). It has also been used to investigate the role of HDAC3 on cocaine-seeking behavior in mice (84). To determine the optimum

dosage for HDAC3i in HCN cells, we performed the MTS assay to determine its cytotoxicity as well as performed western blotting against acetyl-histone H4 lysine 12(ac-H4K12) at different concentrations as a biological readout (Fig. 3.1*C*-*D*). We found that 10 μM minimized cytotoxicity and maximized biological activity. Thus, 10 μM HDAC3i was used to treat HCN cells for 1 day (Fig. 3.1*E*). We performed immunostaining of BrdU after adding BrdU for 1 hr before fixing the cells. Consistent with the effects of knocking down HDAC3 with an shRNA, HCN cells treated with an HDAC3i also showed significant defects in proliferation as indicated by the decreased number of BrdU+ cells.

To confirm that HDAC3 is required for neural stem/progenitor proliferation in multiple rodent species, we also used established primary NSPCs ("neurospheres") from the hippocampus and subventricular zone (SVZ) of 1-month old *Hdac3* wildtype (HDAC3 WT) or floxed (HDAC3 F/F) mice (150). First, we confirmed that Adenovirus (Ad) Cre-GFP infection of *Hdac3* floxed neurospheres could efficiently knockout HDAC3 levels (Fig. 3.1*F*). Using BrdU exposure (1 hr) after 2 days of Ad-Cre-GFP infection, we observed a reduction of proliferation in *Hdac3* floxed neurospheres as indicated by fewer BrdU and GFP double positive cells (Fig. 3.1*G*). Moreover, we did not observe a significant effect on differentiation, at least under neuronal differentiation conditions (Fig. 3.1*H*). Taken together, we showed in primary mouse NSPC cultures and an adult rat hippocampal NSPC line, that the major effect of deletion or inhibition of HDAC3 is a proliferation defect *in vitro*.

Adult NSPCs lacking HDAC3 display specific G2/M phase defects.

Previous studies showed that HDAC3 could regulate the G1/S phase transition in HeLa cells (82), but was only required for S phase progression in mouse embryonic

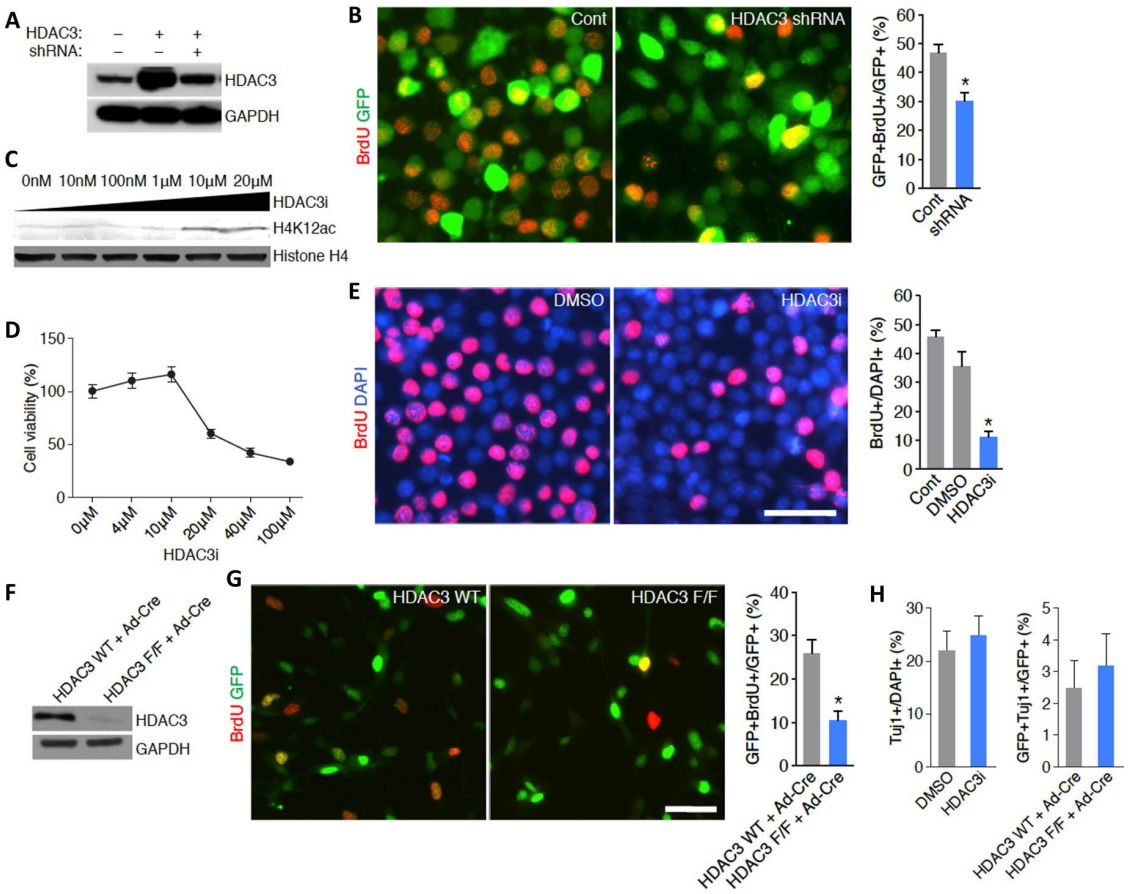


Figure 3.1. Loss of HDAC3 results in decreased proliferation *in vitro*. (*A*) Western blot of lysates from HEK293T cells cotransfected with HDAC3 overexpression plasmid and pllu2g or pllu2g-HDAC3-shRNA plasmid, probed with an HDAC3 antibody. GAPDH was used as a loading control. (*B*) Immunocytochemical staining of BrdU following by 1 hr BrdU pulse labeling in HCN cells infected with Control (Cont) or HDAC3-shRNA-IRES-EGFP lentivirus for 3 days. Quantification of GFP+BrdU+ cells out of the total GFP+ cells is shown. (*C*) Western blot of lysates from HCN cells treated with HDAC3i at different concentrations and probed with an H4K12ac antibody. Histone H4 was used as a loading control. (*D*) MTS assay of HCN cells treated with HDAC3i at different concentrations. (*E*) BrdU staining of 1 hr BrdU pulse labeling in HCN cells treated with either vehicle (DMSO) or HDAC3 inhibitor for 1 day. Quantification of BrdU+ cells out of the total DAPI+ cells is shown. (*F*) Western blot of lysates against HDAC3 from WT and HDAC3 F/F neurospheres treated with Ad-Cre-GFP virus for 2 days. GAPDH was used as a loading control. (*G*) *Hdac3* wildtype (HDAC3 WT) or floxed (HDAC3 F/F) neurospheres were infected with Ad-Cre-GFP virus for 2 days. Immunocytochemical staining of GFP and BrdU following 1 hr BrdU pulse labeling reveals a decrease in the

percentage of BrdU+GFP+ double positive cells out of the total GFP+ cells in HDAC3 F/F neurospheres. (*H*) Left: percentage of Tuj1+ cells out of the total DAPI+ cells in HCN cells treated with either DMSO or HDAC3i in neuronal differentiation medium (RA/FSK) for 4 days. Right: percentage of GFP+Tuj1+ cells out of the total GFP+ cells in HDAC3 WT or F/F neurospheres infected with Ad-Cre virus in neuronal differentiation medium for 4 days. For *B*, *E*, and *G*: * $p < 0.05$. For *B* and *E*: scale bar, 200 μm. For *G*: Scale bar, 100 μm. All experiments were performed at least three independent times.

fibroblast (MEF) cells (155), suggesting that HDAC3 has distinct roles on cell cycle progression in different cell types. To gain further insight into the role of HDAC3 in NSPC proliferation, specifically, in regulating cell cycle progression, we performed cell cycle analysis following propidium iodide (PI) staining of WT and *Hdac3* floxed neurospheres. After infection with Ad-Cre-GFP, HDAC3 deleted neurospheres showed decreased percentage of cells in S phase and increased percentage of cells in G2/M phase compared to WT neurospheres (Fig. 3.2*A*-*B*).

To further confirm the role of HDAC3 in cell cycle progression in adult NSPCs, we treated HCN cells with HDAC3i for 1 day prior to cell cycle analysis. Compared to cells treated with vehicle (DMSO), HDAC3i treated cells exhibited a decrease in S phase and an increase in G2/M phase (Fig. 3.2*C*). To determine whether decreased S phase and increased G2/M phase was a general property of blocking HDACs, we treated HCN cells with the broad HDACi valproic acid (VPA) for 1 day. Interestingly, we observed an increase in G1/G0 phase and a decrease in S phase, with no change in G2/M phase (Fig. 3.2*C*), suggesting that individual HDACs have divergent effects on cell cycle progression.

To determine the primary cause of the observed cell cycle defects after HDAC3 inhibition, we performed cell cycle synchronization in HCN cells using various molecules

to enrich cells in either G1/G0 or G2/M phase. We synchronized HCN cells in G1/G0 phase after addition of bone morphogenic protein 4 (BMP4) to proliferation medium (fibroblast growth factor 2 [FGF2]) for 1 day (43) (Fig. 3.2*D*-*E*). To release HCN cells from G1/G0, BMP4 was withdrawn and cells were treated with either HDAC3i or dimethyl sulfoxide (DMSO) in proliferation medium. To confirm HCN cells were effectively enriched in G1/G0 and could be released back into cell cycle, we performed western blotting with known cell cycle proteins (Fig. 3.2*D*). We observed that both DMSO- and HDAC3i-treated HCN cells entered into S phase with no lag at 12 hr after withdrawal of BMP4, indicating that HDAC3 is not required for G1/S phase transition and entry into S phase (Fig. 3.2*E*).

To examine whether HDAC3 is required for M phase progression, we enriched HCN cells in M phase by treating with nocodazole for 16 hrs before releasing them into HDAC3i or DMSO containing proliferation medium (Fig. 3.2*F*-*G*). To confirm HCN cells were effectively enriched in G2/M and could be released back into cell cycle, we performed western blotting with known cell cycle proteins (Fig. 3.2*F*). Twelve hrs after release from nocodazole, most of the cells treated with HDAC3i were still in G2/M phase, while control-treated cells have entered into G1/G0 phase, similar to cells in proliferation conditions (Fig. 3.2*G*). These data suggest that HDAC3 is required for G2/M phase progression in adult NSPCs and the proliferation defects observed after HDAC3 inhibition primarily due to an impairment of G2/M phase progression.

CDK1 is selectively degraded after loss or inhibition of HDAC3 in adult NSPCs.

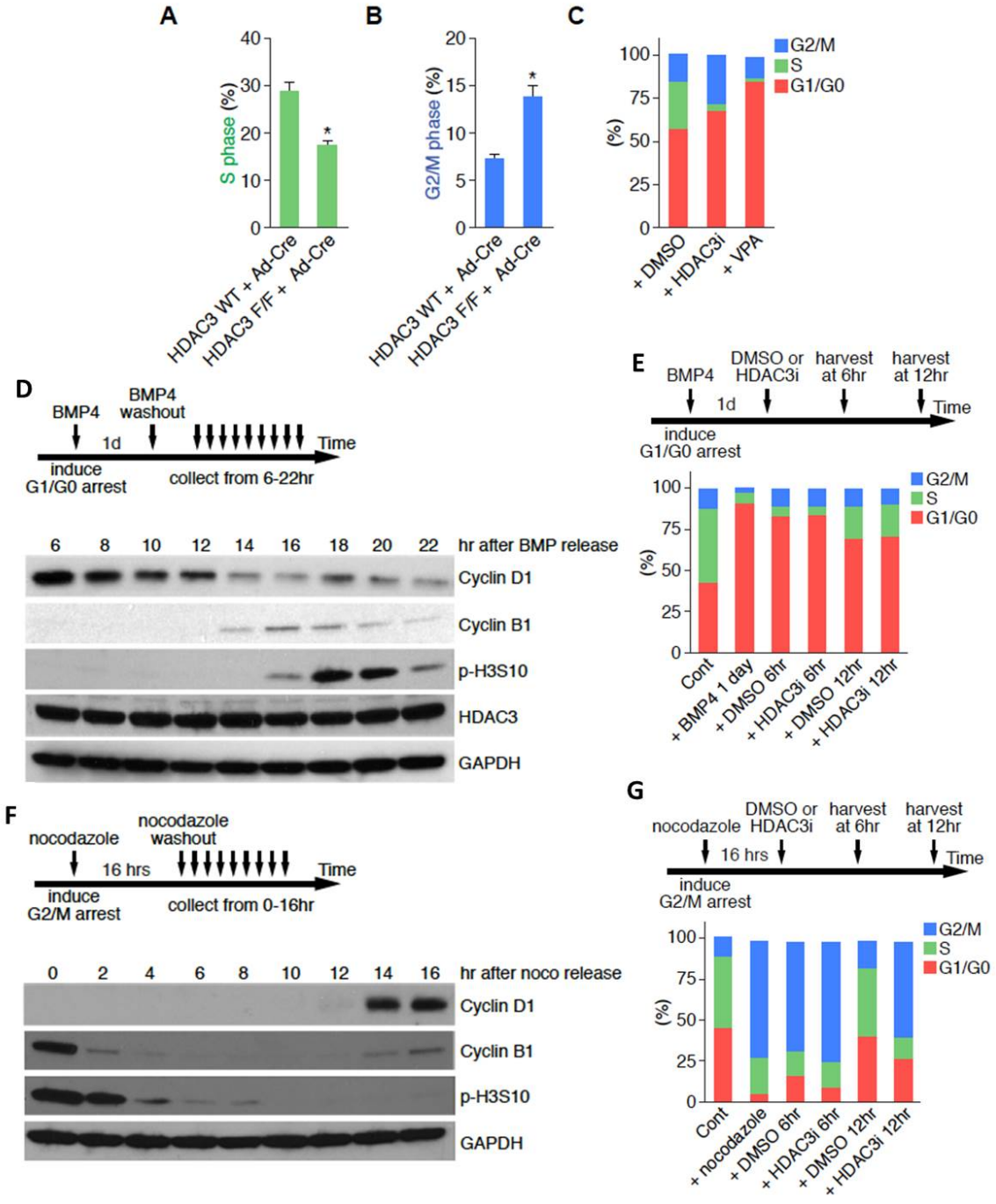


Figure 3.2. HDAC3 controls G2/M phase progression. (*A-B*) Flow cytometry analysis of HDAC3 WT and F/F neurospheres infected with Ad-Cre-GFP virus for 2 days before harvesting and being fixed in 70% ethanol. Quantification of the percentage of cells in S and G2/M phase is shown. (*C*) Cell cycle analysis of HCN cells treated with HDAC3i or VPA for 1 day. The percentage of each cell cycle stage from one representative experiment is shown in bar graph. (*C*) Western blot of lysates from HCN cells released

from G1/G0 into proliferation medium (FGF2) for different lengths of time, and probed with antibodies against HDAC3, G1/S enriched Cyclin D1, G2/M enriched Cyclin B1, and p-H3S10. (*E*) HCN cells were synchronized in G1/G0 phase by addition of BMP4 in proliferation medium for 1 day before switching to proliferation medium containing either DMSO or HDAC3i. Cells were collected at 6 hr and 12 hr and analyzed by flow cytometry. The percentage of each cell cycle stage from one representative experiment is shown in bar graph. (*F*) Western blot of lysates from HCN cells released from G2/M into proliferation medium (FGF2) for different lengths of time, and probed with antibodies against G1/S enriched Cyclin D1, G2/M enriched Cyclin B1, and p-H3S10. GAPDH was used as a loading control. (*G*) HCN cells were synchronized in M phase by addition of nocodazole in proliferation medium for 16 hrs before switching to proliferation medium containing either DMSO or HDAC3i. Cells were collected at 6 hr and 12 hr and analyzed by flow cytometry. The percentage of each cell cycle stage from one representative experiment is shown in bar graph. For A-B: * $p < 0.05$. All cell cycle experiments were replicated at least three independent times.

In order to understand the mechanism by which HDAC3 controls G2/M phase progression, we performed western blotting to detect G2/M phase enriched cyclins and cyclin-dependent kinases (CDKs) in both primary neurospheres and HCN cells. First, we observed decreased HDAC3 levels and increased acetylated histone H4 as expected after Ad-Cre-mediated deletion of HDAC3 in HDAC3 F/F neurospheres (Fig. 3.3*A*). We also observed a decrease in the levels of G2/M CDKs and cyclins, such as CDK1, phospho-CDK1, and cyclin B1, but no change in the levels of the G1/S cyclin A (Fig. 3.3*A).* Treatment with HCN cells with HDAC3i also resulted in decreased CDK1, phospho-CDK1, and cyclin B1 (Fig. 3.3*B*). Interestingly, in both HDAC3 F/F neurospheres and HCN cells, we found an increase in the mitosis marker phospho-histone H3S10 (p-H3S10) after HDAC3 deletion or HDAC3i treatment, consistent with the increased percentage of cells in G2/M phase (Fig. 3.3*A*-*B*).

To gain further mechanistic insight regarding HDAC3, we next examined whether the decline of CDK1 and cyclin B1 levels after HDAC3 deletion or inactivation was due to its roles as an epigenetic repressor of gene transcription. Thus, we used quantitative PCR (qPCR) to investigate a panel of cell cycle genes and found that only *cyclin B1* mRNA levels was reduced after deletion of HDAC3 in neurospheres (Fig. 3.3*C*). Besides deacetylation of nuclear histone tails, HDAC3 has been described to shuttle between the nucleus and cytoplasm and may also have non-histone protein targets (156). To examine whether reduced CDK1 levels after loss of HDAC3 occurred through post-translational mechanisms to control protein stability, we measured CDK1 levels by treating HCN cells with HDAC3i and a protein synthesis inhibitor cycloheximide (CHX) (Fig. 3.3*D-E*). Compared to CDK1 levels in cells treated with HDAC3i alone which decreased by 1 day, we observed decreased CDK1 levels at 12 hr, suggesting that CDK1 has a shorter half-life after HDAC3 inhibition and that control of CDK1 levels by HDAC3 occurred at the post-translational level.

Ubiquitination is the most common post-translational modification to control protein stability (157). To explore whether CDK1 levels in adult NSPCs is subjected to ubiquitin-mediated protein degradation, we first tried to block the proteasome pathway by treating HCN cells with a proteasome inhibitor MG132 together with HDAC3i for 1 day. We observed selective loss of CDK1 after HDAC3i treatment in HCN cells, which was prevented by MG132 treatment, without a change in cyclin A or CDK4 levels (Fig. 3.3*F*). Interestingly, in non-NSPCs such as human HEK 293T cells and Ink4a/Arf knockout mouse glioma cell (SS05 cell line) (139), we did not observe any downregulation of CDK1 after HDAC3i treatment (Fig. 3.3*G*), suggesting that the HDAC3/CDK1 ubiquitination pathway may be selective for NSPCs.

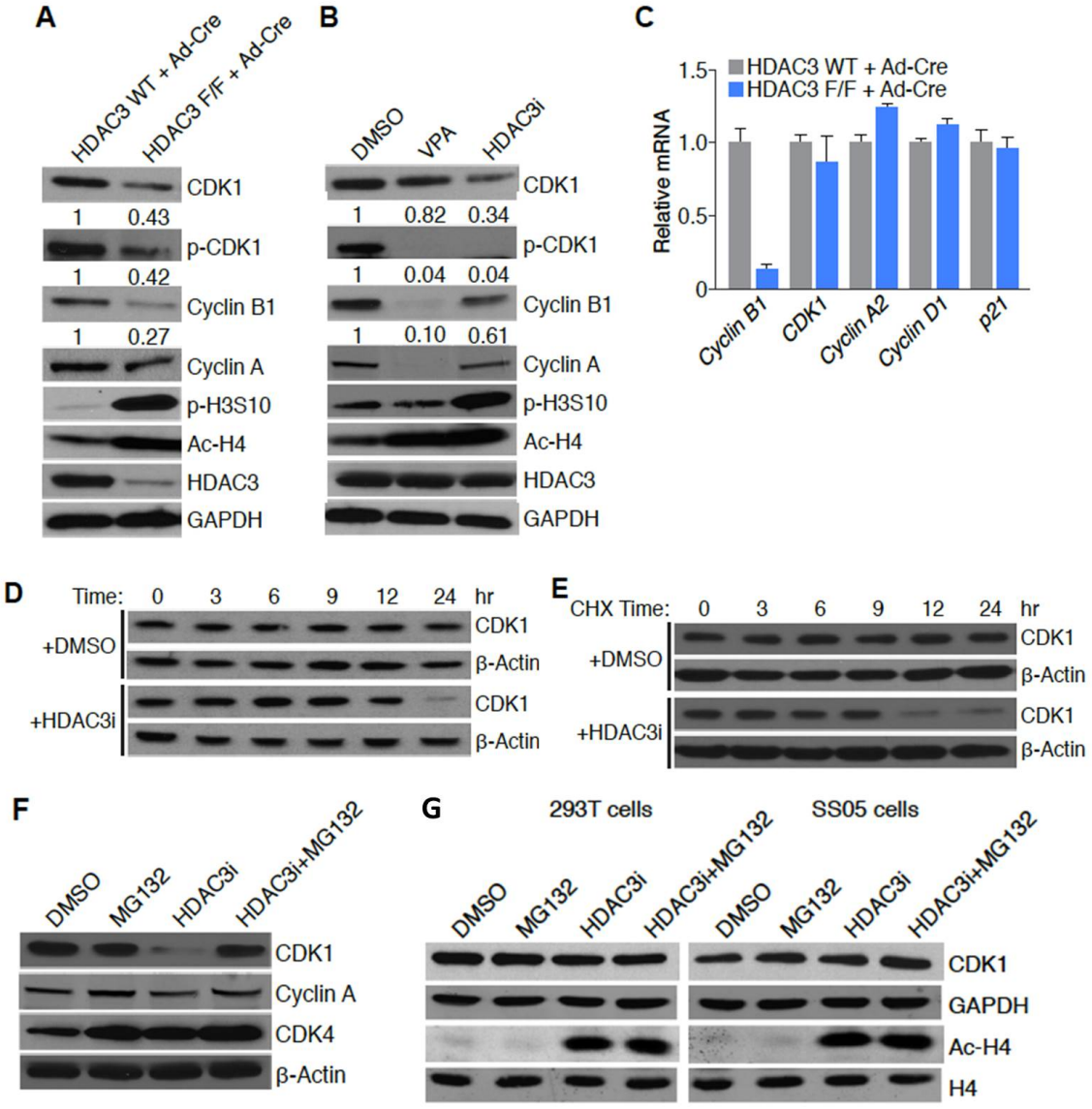


Figure 3.3. G2/M phase-specific protein CDK1 is downregulated after HDAC3 deletion. (*A*) Western blotting against G2/M phase cell cycle proteins in WT and HDAC3 F/F neurospheres infected with Ad-Cre-GFP virus for 2 days and in (*B*) HCN cells treated with DMSO, VPA, or HDAC3i for 1 day. Levels of CDK1, p-CDK1, and Cyclin B1 were quantified. (*C*) qPCR of several cell cycle genes in WT and HDAC3 F/F neurospheres infected with Ad-Cre-GFP virus. These qPCR assays were repeated three independent times. (*D-E*) Western blotting against CDK1 in HCN cells treated with DMSO or HDAC3i together with protein synthesis inhibitor cycloheximide (CHX) for different length of time. (*F*) Western blot of lysates from HCN cells treated with vehicle (DMSO), MG132, HDAC3i, or HDAC3i + MG132. (*G)* Western blotting against CDK1 for

lysates from HEK293T cells or SS05 cells treated with MG132, HDAC3i, or HDAC3i+MG132.

To examine CDK1 ubiquitination in NSPCs, we first treated HCN cells with MG132 and DMSO or HDAC3i, followed by immunoprecipitation of CDK1 and western blotting against ubiquitin. We observed heavily ubiquitinated bands in HDAC3i-treated cells, indicating that CDK1 can be ubiquitinated in HCN cells (Fig. 3.4*A*). These results suggested that CDK1 might be degraded through the ubiquitination pathway after loss or inhibition of HDAC3 in NSPCs.

To further examine whether HDAC3 controls ubiquitination of CDK1, we overexpressed HA-tagged CDK1 and Flag-tagged ubiquitin with or without a cDNA overexpressing full-length HDAC3 in HCN cells (Fig. 3.4*B*). Following immunoprecipitation of CDK1 and western blotting against ubiquitin, we observed reduced CDK1 ubiquitination after HDAC3 overexpression, indicating that HDAC3 is sufficient to prevent ubiquitination of CDK1 in adult NSPCs.

Two possible mechanisms by which HDAC3 regulates ubiquitination and stability of CDK1 are through regulation of ubiquitin-related genes (158) or through physical interaction and deacetylation of CDK1. To examine the first possibility, we examined the levels of Ubqln2, Ube2e3, Ube2K, Usp18, Usp28, and Sumo2 and did not detect any differences in gene expression between HDAC3 WT and KO neurospheres (Fig. 3.4*C*). Next, to examine a possible interaction between HDAC3 and CDK1, we immunoprecipitated CDK1 and performed western blotting with HDAC3 in HCN cells (Fig. 3.4*D*). Since HDAC3 could interact with CDK1, we hypothesize that CDK1 may be deacetylated by HDAC3. To examine this, we immunoprecipated CDK1 and performed western blotting with the pan acetyl-lysine antibody (Fig. 3.4*E*). We observed

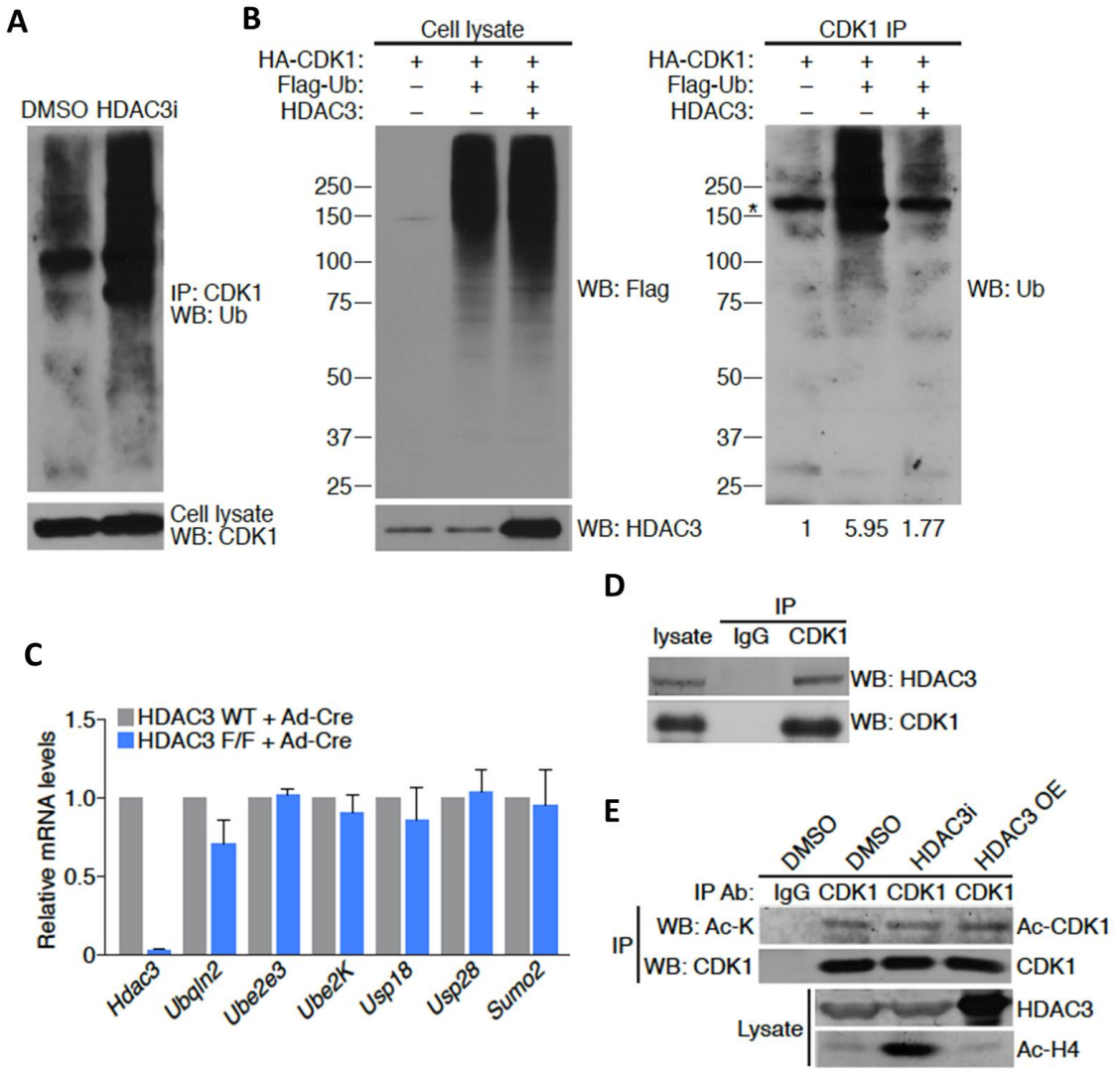


Figure 3.4. HDAC3 controls CDK1 stability by regulating ubiquitin levels. (*A*) Immunoprecipitation of CDK1 and western blotting against ubiquitin in HCN cells treated with MG132 and DMSO or HDAC3i. Western blotting of CDK1 from cell lysate is shown in bottom panel. (*B*) Left panel: western blotting against Flag in HCN cells co-electroporated with HA-CDK1, Flag-ubiquitin (Ub), and HDAC3. Western blotting against HDAC3 from cell lysate is shown in bottom left panel. Right panel: immunoprecipitation of CDK1 and western blotting against ubiquitin in HCN cells co-electroporated with HA-CDK1, Flag-Ub, and HDAC3 overexpression plasmids. Levels of ubiquitinated CDK1 were quantified. * denotes a non-specific band. (*C*) qPCR of several ubiquitination related genes in HDAC3 WT and F/F neurospheres infected with Ad-Cre-GFP virus. These qPCR assays were repeated three independent times. (*D*) Immunoprecipitation of

CDK1 and western blotting against HDAC3 in HCN cells. (*E*) Immunoprecipitation of CDK1 and western blotting against acetyl-lysine in HCN cells treated with DMSO, HDAC3i, or electroporated with HDAC3 overexpression plasmids. All the western blot experiments were replicated three independent times.

comparable levels of CDK1 acetylation after HDAC3i treatment or overexpression of HDAC3 compared to DMSO treatment (controls) (Fig. 3.4*E*), suggesting that HDAC3 is not necessary for CDK1 acetylation. Taken together, these results suggest that HDAC3 plays a role in stabilizing CDK1 by regulating ubiquitin levels for proper G2/M phase progression in adult NSPCs.

HDAC3 is broadly expressed in adult NSPC and neuronal populations.

We have shown that HDAC3 is required for the proliferation of NSPCs *in vitro*. To begin to investigate the role of HDAC3 during the course of adult neurogenesis *in vivo*, we determined the expression pattern of HDAC3 in adult mouse brain, particularly within the SGZ of the dentate gyrus. We performed immunohistochemical staining using an antibody recognizing HDAC3 together with stage-specific markers. Radial glia-like cells (RGLs also known as Type 1 cells) can be identified using a set of immunohistological markers and morphological criteria (24). We observed HDAC3 expression within glial fibrillary acidic protein (GFAP)+Sox2+ RGL and GFAP-Sox2+ TAP stem/progenitor cells (Fig. 3.5*A*). We quantified the percentage of RGL cells that expressed HDAC3 and found HDAC3 is expressed in a large proportion of RGLs (94.1 ± 3.6%). We also detected HDAC3 in doublecortin (DCX)+ immature dentate granule cells as well as in postmitotic NeuN+ neurons throughout hippocampus and cortex (Fig. 3.5*B*-C). Moreover, HDAC3 was found in MCM2+ proliferating cells in SGZ, consistent with its possible role in regulating proliferation *in vivo* as it does *in vitro* (Fig. 3.5*D*). Due to the broad expression of HDAC3 in adult NSPC and neuronal populations and since HDAC3 null mice are

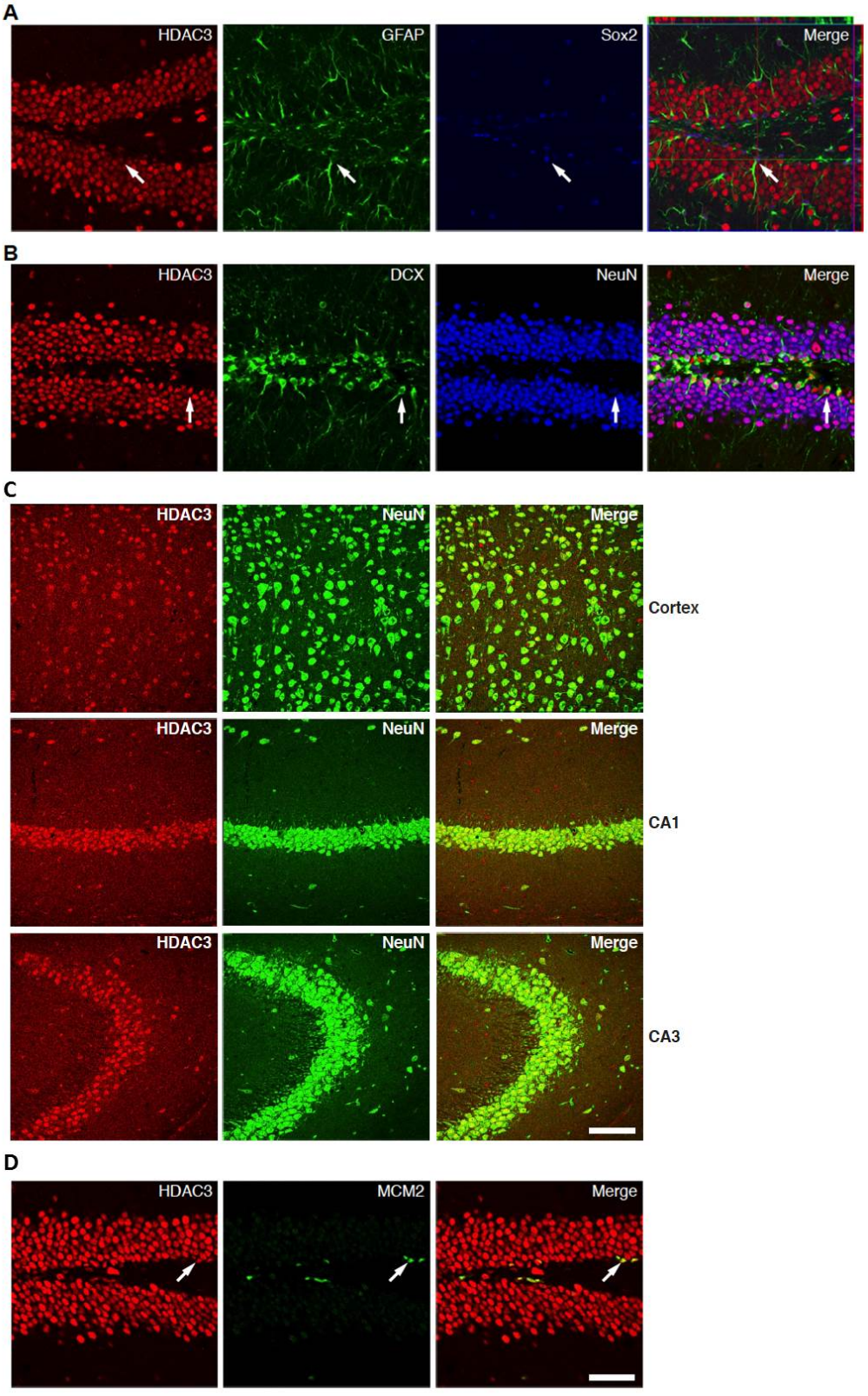


Figure. 3.5. HDAC3 is broadly expressed in adult dentate gyrus. (*A*) Immunostaining of HDAC3, GFAP (a marker of RGLs), and Sox2 (a marker of RGLs and TAPs) of brain

sections from P30 WT mice. HDAC3 is expressed in GFAP+Sox2+ RGL cells (arrow). (*B*) Immunostaining of HDAC3, DCX (marker of immature neurons), and NeuN (marker of mature neurons). HDAC3 is expressed in both DCX+NeuN- immature neurons (arrow) and DCX-NeuN+ mature neurons. (*C*) Immunostaining of HDAC3 and MCM2 (a marker of proliferation). Arrow indicates colocalization of HDAC3 and MCM2. (*D*) Immunostaining of HDAC3 and NeuN (a marker of mature neurons) in cortex, CA1, and CA3. For *A*-*D*: scale bar, 50 μm. One representative image from three independent staining is shown.

reported to die at embryonic stage 9.5 (E9.5) due to gastrulation defects (150), we decided to employ an inducible conditional knockout (cKO) strategy to determine the cell autonomous requirement of HDAC3 in adult NSPCs *in vivo*.

Conditional knockout of HDAC3 results in reduced proliferation of adult NSPCs and decreased neurogenesis.

To evaluate the impact of deleting HDAC3 in adult NSPCs and their progeny *in vivo*, we crossed *Hdac3* floxed mice with a tamoxifen (TAM)-inducible *Nestin-CreER*^{T2} allele (*Hdac3* cKO mice) and sacrificed mice at various timepoints after TAM (Fig. 3.6*A*). These mice were also bred to a *R26R-YFP* reporter allele so YFP+ recombined cells can be used as a surrogate marker for cells recombined and deleted for HDAC3 (149). To validate the efficiency of HDAC3 recombination after TAM treatment in adult HDAC3 cKO mice, we performed PCR of HDAC3 genomic region from primary hippocampal neurospheres isolated from TAM-injected adult *Hdac3* floxed mice and was unable to detect *Hdac3* gene expression consistent with efficient recombination and deletion of HDAC3 (Fig. 3.6*B*).

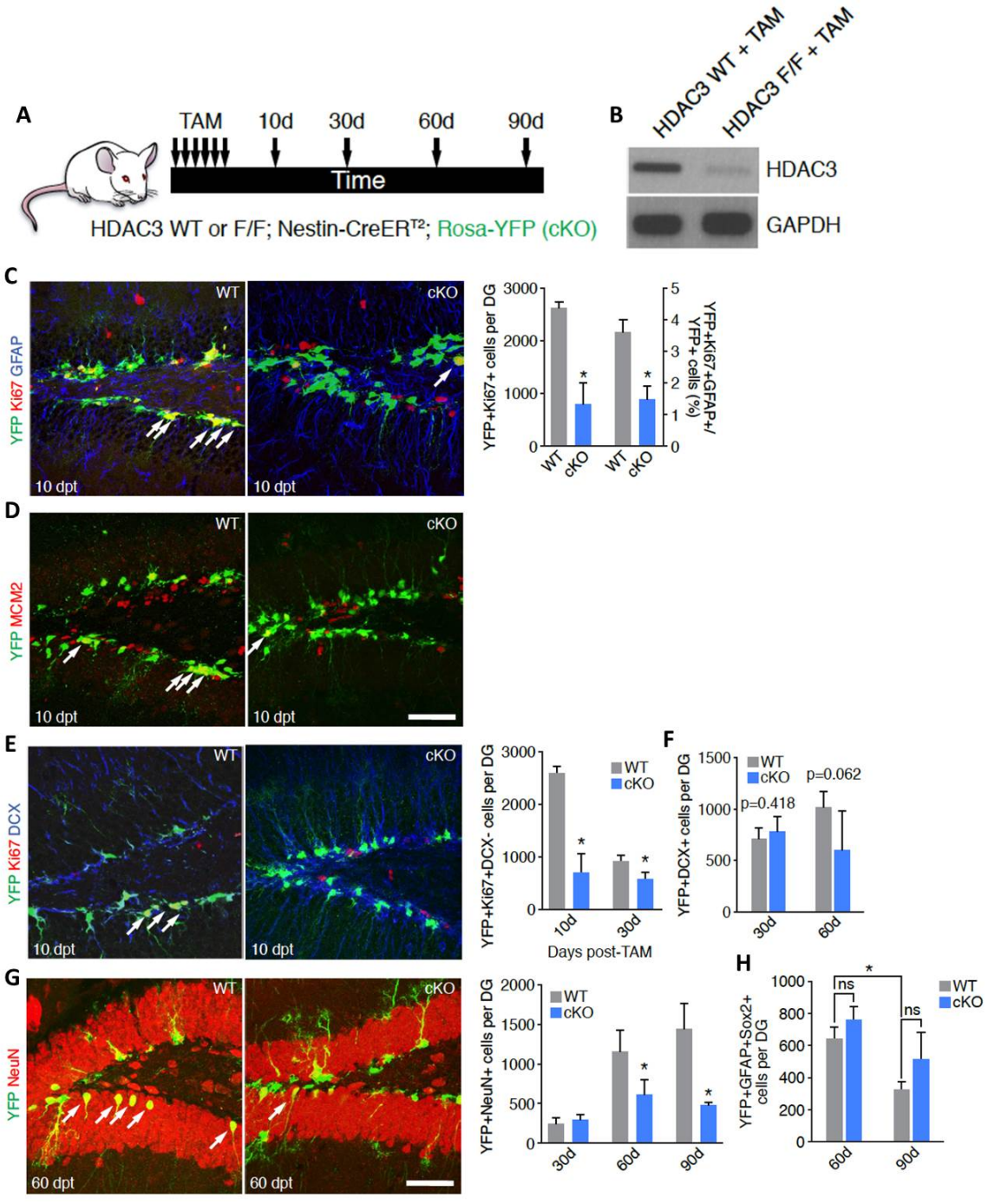


Figure 3.6. Loss of HDAC3 results in decreased proliferation *in vivo*. (*A*) Schematic of tamoxifen (TAM) injection and collection of brain tissue. (*B*) Genomic PCR of *Hdac3* in neurospheres isolated from WT and *Hdac3* cKO mice. (*C*) Immunostaining of YFP, Ki67, and GFAP in *Hdac3* WT and cKO mice 10 days post-TAM (dpt) and quantification of YFP+Ki67+ proliferating cells (arrows) and YFP+Ki67+GFAP+ RGL cells. (*D*) Representative immunostaining of YFP and MCM2 in *Hdac3* WT (n=3) and cKO mice

(n=3) at 10 dpt. Arrows indicate YFP+MCM2+ double positive TAP cells. Scale bar, 50 μm. (*E*) Immunostaining of YFP, Ki67, and DCX in WT and cKO mice 10 dpt and quantification of YFP+Ki67+DCX- TAPs (arrows) at 10 and 30 dpt. (*F*) Quantification of YFP+DCX+ cells in *Hdac3* WT and cKO mice at 30, and 60 dpt. (*G*) Immunostaining of YFP and NeuN in WT and cKO mice 60 dpt and quantification of YFP+NeuN+ cells (arrow) at 30, 60, and 90 dpt. (*H*) Quantification of YFP+GFAP+Sox2+ cells in *Hdac3* WT and cKO mice at 60 and 90 dpt. ns = not significant. For *C*, *E*, *G*, and H: *p<0.05. For *C*-*E*, and *G*: scale bars, 50 μm. Three HDAC3 WT mice 90 dpt were used for the analysis. At least six mice of each genotype were used in other experiments.

To examine the potential ability of HDAC3 to control proliferation *in vivo*, we sacrificed *Hdac3* cKO at an early timepoint, such as 10 days post TAM (dpt). We observed a significant reduction in the number of YFP+Ki67+ and YFP+MCM2+ proliferating cells in the SGZ of *Hdac3* cKO mice compared to WT littermates (Fig. 3.6*C*-*D*). Moreover, further analysis of YFP+ cells with cell type-specific markers revealed that both the percentage of proliferating YFP+GFAP+Ki67+ RGL cells out of total YFP+ cells and the number of YFP+Ki67+DCX- TAPs is reduced in *Hdac3* cKO mice compared to WT mice at 10 dpt (Fig. 3.6*C* and *E*), indicating that HDAC3 is required for the proliferation of RGL cells as well as TAPs.

Finally, one prediction of a defect in NSPC proliferation and cell cycle progression due to conditional deletion of *Hdac3* is a change in neurogenesis over time. Thus, we examined the number of immature and mature dentate neurons in *Hdac3* cKO mice between 30-90 dpt by quantification of immature YFP+DCX+ and mature YFP+NeuN+ cells. We observed a trend toward decrease in the number of YFP+DCX+ cells at 60 dpt, although this was not significant (p-value=0.062) (Fig. 3.6*F*). Consistent with the reduction in YFP+Ki67+ cells at 10 dpt in HDAC3 cKO mice, we observed a decrease in the number of YFP+NeuN+ adult-born neurons at 60 and 90 dpt (Fig. 3.6*G*).

To determine if the decreased generation of adult-born neurons after *Hdac3* deletion was due to a failure to maintain the NSC pool, we quantified the number of YFP+GFAP+Sox2+ RGLs at 60 and 90 dpt. Interestingly, while the number of YFP+GFAP+Sox2 RGLs decreased between 60 and 90 dpt in WT mice as expected, we did not observe a significant difference in the number of YFP+GFAP+Sox2+ RGLs in *Hdac3* cKO mice compared to WT at 60 and 90 dpt (Fig. 3.6*H*). These results suggest that the decreased neurogenesis found in *Hdac3* cKO mice could be due to the decreased proliferation of RGLs and TAPs and not from a change in the total number of RGLs. Together these results support the major role of HDAC3 is to regulate adult NSPC proliferation and cell cycle progression to ensure the continuous generation of adult-born neurons over time.

Discussion

The immense cellular heterogeneity within the brain represents a formidable challenge for the development of HDAC inhibitors as potential therapeutics to treat brain disorders. Moreover, the function of various HDAC members in different progenitor, neuronal or glial cell types, some having redundant properties, provides rationale for uncovering the roles of specific HDACs in the nervous system. In this study, we showed that HDAC3 is required for the proliferation of adult NSPCs (both RGLs and TAPs), ultimately resulting in decreased generation of mature dentate neurons. Surprisingly, we did not observe a significant difference, although there was a trend towards reduction of immature YFP+DCX+ at 60 dpt, suggesting that DCX+ cells may increase their proliferation to compensate for the early reduction of YFP+Ki67+ cells. We also demonstrated that HDAC3 is important to regulate CDK1 levels for proper G2/M phase progression. We propose that wherein WT NSPCs, HDAC3 stabilizes CDK1 to promote normal cell cycle progression, in NSPCs lacking or inactive for HDAC3, CDK1 is

degraded through the ubiquitin-proteasome pathway, resulting in G2/M phase progression defects. Our results suggest that inhibition of HDAC3 may be beneficial for preventing brain malignancies as well as treating certain neurological diseases, such as epilepsy, which may arise from proliferation of NSPCs after seizures.

HDAC3 plays a role in adult NSPC proliferation, specifically in G2/M phase progression.

Previously, we and others showed that application of pharmacological inhibitors of class I HDACs leads to enhanced neuronal differentiation within adult hippocampus and developing forebrain (159, 160). However, some reports indicate that neurogenesis in certain brain regions is decreased after HDAC inhibitor treatment (161). Whether these studies indicate context-dependent differences based on the development stage and/or brain region provides rationale for additional studies of the individual roles of HDACs in neurogenesis. HDACs 1 and 2 appear to function redundantly during neurodevelopment to promote cell survival and neuronal migration (162), while deletion of HDAC2 alone in adult brain is sufficient to mediate cell death and defective neuronal maturation (163). In contrast to HDACs 1 and 2, which are enriched in neural progenitors and mature neurons respectively (163, 164), we observe HDAC3 is broadly expressed in adult brain, including hippocampal NSPCs, neuroblasts, and mature neurons.

Deletion of HDACs 1 and 2 in fibroblasts blocks cell cycle in G1 phase by increasing the mRNA levels of CDK inhibitors *p21* and *p57* (165). In adult NSPCs, *p21* does not increase after removal of HDAC3, which is consistent with our observation that G1/S phase progression is not primarily affected. We have shown that HDAC3 specifically controls G2/M phase progression in adult NSPCs. In other cell types, such as hematopoietic progenitor cells and mouse embryonic fibroblasts, loss of HDAC3 will

result in S phase progression and DNA replication defects (81). The discrepancies in cell cycle defects after loss of HDAC3 may reflect differences in the level of HDAC3 at different stages of the cell cycle. For instance, in Hela cells, the levels of HDAC3 dropped in metaphase due to proteasome degradation (82). However, in NSPCs, the levels of HDAC3 remained consistently high throughout the cell cycle (Supp. Fig. 2*B*). Therefore, we do not believe that HDAC3 control of cell cycle progression in mainly due to changes in HDAC3 levels, at least in adult NSPCs.

HDAC3 mediates G2/M progression through post-translational control of CDK1 in adult NSPCs.

How does HDAC3 specifically control G2/M progression? Since HDACs do not bind to chromatin during M phase (166), one possibility is that HDAC3 may have non-histone substrates in adult NSPCs. Indeed, HDAC3 was previously reported to control mitosis by targeting A-Kinase-Anchoring Proteins AKAP96 and aurora B kinase in 293T cells (167). However, when HDAC3 is deleted in 293T cells, an increase in percentage of G2/M phase is correlated with a decrease in levels of aurora B kinase substrate, p-H3S10, which is opposite to what we observed in NSPCs lacking HDAC3 (Fig. 3.3*A*). Thus, it is reasonable to postulate that HDAC3 controls G2/M phase in adult NSPCs through targets other than AKAP96 and aurora B, and depending on the cell type, HDAC3 may have different targets to control the cell cycle.

Based on our observation that HDAC3 is required for maintaining CDK1 levels, we postulate that HDAC3 regulates the stability of CDK1, thus controlling G2/M progression. Our results also indicate that HDAC3 is required for preventing CDK1 ubiquitination. The exact mechanism by which HDAC3 regulates ubiquitination of CDK1 is unknown. In yeast, CDK1 can be acetylated at lysine 33, which is essential for proper

growth (168). Moreover, a recent study using HeLa cells showed that HDAC3 deacetylates and prevents the ubiquitination of cyclin A (82). However, in our study, while we detected acetylated-CDK1 in HCN cells, we did not observe any differences in CDK1 acetylation levels after HDAC3 inhibition or overexpression, possibly due to regulation by other HDACs. It would be interesting in future studies to examine the detailed mechanisms by which HDAC3 controls CDK1 ubiquitination.

Therapeutic implications of HDAC3 inhibitors.

Broad HDAC inhibitors (such as VPA) can mediate proneurogenic differentiation of NSPCs as well as spinal cord graft improvement (159, 169). Here we have shown that an HDAC3 inhibitor specifically blocks adult NSPC proliferation, instead of inducing neuronal differentiation. One potential advantage of HDAC3 inhibitors over broad HDAC inhibitors is to pharmacologically regulate proliferation of adult NSPCs, such as in post-seizure aberrant neurogenesis, which is a fundamental problem in epilepsy (170). Moreover, hyperproliferation of cancer cells is correlated with overexpression of HDAC3 (80) and quiescent adult NSPCs have been suggested to be a cellular source of brain tumors (171). Thus, we speculate that inhibition of HDAC3 may decrease the proliferation of proliferating cancer cells as well as activated brain cancer stem cells, which may be a more promising strategy than targeting proliferating cells alone.

On the other hand, activation of HDAC3 may promote the proliferation of adult NSPCs. Consistent with this idea, transgenic mice overexpressing HDAC3 was sufficient to induce cardiomyocyte proliferation (172). Our results suggest that development of HDAC3 activators may help in the pharmacotherapy of neurodegenerative diseases. Sequential use of an HDAC3 activator to boost the endogenous NSPC pool and then treatment of a broad HDAC inhibitor to promote neuronal differentiation may be more

effective for spinal cord repair while avoiding possible immunological complications from exogenous stem cell transplantation. Thus, combined use of HDAC-selective activators and broad-acting inhibitors may extend the therapeutic applications of HDAC inhibitors.

In summary, we have identified a crucial role for HDAC3 in adult NSPC proliferation. Mechanistically, HDAC3 is essential for proper G2/M progression, at least in part through post-translational regulation of CDK1. Our work confirms that detailed analysis of individual HDACs is important for translating HDAC inhibitors into the clinic.

Chapter 4

Class IIa HDACs regulation of adult hippocampal neurogenesis

Class I HDACs have been widely studied due to their significance in regulation of gene transcription. The clinical promise of specific Class I HDAC inhibitors also drives this research. However, Class IIa HDACs have received much less attention due to lack of intrinsic deacetylase activities and class-specific inhibitors, making it challenging to assess their function. Several groups have been intensively working on this bottleneck (173, 174). Tempero and GSK pharmaceuticals recently developed a trifluoromethyloxadiazole (TFMO) compound, TMP269, through high-throughput screening of 2 million individual compounds (Fig. 4.1*A*) (175). It shows promising selectivity against Class IIa HDACs (Fig. 4.1*B*). This compound becomes a good tool to study the function of Class IIa HDACs in various systems, including adult neurogenesis. The data presented in this chapter is currently in preparation for submission.

Inhibition of Class IIa HDACs does not affect proliferation and neuronal differentiation of adult NSPCs *in vitro*.

To rapidly determine whether TMP269 was required for adult NSPCs *in vitro*, I first determined the optimal dosage of TMP269 in HCN cells. I performed the MTS assay to determine its cytotoxicity at different concentrations (Fig. 4.1*C*) and found that 5 μM minimized cytotoxicity. Thus, 5 μM TMP269 was used to treat HCN cells for 1 day (Fig. 4.1*D*). We performed immunostaining of BrdU after adding BrdU for 1hr before fixing the cells. HCN cells treated with TMP269 showed no obvious defects in proliferation as indicated by comparable number of BrdU+ cells (Fig. 4.1*E*). To test whether TMP269 controlled neuronal differentiation as broad HDAC inhibitors, such as VPA and

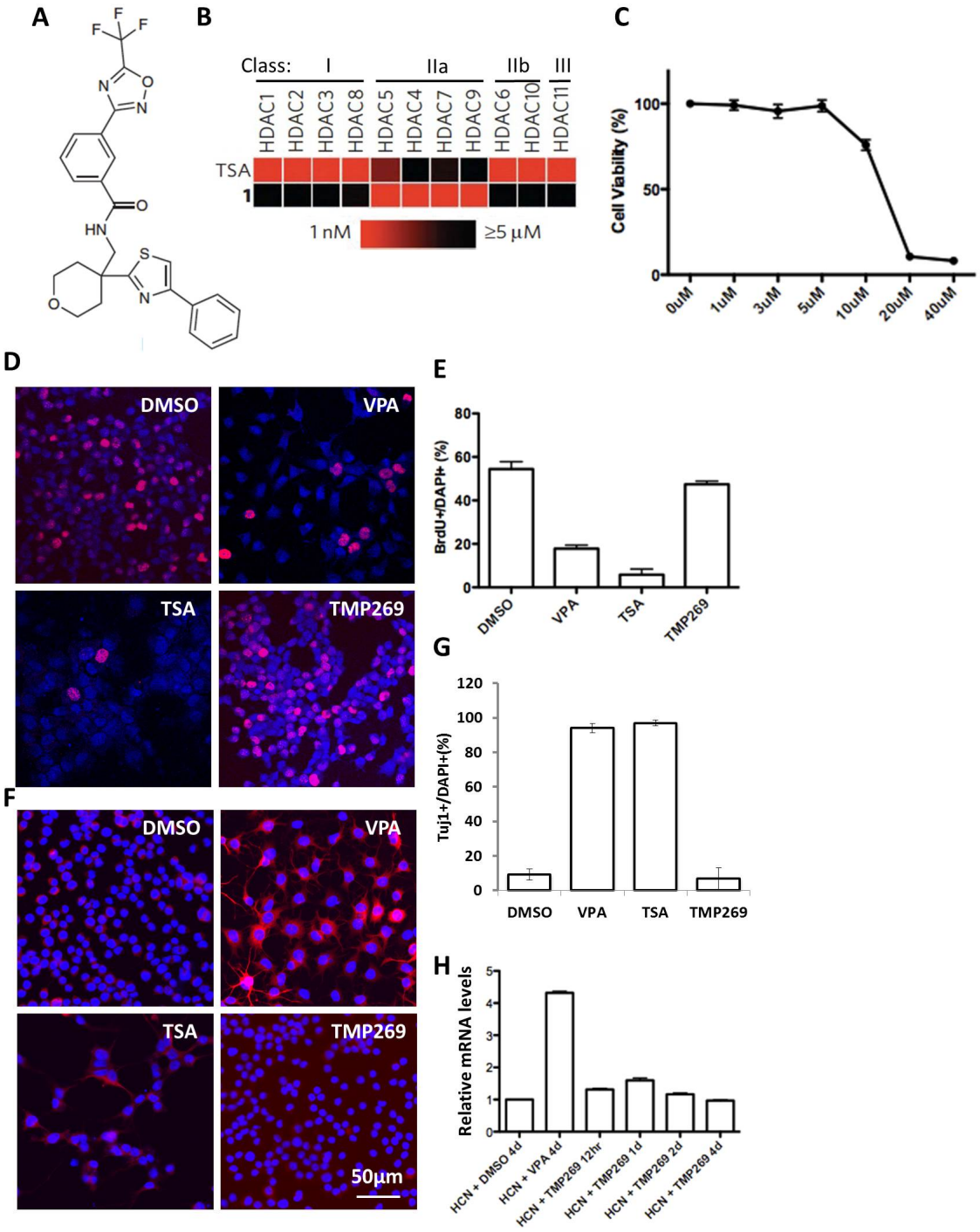


Figure 4.1. Class IIa HDAC inhibitor is not required for proliferation and neuronal differentiation of NSPCs *in vitro*. (*A*) Chemical structure of TMP269. (*B*) Heatmap of Ki of TMP269 against HDACs. (*C*) MTS assay of HCN cells treated with TMP269 at different concentrations. (*D*) BrdU staining of 1 hr BrdU labeling in HCN cells treated with DMSO, VPA, TSA, or TMP269 for 1 day. (*E*) Quantification of BrdU+ cells out of the total

DAPI+ cells. (*F*) Tuj1 staining of HCN cells treated with DMSO, VPA, TSA, or TMP269 in RA/FSK containing medium for 4 days. (*G*) Quantification of Tuj1+ cells out of the total DAPI+ cells. (*H*) qPCR of Tuj1 in HCN cells treated with DMSO, VPA, or TMP269 in RA/FSK containing medium for indicated length of time. A-B, adapted from Lobera et al. (2013) Nature Chemical Biology.

trichostatin A (TSA), we treated HCN cells with RA/FSK for 4 days and performed immunostaining of Tuj1 (Fig. 4.1*F*). We still did not observe a significant change in the levels of Tuj1 (Fig. 4.1*G*), which was confirmed by qPCR (Fig. 4.1*H*). These results indicate that inhibition of Class IIa HDACs may not affect adult neurogenesis *in vitro*, which is not surprising considering the fact that they lack intrinsic deacetylase activities (87).

HDAC5 is highly expressed in the neuronal population in adult dentate gyrus.

We next examined whether deletion of Class IIa HDACs will affect adult hippocampal neurogenesis *in vivo*. Since there are four HDACs in Class IIa, we first determined which HDAC is the most highly expressed in adult dentate gyrus. In the hippocampus of rat brains, the levels of HDAC4 and HDAC5 were much higher than HDAC7 and HDAC9 (176), we thus focused on these two HDACs in mouse dentate gyrus.

Because commercial HDAC4 and 5 antibodies did not work well for staining on tissues from WT mice, we utilized sections from mice with a replacement of LacZ-neomycin resistant cassette in Hdac4 and 5 genes to study their expression (91, 94). LacZ could then be used as a readout for in vivo expression of these two HDACs. We performed immunohistochemical staining using an antibody recognizing LacZ on

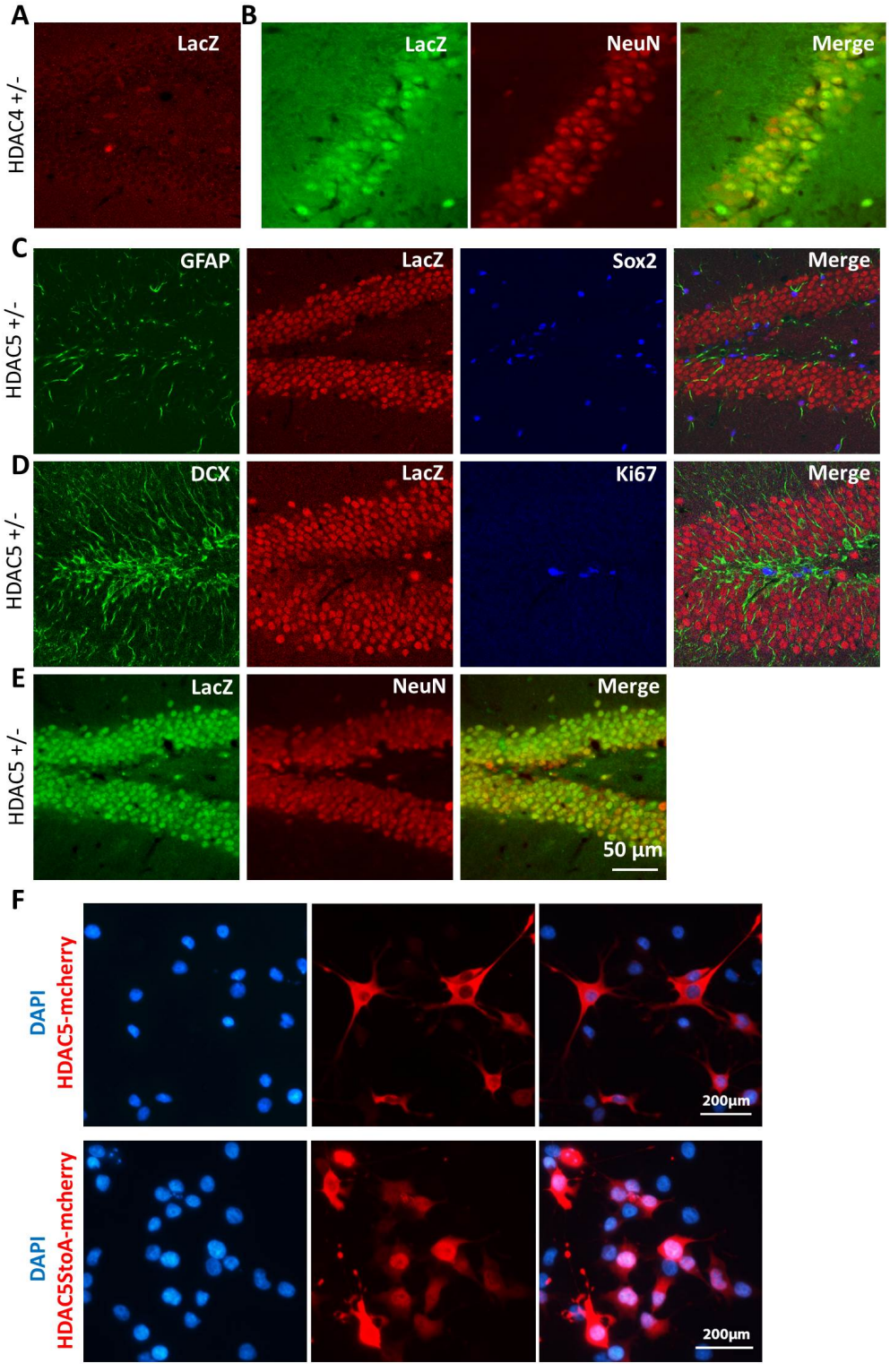


Figure 4.2. HDAC5 is expressed in immature and mature neurons in adult dentate gyrus. (*A*) Immunostaining of LacZ in dentate gyrus of brain sections from P60 Hdac4 heterozygous mice. (*B*) Immunostaining of LacZ and NeuN (a marker of mature

neurons) in CA3. (*C*) Immunostaining of LacZ, GFAP (a marker of RGLs), and Sox2 (a marker of RGLs and TAPs) of brain sections from P60 Hdac5 heterozygous mice. (*D*) Immuostaining of LacZ, DCX (a marker of immature neurons), and Ki67 (a marker of proliferation). (*E*) Immunostaining of LacZ and NeuN (a marker of mature neurons). (*F*) Shown are representative images of HCN cells electroporated with pCAG-HDAC5-mcherry or pCAG-HDAC5 StoA (S259A and S498A)-mcherry and incubated in RA/FSK containing medium for 4 days. For A-C: scale bar, 50 μm.

sections from Hdac4 heterozygous mice. Surprisingly, we did not observe any LacZ expression in the dentate gyrus (Fig. 4.2*A*), but in the CA3 region (Fig. 4.2*B*). We also stained LacZ together with stage-specific markers on sections from Hdac5 heterozygous mice. We did not observe LacZ expression within GFAP+Sox2+ RGLs and GFAP-Sox2+ TAPs (Fig. 4.2*C*). We could detect LacZ in DCX+Ki67- immature neurons as well as in postmitotic NeuN+ neurons in the dentate gyrus (Fig. 4.2*D*-*E*). Since LacZ staining only reveals the cellular localization in tissues, we overexpressed Hdac5 fused to mcherry fluorescent protein in HCN cells to study the subcellular localization of HDAC5 in neurons. We observed most HDAC5 in the cytoplasmic, while S259A and S498A mutated HDAC5 stayed in the nucleus (Fig. 4.2*F*), suggesting that HDAC5 is expressed in the cytoplasm of immature and mature neurons in adult dentate gyrus.

HDAC5 restricts neuronal differentiation of adult NSPCs *in vivo.*

To evaluate the impact of deleting HDAC5 in adult NSPCs and their progeny *in vivo*, we first injected BrdU for 2 hrs in Hdac5 WT or KO mice to evaluate the proliferation. We observed an increasing trend in the number of BrdU+ cells in dentate gyrus, although this was not significant (p-value=0.065) (Fig. 4.3*A*). To evaluate

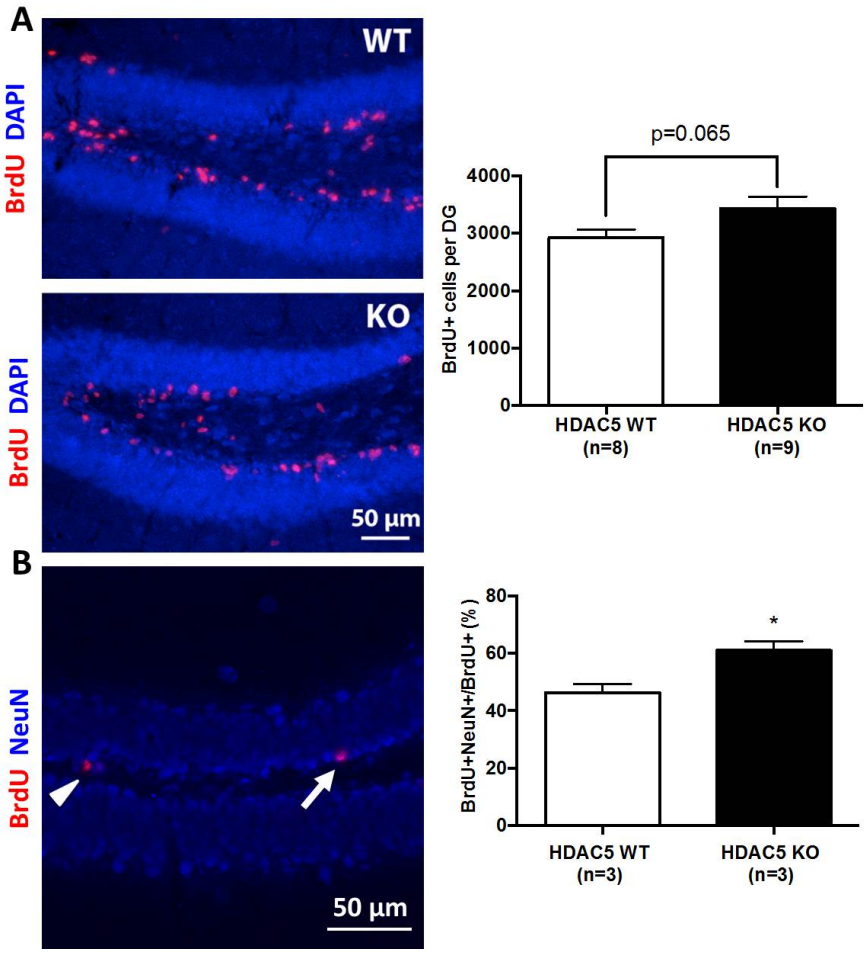


Figure 4.3. HDAC5 restricts neuronal differentiation *in vivo*. (*A*) Immunostaining of BrdU and DAPI in P15 Hdac5 WT and KO mice injected with BrdU for 2 hours before sacrifice and quantification of total BrdU+ cells per dentate gyrus. (*B*) Immunostaining of BrdU and NeuN in Hdac5 WT and KO mice sacrificed 30 days after 7 days BrdU injection and quantification the percentage of BrdU+NeuN+ (arrow) cells out of the total BrdU+ cells. Arrowhead indicates a BrdU+NeuN- cell on a section from Hdac5 WT mice.

neuronal differentiation, we injected BrdU into mice for seven days, and pulse chase BrdU for 30 days after last injection. We observed an increase in the percentage of BrdU+NeuN+ cells out of the total BrdU+ cells (Fig. 4.3*B*), suggesting that HDAC5 may be involved in restricting neuronal differentiation *in vivo*.

To determine whether HDAC5 is required for neuronal differentiation *in vitro*, we established primary neurospheres from the hippocampus and subventricular zone of P15 Hdac5 WT or KO mice. We observed an increase in the level of two neuronal markers, Map2ab and Tuj1 in Hdac5 KO neurospheres in both growth factor withdrawal and neuronal differentiation conditions (Fig. 4.4*A*). This was confirmed by qPCR analysis of Tuj1 (Fig. 4.4*B*). The activity of neuronal protein NeuroD was also increased in Hdac5 KO neurospheres as indicated by NeuroD luciferase reporter assay (Fig. 4.4*C*). HDAC5 is known to repress MEF2 activity in the nucleus, we then examined whether Mef2 activity was altered in Hdac5 KO neurospheres by electroporating desMEF-luciferase plasmid. We observed an increase in the activity of MEF2 in Hdac5 KO neurospheres (Fig. 4.4*D*), which was not due to an increase in the level of Mef2 genes (Fig. 4.4*E*). These results suggest that HDAC5 is sufficient for neuronal differentiation of NSPCs *in vitro*.

To examine whether HDAC5 is necessary for neuronal differentiation *in vitro*, we electroporated Hdac5 overexpression plasmids into HCN cells (Fig. 4.4*F*). We observed a decrease in the level of Tuj1 (Fig. 4.4*G*). Taken together, HDAC5 is both necessary and sufficient for neuronal differentiation of NSPCs *in vitro*.

Discussion

The preliminary results of this study reveal an essential role of a Class IIa HDAC, HDAC5 in adult hippocampal neurogenesis. Inhibition of Class IIa HDACs by small

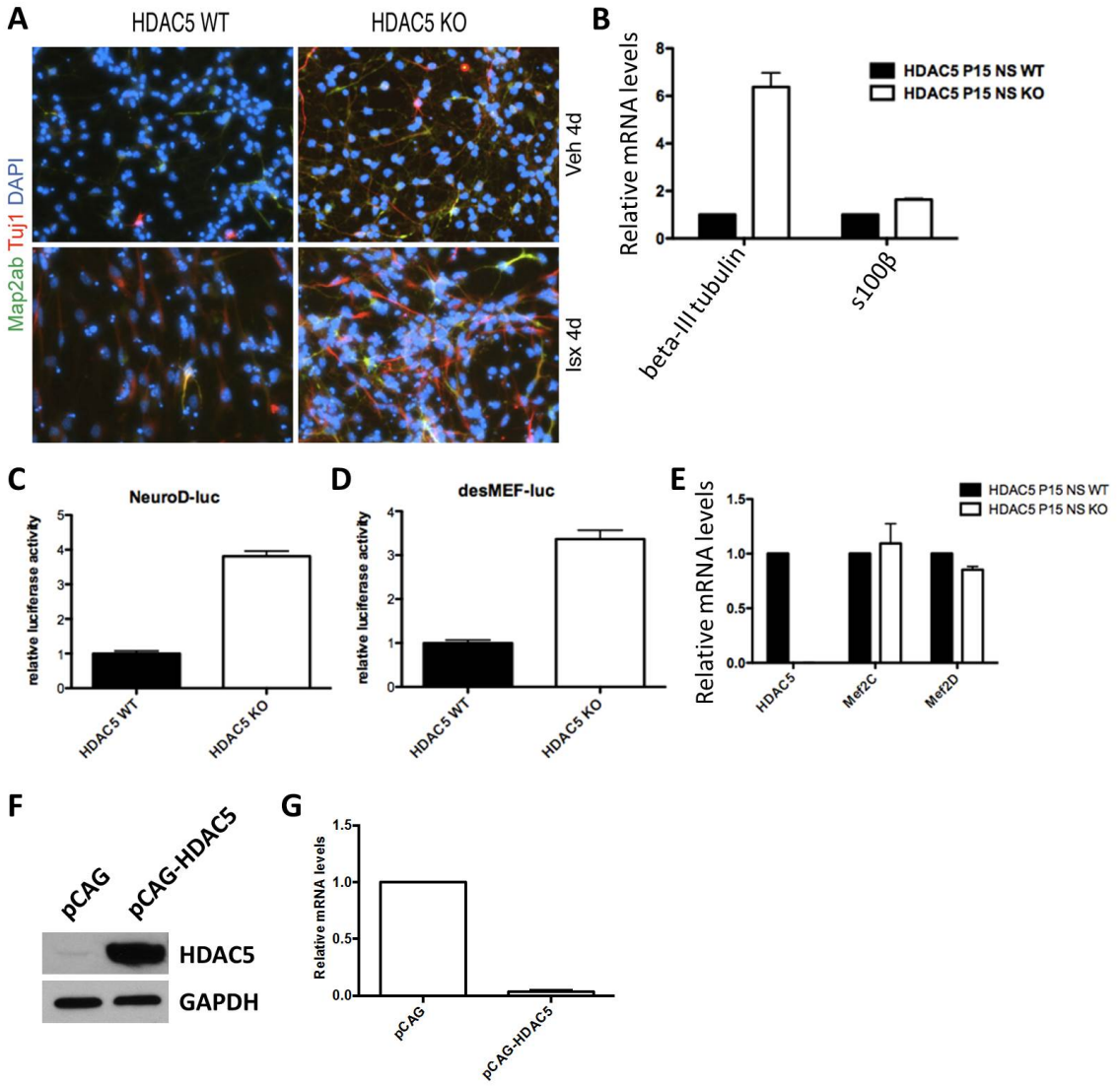


Figure 4.4. HDAC5 is sufficient and necessary for neuronal differentiation *in vitro*. (*A*) Immunocytochemical staining of Map2ab, Tuj1 in Hdac5 WT or KO neurospheres treated with Veh or Isx-9 for 4 days. (*B*) qPCR analysis of Tuj1 and S100β in Hdac5 WT or KO neurospheres cultured in medium without growth factors. (*C*) Luciferase assay of Hdac5 WT or KO neurospheres electroporated with NeuroD-luciferase plasmid. (*D*) Luciferase assay of HDAC5 WT or KO neurospheres electroporated with desMEF-luciferase plasmid. (*E*) qPCR of Hdac5, Mef2C, and Mef2D in Hdac5 WT or KO neurospheres. (*F*) Western blotting against HDAC5 in HCN cells electroporated with pCAG or pCAG-Hdac5 overexpression plasmids and incubated in medium without growth factors for two days. (*G*) qPCR of beta-III tubulin in HCN cell electroporated with

pCAG or pCAG-Hdac5 overexpression plasmids and incubated in medium without growth factors for two days.

molecule TMP269 does not affect proliferation and neuronal differentiation of NSPCs *in vitro*. In Class IIa HDACs, HDAC5 is the most highly expressed one in immature and mature neurons in adult dentate gyrus, whereas it cannot be detected in NSPCs and neuroblasts. After removal of Hdac5 *in vivo* and *in vitro*, we observed increased neuronal differentiation of NSPCs.

It is known that the deacetylase domain of Class IIa HDACs is not functional. It is proposed to serve as a "reader" for acetyllysine in histones or other proteins, but not as an "eraser" to remove the acetyl group. Because the crystal structure shows that TMP269 occupies the catalytic pocket of Class IIa HDACs, these HDACs cannot function as a "reader" in NSPCs after treatment of TMP269. In this study, we showed that inhibition of Class IIa HDACs does not affect proliferation and neuronal differentiation of adult NSPCs *in vitro*, suggesting that this "reader" function is not necessary for adult NSPCs. However, it does not rule out the possibility that this proposed "reader" function of Class IIa HDACs may play an important role in other biological processes.

Most HDAC5 is expressed in the cytoplasm of immature neurons. Nuclear HDAC5 can bind to MEF2 to repress its transcription of target pro-neuronal genes. We believe that increased neuronal differentiation of NSPCs in Hdac5 KO mice is mostly due to loss of nuclear HDAC5, but not cytoplasmic HDAC5, because we observed increased MEF2 activity in Hdac5 KO neurospheres. Nevertheless, the function of cytoplasmic HDAC5 in immature and mature neurons is still unknown.

Future directions

There are several future directions emerging from our discovery that HDAC5 restricts neuronal differentiation of NSPCs. First, we showed that TMP269, a recently identified Class IIa specific inhibitor, might not affect proliferation and cell fate specification of neural stem cells. However, this is not conclusive yet due to a lack of positive control for the inhibitor. Acetylated histones are commonly used as a control for Class I specific inhibitor, but an acetylated substrate that is specific for Class IIa HDACs and universal for different cell types has not been identified. Discovery of this substrate will facilitate the current HDAC5 study in adult neurogenesis as well as all Class IIa HDACs related studies.

Second, we used Hdac5 knockout mice instead of Hdac5 conditional knockout mice in this study. One caveat in using these mice is the possible compensatory effect by other Class IIa HDACs. Examination of protein levels of other Class IIa HDACs should be conducted to exclude this possibility.

Third, HDAC5 is critical for neuronal survival in cerebellar granule neurons *in vitro* (177), however, information about cell death is still lacking in the current study. Staining of activated caspase 3 on sections from Hdac5 WT and KO mice is necessary to answer that question.

Finally, as shown in Chapter 5, conditional deletion of MEF2 in neural stem/progenitor cells and their progeny results in defects in dendritic morphology, whereas Isx-9 mediated up-regulation of MEF2 leads to more complex dendrites, suggesting that MEF2 is involved in dendritic development. In Hdac5 knockout neurospheres, the activity of MEF2 increases as revealed by luciferase assay. It is interesting to examine the dendrites of newborn neurons in Hdac5 WT and KO mice.

In summary, one of the most abundant Class IIa HDACs, HDAC5 can restrict neuronal differentiation of NSPCs *in vivo* and *in vitro*. Mechanistically, HDAC5 binds MEF2 in the nucleus to repress MEF2-dependent pro-neuronal target gene expression. Further examination of these Hdac5 KO mice and possible mechanisms will strengthen the current conclusion.

Chapter 5

Mef2 regulation of adult hippocampal neurogenesis

As described in Chapter 1, Mef2 proteins are enriched in several organs, such as heart, spleen, and brain. It is well documented that the Mef2 transcription factor is essential for myogenesis. In the adult brain, Mef2 is only known for neuronal survival, excitatory synapse elimination, and learning and memory. However, its role in neurogenesis remains to be elucidated. In this chapter, I will describe our studies to examine the role of Mef2 in adult neurogenesis by using both pharmacological and genetic approaches. The work presented in this chapter is collaborative with Sarah Latchney and David Petrik, two postdocs in Dr. Amelia Eisch's lab.

Role of Mef2 in Isx-9 induced neurogenesis

The data presented in this section have been published as a research communication in *The FASEB Journal* (178).

Our lab has shown that a synthetic small molecule, 3, 5-disubstituted isoxazole 9 (Isx-9) (Fig. 5.1*A*), could strongly induce neuronal differentiation of HCN cells *in vitro* via a neurotransmitter-like evoke Ca^{2+} signal which leads to HDAC5 export from the nucleus and activation of pro-neuronal genes critical for neurogenesis (136), however, it was still unknown whether Isx-9 would promote neurogenesis and even result in functional improvement in mouse. Given the prior work with Isx-9 *in vitro*, we hypothesized that in vivo Isx-9 would predominantly enhance proneuronal differentiation. Specifically, we hypothesized that Isx-9 would not change the number of Ki67+ SGZ progenitors but would enhance the number of immature neurons immunopositive for DCX, an intermediate filament expressed in neuroblasts and immature SGZ neurons. Postdoc researcher Dr. David Petrik from Amelia Eisch's lab first showed that Isx-9 could cross

the blood brain barrier. We then injected Isx-9 dissolved in β-cyclodextrin i.p. into 8- to 11-week old Nestin-GFP mice. Isx-9 transiently increased the number of SGZ Ki67+ cells 1 and 12 days after Isx-9 by around 50 and 86% relative to Veh (Fig. 5.1*B*-*C*), but returned to Veh levels by 30 days.

As Ki67+ cells represent both uncommitted TAP cells (Ki67+DCX-) and neuronally committed neuroblasts (Ki67+DCX+), we next analyzed cellular colocalization of Ki67 with DCX. Confocal phenotyping revealed a statistically significant increase in the proportion of Ki67+DCX+/Ki67+ SGZ cells 12 days post-treatment (Fig. 5.1*D*). These data suggest that Isx-9 increases the proportion of proliferating neuroblasts. To further analyze how Isx-9 affects cells in these early stages of adult neurogenesis, mice received an injection of the thymidine analog BrdU to label dividing cells either before (Fig. 5.1*E*) or after (Fig. 5.1*F*) Veh or Isx-9 and were killed 2 hrs or 30 days later. BrdU +cells were then visualized and counted. While Isx-9 did not change the number of BrdU+ cells when BrdU was given before treatment (Fig. 5.1*G*), Isx-9 increased the number of BrdU+ cells 2 hrs and 30 days later when BrdU was given after treatment (Fig. 5.1*H*). Taken together with Fig. 5.1*D*, these data suggest that Isx-9 increases proliferation of SGZ neuroblasts.

While BrdU labels dividing cells and most surviving BrdU+ cells in the adult DG GCL become neurons, a small proportion of them also become astrocytes. Therefore, to assess the phenotype of the surviving BrdU+ cells, we used confocal microscopy to determine the colocalization of BrdU+ cells with the neuronal protein NeuN, the astrocytic/NSC protein GFAP, and the neuroblast/immature neuron protein DCX (Fig. 5.1*I*-*K*). There were almost no BrdU+NeuN+ cells 2 hrs post-BrdU in Veh or Isx-9 treated mice, as expected (Fig. 5.1*I*). However, there was a statistically significant increase in

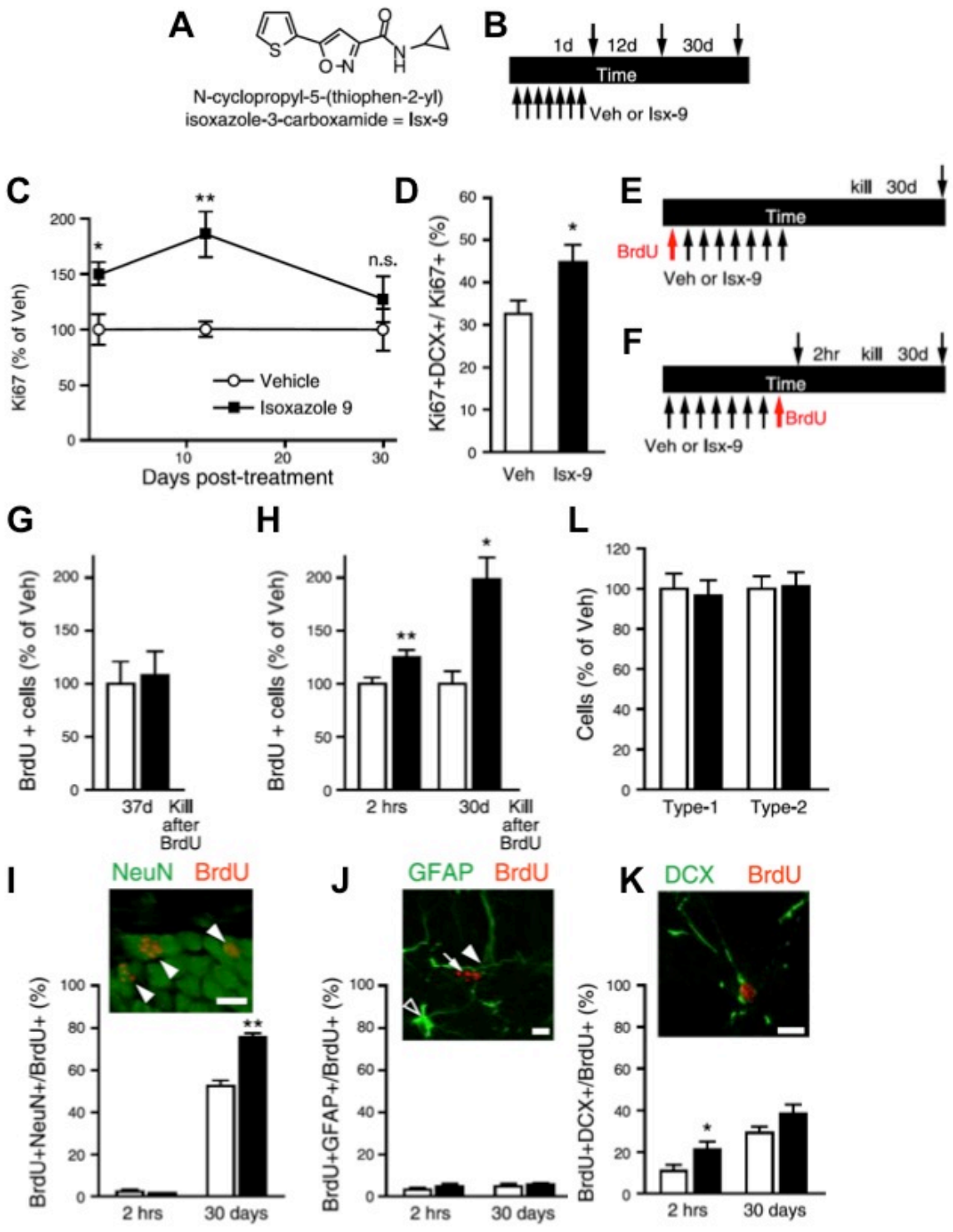


Figure 5.1. Isx-9 increases proliferation of neuroblasts and differentiation of immature neurons in hippocampal SGZ. (*A*) Chemical structure and nomenclature of Isx-9. (*B*) Timeline (applies to C-D) of 7d Veh or Isx-9 adminstration to Nestin-GFP mice (arrows under timeline) and neurogenesis analysis 1, 12, or 30 days later (arrows above

timeline). (*C*) Quantification of Ki67+ cells 1, 12, and 30 days after Veh or Isx-9. (*D*) Twelve days post-treatment, Isx-9 also increased the proportion of Ki67+ cells that were also DCX+. (*E-F*) Timelines show 3 paradigms of BrdU injection used in panels G-H to test the influence of Isx-9 on neurogenesis. (*G*) BrdU given before treatment used to examine survival of BrdU+ cells. (*H*) BrdU given after treatment used to examine proliferation (mice killed 2 hrs later) or survival (mice killed 30 days later) of BrdU+ cells. Isx-9-treated mice had more BrdU+ cells than Veh when BrdU is given after but not before Veh or Isx-9. (*I-K*) Confocal phenotyping revealed an Isx-9 induced increase in the proportion of BrdU+NeuN+/BrdU+ neurons at 30 days post-BrdU (*I*), no change in proportion of BrdU+GFAP+/BrdU+ at either time point (*J*), but increased proportion of BrdU+DCX+/BrdU+ cells at 2 hr, not 30 day (*K*). (*L*) Proportion of Nestin-GFP+ Type-1 and Type-2 cells was not changed after Isx-9. *I-K*: scale bars, 10 μm. *P<0.05, **P<0.01; unpaired *t* test.

the percentage of BrdU+NeuN+/BrdU+ cells in Isx-9 vs. Veh 30 days post-BrdU. In contrast, there was no change in the percentage of BrdU+GFAP+/BrdU+ cells at either time point (Fig. 5.1*J*), consistent with no obvious change in DG GFAP+ cell number in the DG (data not shown). Isx-9 also enhanced the proportion of BrdU+DCX+/BrdU+ cells 2 hrs but not 30 days post-BrdU (Fig. 5.1*K*). These results suggest that Isx-9 increases the proliferation of neuronally committed SGZ cells and leads to a subsequent enhancement in the number of adult-generated GCL neurons.

A key facet of the SGZ neurogenesis cellular cascade is the NSC/Type-1 cell, which is the putative source of adult-generated GCL neurons. NSCs rarely or slowly divide, so they are rarely labeled by either Ki67 or BrdU. Therefore, to assess whether Isx-9 influenced NSCs and to confirm the lack of influence of Isx-9 on Type-2a cells, we exploited the ability of nestin-GFP reporter mice to reveal the number of radial-glial-like Type-1 NSCs and dividing Type-2 progenitors labeled with GFP under control of the intermediate filament nestin (179). Morphological dissection of GFP+ cells into Type-1

QNPs and Type-2 GFP+ cells via stereology revealed no change in either cell type number at the 12-day time point (Fig. 5.1*L*). Furthermore, we did not observe any difference between the total number of GFP+ cells (Type-1 and Type-2 combined) in the SGZ at multiple time points after Isx-9 or Veh treatment (data not shown). Our findings allowed us to verify our *in vitro* inspired hypothesis of proneuronal differentiation and notably expand it within the *in vivo* context: Isx-9 enhances the proliferation of neuroblasts and adult neurogenesis without exhausting the NSC pool.

Based on our *in vitro* finding that Isx-9 enhanced neuroblast proliferation, we next assessed whether Isx-9 influences the number of maturation of immature adult-born neurons via DCX+ cell quantification. There was a transient increase in the total number of SGZ DCX+ cells of mice given Isx-9 vs. Veh at the 1, 12, and 30 day time points (Fig. 5.2*A*), with a return to Veh levels by 60 d (Fig. 5.2*B*). Isx-9 also resulted in a greater proportion of DCX+ neurons 12 and 30 days post-treatment (Fig. 5.2*C*) but returned to Veh levels by 60 days. During DCX+ neuron quantification, we noted a striking qualitative difference in DCX dendrite length in mice given Isx vs. Veh. To quantify this, we performed a detailed analysis of the dendritic trees of DCX+ cells 12 days post-treatment. Indeed, Isx-9 increased dendritic complexity, the total length, and area of the dendritic trees of DCX+ cells (Fig. 5.2*D*). Taken together, Isx-9 increased the number of DCX+ neurons and the complexity of their dendritic trees.

Since both adult neurogenesis and immature neuron dendritic complexity are linked with hippocampal function (39, 180), we explored the functional effect of the Isx-9-induced increase in neurogenesis and dendritic complexity. We first evaluated the effect of Isx-9 on the Morris water maze, a test that allows assessment of hippocampus-dependent learning and memory (181). To maximize the response to Isx-9 and to allow

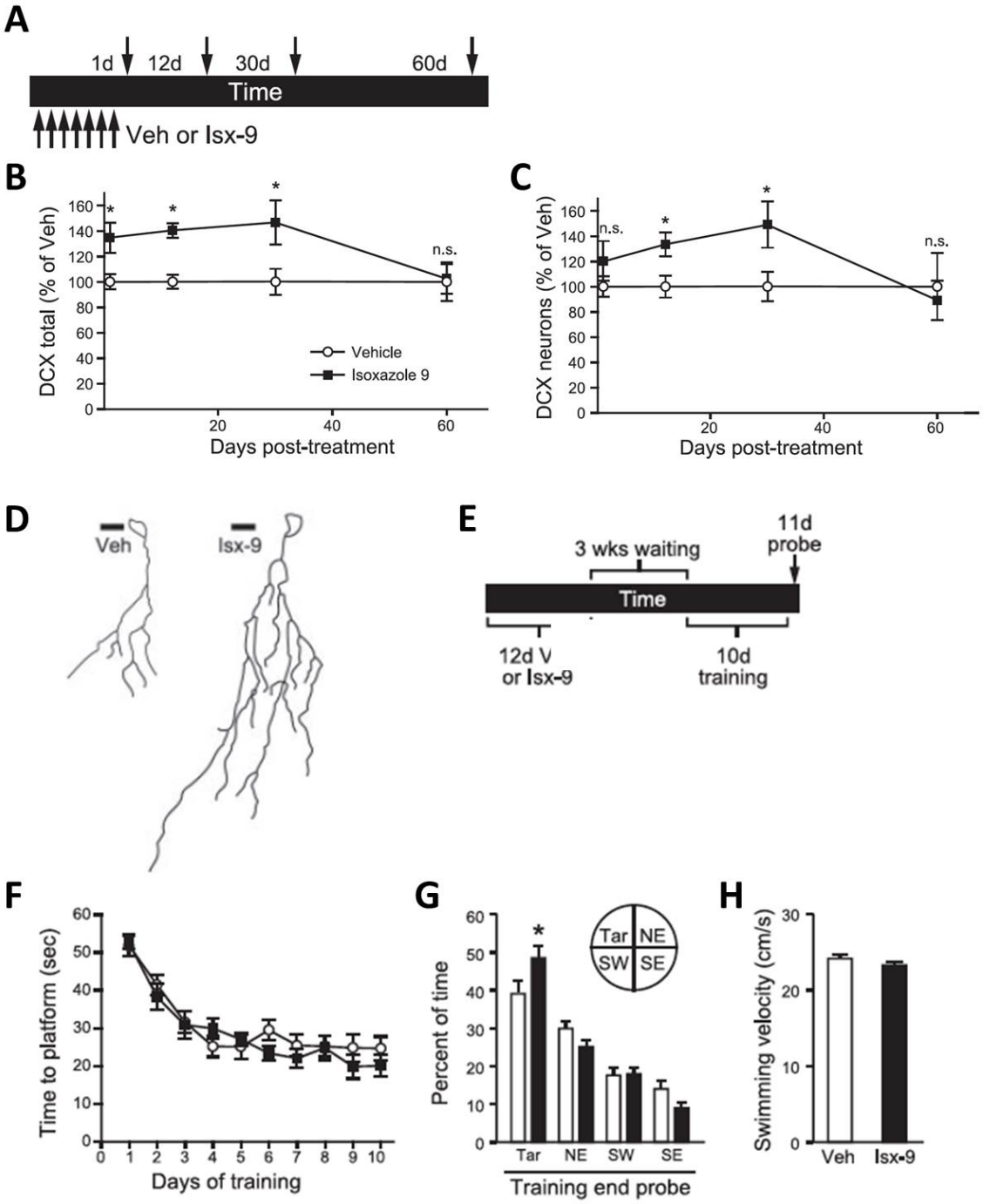


Figure 5.2. Isx-9 increases differentiation of immature neurons and improves memory. (*A*) Timeline for panels B-C. Nestin-GFP mice were given Veh or Isx-9 for 7d (arrows under timeline) and DCX+ SGZ cells were quantified at 1, 12, 30, and 60 days post-treatment (arrows above timeline). (*B*-*C*) Isx-9 mice had more DCX+ cells (*B*), and DCX+ immature neurons (*C*) at 12, and 30 days post-treatment. (*D*) Representative

dendritic tracings and photomicrographs of DCX+ neurons in Veh or Isx-9 treated mice. (*E*-*G*) MWM training after 12 days of Veh or Isx-9 (*E*) revealed no difference in learning (*F*); however, Isx-9-treated mice spent more time in the target quadrant during the probe test on day 11 (*G*) when the platform was removed. (*H*) There was no difference in swim speed between Veh and Isx-9. Scale bar in *D*, 10 μm. n.s.= not significant. *p<0.05; unpaired *t* test.

the adult-generated neurons sufficient time to incorporate into hippocampal circuitry, mice were given 12 days of Isx-9 or Veh and were trained on MWM 3 weeks later (Fig. 5.2*E*). There was no overall effect of Isx-9 on learning during training (Fig. 5.2*F*). However, during the probe test, mice given Isx-9 spent significantly more time in the target quadrant (Fig. 5.2*G*), showing a magnitude of change and pattern of probe results similar to the published literature (182, 183). A possible explanation for improved water maze performance (and for increased neurogenesis) might be enhanced locomotion. However, mice did not show any change in swim speed (Fig. 5.2*H*) or in locomotion during and after administration of Isx-9 vs. Veh (data not shown).

To gain molecular insight regarding Isx-9's ability to increase memory and neurogenesis *in vivo*, we used microarray and qPCR to identify Isx-9-induced alterations in gene expression in SGZ progenitors (Fig. 5.3*A*). We used Nestin-GFP mice for this endeavor, since Isx-9 induced changes in gene expression in earlier stages of neurogenesis might precede or contribute to Isx-9 induced changes in neurogenesis we see in the later stages of neurogenesis (Fig. 5.1*D*, 5.2*A*-C). GFP+ cells from Nestin-GFP hippocampi were sorted (FACS), and their mRNAs were amplified and subjected to genome-wide Affymetrix gene chip analysis. There were 874 genes with ≥ 2-fold expression level changes after Isx-9 vs. Veh, and 121 genes with ≥ 3-fold expression level changes after Isx-9 vs. Veh. Many target genes encoded transcription factors

critical for neuronal progenitors (e.g. Zic3; (184)) or molecules important for neural stem cells and progenitors (e.g. Smad7; (185)). Ingenuity software analysis showed that Isx-9 regulates biofunction pathways involved in cellular growth and development as well as intercellular signaling and movement, which collectively may suggest, among other factors, involvement of transcription factors, such as Mef2. Indeed, closer analysis of the array results showed that Mef2C was up-regulated 2.05-fold in the GFP+ cells from mice given Isx-9 vs. Veh. While *in vitro* studies showed Mef2C up-regulated in response to Isx-9 (136), this was the first *in vivo* link among adult neurogenesis, Mef2, and Isx-9. To further understand this link, we first confirmed the Isx-9-induced increase in Mef2C by qPCR in a new set of hippocampi from Nestin-GFP mice (Fig. 5.3*B*). We then set out to determine whether the Isx-9 induced up-regulation of neurogenesis involved MEF2 function.

To explore whether Isx-9 requires MEF2C for its ability to increase adult neurogenesis, we had to take into account that the adult DG also expresses other family members, including MEF2A and 2D. We used out TAM-sensitive nestin-expressing NSPCs and their progeny. Crossing Nestin-CreER^{T2} mice with R26R-YFP reporter mice allowed us to visualize the recombined cells by YFP expression. To exclude any possible compensation by Mef2A and Mef2D after the deletion of Mef2C, we used Nestin-CreER^{T2} mouse line to inducibly delete all three Mef2 genes in nestin expressing neural stem/progenitor cells and their progenies (Mef2a/c/d KO). Two control groups were used here: Nestin-CreER^{T2}/Rosa-YFP ("WT") and Mef2A F/F; Mef2C +/+; Mef2D F/F; Nestin-CreER^{T2}/Rosa-YFP (Mef2a/d KO). Crossing Nestin-CreER^{T2} mice with Rosa-YFP reporter line allowed us to visualize the recombined cells by YFP expression. We injected Tamoxifen for five days to delete Mef2 genes before Isx-9 or vehicle injection for 7 days. 12 days after the last injection of Isx-9 or vehicle, we sacrificed mice,

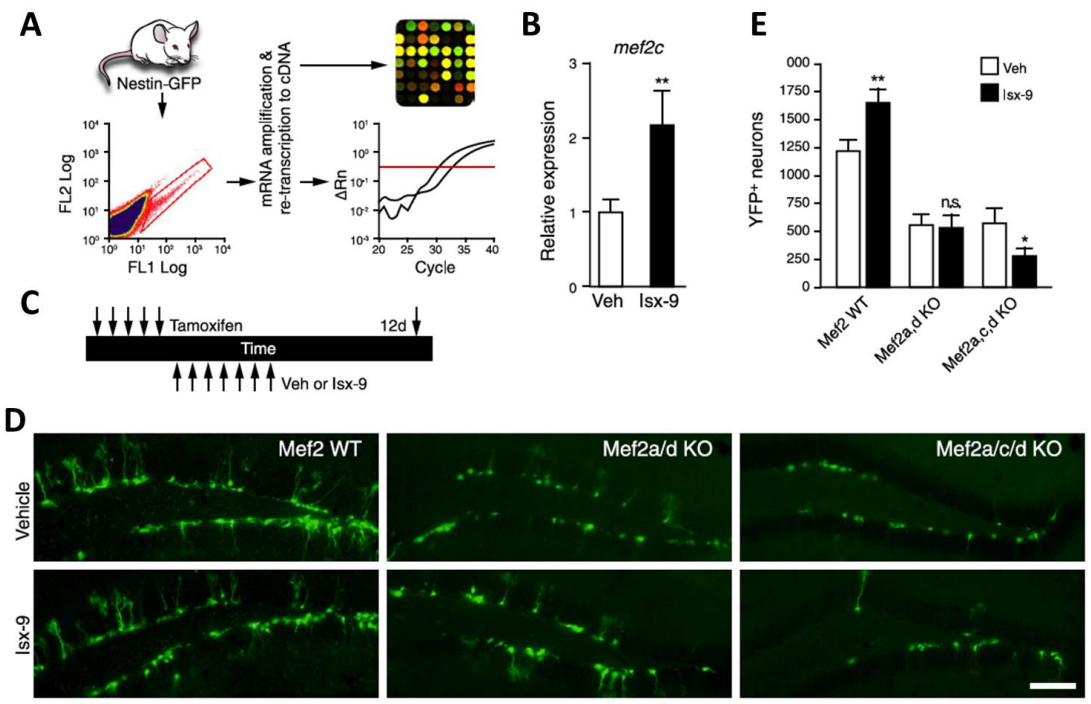


Figure 5.3. Mef2 is required for Isx-9 induced neurogenesis. (*A*) Cells from Nestin-GFP mice were FACSorted, and their RNA was amplified, reverse-transcribed, and used for a gene chip or qPCR. (*B*) Isx-9 induced up-regulation of Mef2C was confirmed by qPCR. (*C*) To assess whether Mef2 was necessary for Isx-9-induced enhancement of adult hippocampal neurogenesis, Nestin-CreER^{T2}/R26R-YFP-WT, Mef2a/d$^{flox/flox}$ or Mef2a/c/d$^{flox/flox}$ transgenic mice were given Tam to induce Cre-mediated recombination and then given Veh or Isx-9. (*D*) Representative photomicrographs show YFP+ DG cells in mice that are Mef2-WT (left column), Mef2a/d-KO (middle column), and Mef2a/c/d-KO (right column) 12 d after 7 d administration of Veh (top panels) or Isx-9 (bottom panels). Scale bar, 50 μm. (*E*) Isx-9 increased YFP+ neuron numbers in Mef2-WT mice, but decreased YFP+ neuron numbers in Mef2a/c/d-KO mice. *p<0.05, **p<0.01; paired *t* test.

immunostained and quantified the number of YFP+ mature neurons by morphology (Fig. 5.3*C*). In the WT group, we did observe an increase of the number of YFP+ neurons after treatment of Isx-9, which was consistent with previous data. However, in Mef2a/d

KO and Mef2a/c/d KO mice, Isx-9 did not increase the number of YFP+ neurons (Fig. 5.3*D*-*E*), suggesting that Isx-9 induced increase in adult hippocampal neurogenesis requires MEF2 transcription factors in nestin-expressing NSCs and their progeny.

Interestingly, we also observed a drop in the number of YFP+ neurons in vehicle treated Mef2 tKO mice compared to WT mice, suggesting that baseline adult hippocampal neurogenesis requires intrinsic intact MEF2 signaling, highlighting a completely novel role for MEF2 in the brain. Thus, our next question focuses on the requirement of the MEF2 family of genes in baseline neurogenesis.

Localization and levels of MEF2 in adult dentate gyrus

To begin to investigate the role of MEF2 during the course of adult neurogenesis *in vitro* and *in vivo*, we examined the expression pattern and levels of each Mef2 gene in adult mouse brain, particularly within the SGZ of the dentate gyrus.

To determine the expression pattern of MEF2A, 2C, and 2D in adult hippocampal neurogenesis, I first tested the specificity of several commercial Mef2 gene-specific antibodies *in vitro*. I established four different primary neurosphere lines from the hippocampus and SVZ of 1-month old $Mef2A^{F/F}2C^{F/F}2D^{F/F}$, $Mef2A^{F/F}$, $Mef2C^{F/F}$, and $Mef2D^{F/F}$ mice. Deletion of Mef2 genes by infection of adeno-Cre-GFP virus to these neurospheres would be a good tool to study MEF2 antibody specificity, because ideally, specific Mef2 gene-specific antibody will only detect the deletion of tKO and that gene. With the use of following MEF2 antibodies: MEF2A (H300), MEF2C (E17) from Santa Cruz, and MEF2D from BD Biosciences (Fig. 5.4), we observed efficient recombination of all Mef2 lines, resulting in a truncated form of MEF2A, 2C, and 2D proteins.

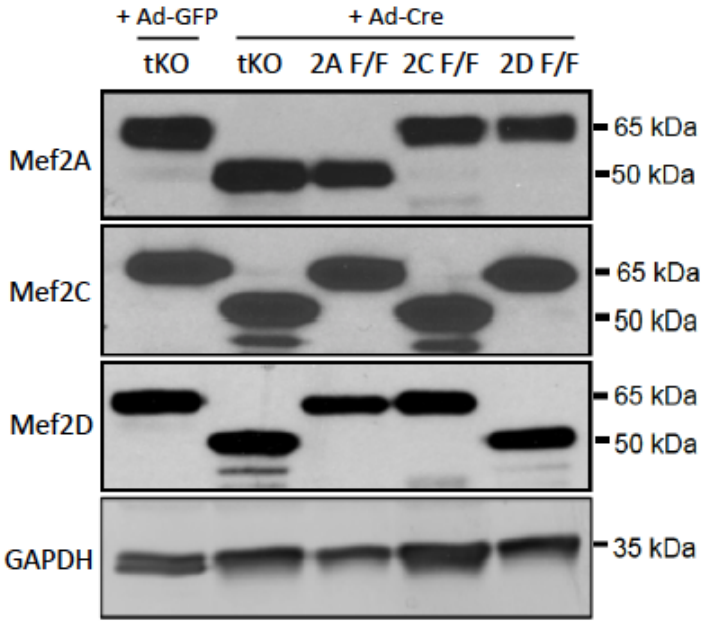


Figure 5.4. Validation of specificity of MEF2 antibodies. Western blotting against Mef2A, 2C, and 2D in neurospheres derived from $Mef2A^{F/F}2C^{F/F}2D^{F/F}$ (lanes 1-2), $Mef2A^{F/F}$ (lane 3), $Mef2C^{F/F}$ (lane 4), and $Mef2D^{F/F}$ (lane 5) mice and infected with Ad-GFP (lane 1) or Ad-Cre-GFP virus (lane 2-5) for 2 days. GAPDH was used as a loading control.

Next, we performed IHC staining using these Mef2 gene-specific commercial antibodies together with stage-specific markers to determine the expression pattern of MEF2. We did not observe any MEF2 expression within GFAP+Sox2+ RGLs (Fig. 5.5*A*). Because GFAP+Sox2+ cells in dentate gyrus could be either neural stem cells or astrocytes, we then used sections from Nestin-GFP reporter mice, which specifically label Nestin+ cells. We did not observe any MEF2 expression in GFP+ cells. Moreover, MEF2 was also not expressed in any Ki67+ or BrdU+ proliferating cells (Fig. 5.5*B*). We further detected 62% of MEF2A, 42% of MEF2C, and 33% of MEF2D out of the total DCX+ immature dentate granule cells (Fig. 5.5*C*; quantification done by Sarah Latchney). Noticeably, MEF2A is more enriched in immature neurons compared to MEF2C, and 2D. Finally, MEF2 is also expressed in postmitotic NeuN+ neurons and prox1+ neurons. In summary, MEF2 family is not expressed in NSPCs, TAPs, but only in immature and mature neurons (Fig. 5.5*D*).

Colocalization experiments could only reveal whether MEF2 proteins were present in one specific cell population, but not about the levels of these Mef2 genes. Since MEF2 is only expressed in immature and mature neurons in the dentate gyrus, I sorted out GFP+ cells from hippocampus of three 6-week-old prox1-GFP transgenic mice (186) by flow cytometry. Prox1 was enriched in GFP+ cells compared to GFP- cells. QPCR analysis of the levels of three Mef2 genes showed that Mef2A was the most highly expressed, while Mef2D was the least abundant (Fig. 5.5*E*), suggesting that Mef2A may plays the most important role among these three Mef2 genes in dentate gyrus.

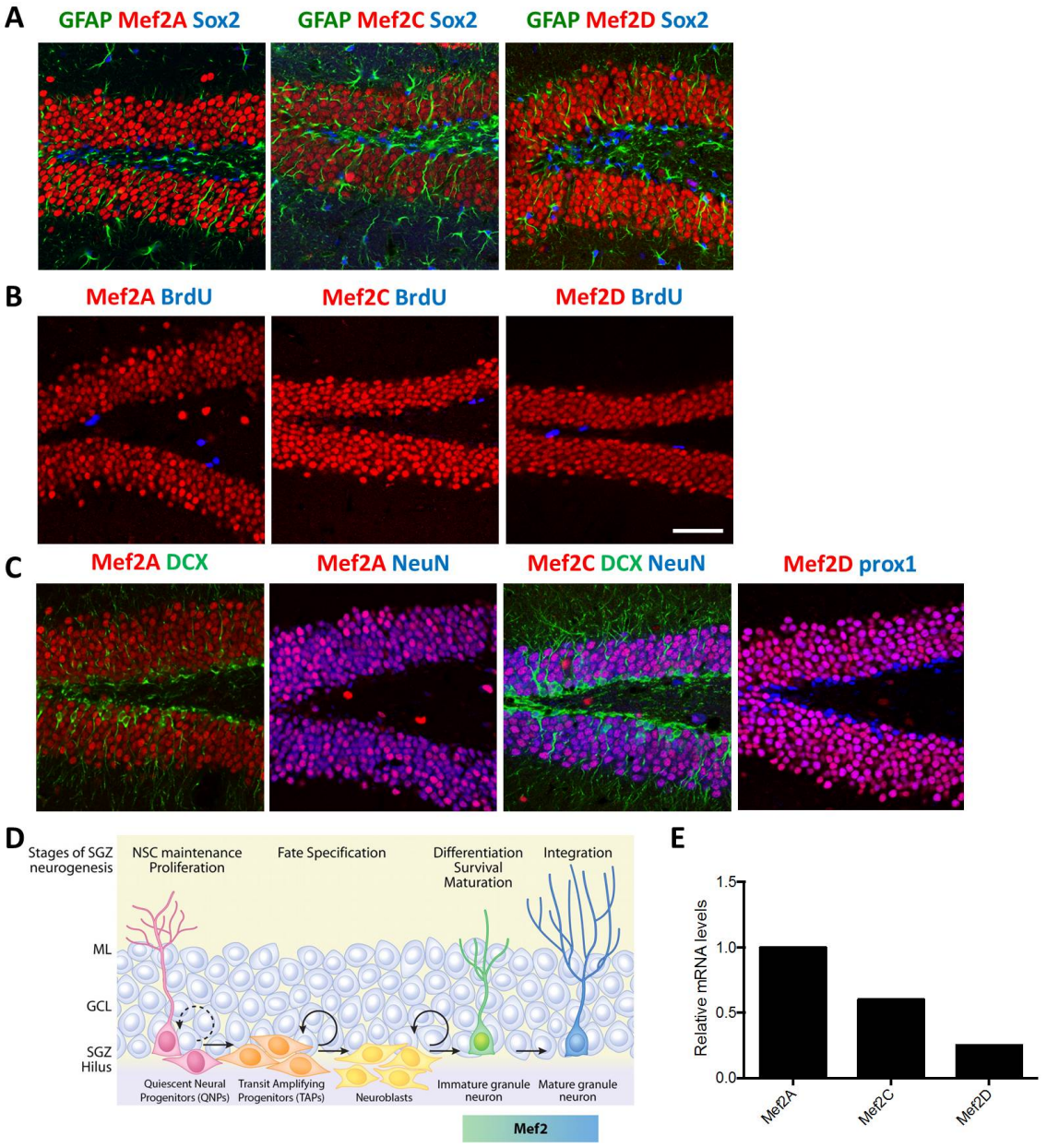


Figure 5.5. MEF2 is expressed in immature and mature neurons in adult dentate gyrus. (*A*) Immunostaining of GFAP (a marker of RGLs), MEF2, and Sox2 (a marker of RGLs and TAPs) of brain sections from P30 WT mice. (*B*) Immunostaining of MEF2, DCX (a marker of immature neurons), NeuN (a marker of mature neurons), and prox1 (a marker of immature and mature neurons). (*C*) Immuostaining of MEF2 and BrdU. For *A*-*C*, scale bar: 50 μm. (*D*) YFP+ cells undergo distinct stages of neurogenesis, beginning as quiescent neural progenitors and differentiating into transit amplifying progenitor and ultimately post-mitotic neurons. MEF2 expression is restricted to immature and mature

granule neurons. (*E*) Quantitative PCR analysis of levels of Mef2A, 2C, and 2D in GFP+ cells sorted from 6-week-old prox1-GFP mice (n=3).

Function of MEF2 *in vitro*

To first determine whether MEF2 is required for neurogenesis, we first established primary neurospheres from the hippocampus and SVZ of 1-month old WT and Mef2A$^{F/F}$2C$^{F/F}$2D$^{F/F}$ mice. We confirmed that Ad-Cre-GFP virus could knockout each Mef2 gene in Mef2 triple floxed neurospheres. We found confirmed efficient removal of all three individual Mef2 genes from quantitative PCR analysis (Fig. 5.6*A*).

MEF2 is known for its roles in neuronal survival (117). It is difficult to analyze the neuronal differentiation if massive cell death occurs after deletion of three Mef2 genes, thus, I performed MTS assay to examine cell survival rate of Mef2 triple floxed neurospheres infected with Ad-Cre-GFP virus in different medium. In neuronal differentiation condition (RA/FSK), most of the cells died at two days post infection (data not shown). However, in growth factor withdrawal conditions, Mef2 deleted neurospheres showed comparable survival rate to WT neurospheres (Fig. 5.6*B*). Thus, to explore the function of MEF2 in neuronal differentiation *in vitro*, I infected WT or Mef2 triple floxed neurospheres with Ad-GFP or Ad-Cre-GFP virus in medium without growth factor for 2 days. Considering the intrinsic differences of neuronal differentiation capacity generated during passaging, I normalized beta-III tubulin levels of Ad-Cre-GFP infection to GFP before comparing the differences between WT and Mef2 triple floxed neurospheres. A decrease in beta-III tubulin levels was observed in Mef2 triple floxed neurospheres (Fig. 5.6*C*), indicating that MEF2 was required for neuronal differentiation *in vitro*.

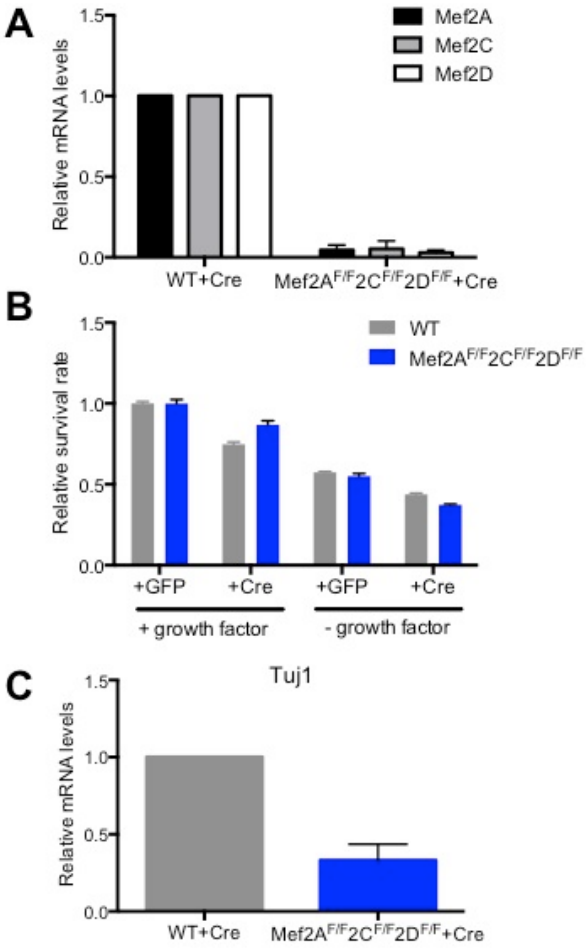


Figure 5.6. MEF2 is required for neuronal differentiation of NSPCs *in vitro*. (*A*) Quantitative PCR analysis of the expression levels of Mef2A, 2C, and 2D in WT and Mef2 triple floxed neurospheres infected with Ad-Cre-GFP virus for 2 days. (*B*) MTS assay of WT and Mef2 triple floxed neurospheres infected with Ad-GFP or Ad-Cre-GFP virus in growth factor containing or withdrawal medium. (*C*) Quantitative PCR analysis of the expression levels of neuronal gene Tuj1 in WT and Mef2 triple floxed neurospheres infected with Ad-Cre-GFP virus. Error bars indicate ± SEM.

Function of MEF2 in adult hippocampal neurogenesis *in vivo*

We have shown that MEF2 is required for neuronal differentiation of NSPCs *in vitro*. To evaluate the impact of deleting Mef2 in adult NSPCs and their progeny *in vivo*, we crossed Mef2A$^{F/F}$2C$^{F/F}$2D$^{F/F}$ mice with tamoxifen-inducible *Nestin-CreER^{T2}* mice (Mef2 iKO mice) and sacrificed mice at various timepoints after TAM (Fig. 5.7*A*). These mice were also bred to R26R-YFP reporter mice so YFP+ recombined cells can be used as a surrogate marker for cells recombined and deleted for Mef2.

Since MEF2 is only expressed immature and mature neurons, we sacrificed Mef2 WT and iKO mice at 19, 30, and 60 days post-TAM, and examined the number of mature neurons (YFP+DCX-NeuN+) and immature neuroblasts (YFP+DCX+NeuN-). Surprisingly, we observed similar accumulation of mature neurons and similar number of immature neuroblasts (Fig. 5.7*B*-*E*). Since Isx-9 could increase the dendritic complexity of DCX+ cells, we examined whether deletion of Mef2 would lead to any morphological defects in immature neurons. We observed stunted dendritic development in Mef2 iKO mice at different time points (Fig. 5.7*F*-*G*). Surprisingly, we also found that DCX+YFP- cells displayed increased dendritic development over time (Fig. 5.7*H*-*I*), indicating a non-cell autonomous effect. To examine whether the other populations would be affected through this mechanism, we quantified the number of YFP+ Type-1 and progenitor cells. We also found an increase in these two populations in iKO mice (Fig. 5.7*L*-*M*), while no difference was observed in WT mice (Fig. 5.7*J*-*K*).

As MEF2 is known to control cell survival of neurons, we examined cell death rate by activated caspase 3 (AC3) staining. However, we did not observe significant increase in iKO mice over time (Fig. 5.7*N*, *P*). Moreover, the volume of GCL did not

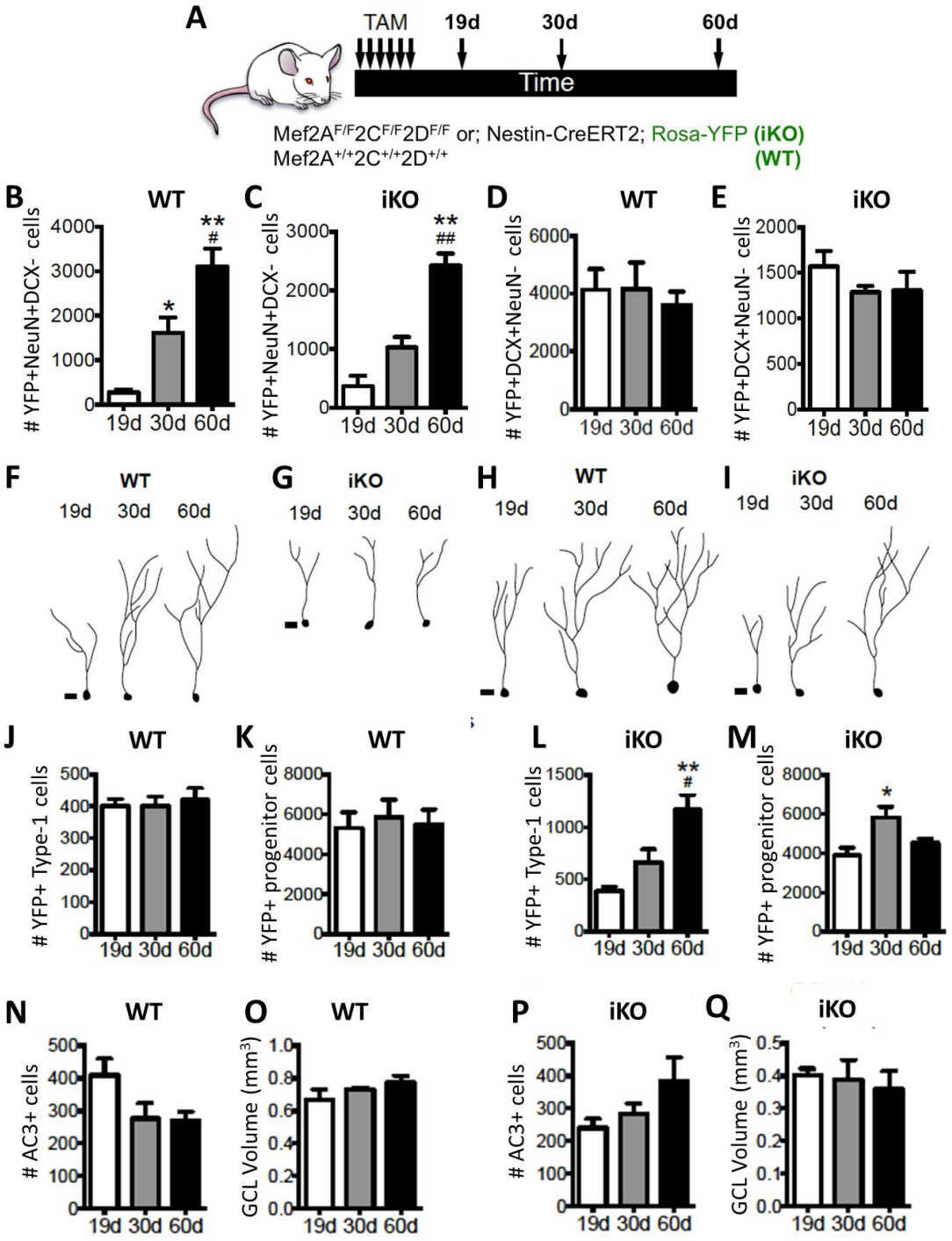


Figure 5.7. Loss of MEF2 results in stunted dendritic development. (*A*) Schematic of tamoxifen (TAM) injection and collection of brain tissue. (*B-C*) Quantification of YFP+DCX-NeuN+ mature neurons in WT (*B*) and iKO mice (*C*). (*D-E*) Quantification of YFP+DCX+NeuN- cells in WT (*D*) and iKO mice (*E*). (*F-G*) Representative dendritic tracings and photomicrographs of YFP+ neurons in WT mice (*F*) and iKO mice (*G*) 19,

30, and 60 days post-TAM. Scale bar, 10 μm. (*H*-*I*) Representative dendritic tracings of DCX+YFP- neurons in WT (*H*) and iKO mice (*I*) 19, 30, and 60 days pos-TAM. Scale bar, 10 μm. (*J*-*K*) Quantification of YFP+ Type-1 stem cells (*J*) and YFP+ progenitors (*K*) in WT mice. (*L*-*M*) Quantification of YFP+ Type-1 stem cells (*L*) and YFP+ progenitors (*M*) in iKO mice. (*N*-*O*) Quantification of total AC3+ cells (*N*) and GCL volume (*O*) in WT mice. (*P*-*Q*) Quantification of total AC3+ cells (*P*) and GCL volume (*Q*) in iKO mice. Data are reported as mean +/- s.e.m and analyzed by 1-way ANOVA with Tukey post hoc test. *p<0.05, **p<0.01.

change over time (Fig. 5.7*O*, *Q*). Taken together, we observed stunted dendritic development in Mef2 iKO mice over time while there is still an apparent increase in the number of immature and mature neurons over time. Interestingly, Type-1 cells and progenitor cells, which have no detectable MEF2 expression, show increased numbers over time, probably due to a non-cell autonomous effect. Taken together, we showed that deletion of MEF2 both *in vivo* and *in vitro* leads to an overall reduction of neurogenesis over time.

Discussion

In this study, we used a disubstituted isoxazole, Isx-9, to promote hippocampal neurogenesis in adult mice *in vivo*. We found that Isx-9 boosts proliferation and neuronal differentiation of SGZ neuroblasts and adult neurogenesis in a cell-intrinsic MEF2-dependent manner, which ultimately correlates with improved spatial memory. We further showed that MEF2 is only expressed in immature and mature neurons in adult hippocampal dentate gyrus. We also observed stunted dendritic development in mice lacking MEF2. Surprisingly, the number of NSPCs is also affected probably through a non-cell autonomous regulatory mechanism. Thus, in addition to discovering the novel *in*

vivo effects of Isx-9, our studies demonstrate a completely new molecular pathway through MEF2 to govern the production of adult-generated neurons.

We first present Isx-9 as a new neurogenesis inducer to enhance adult neurogenesis *in vivo*. Isx-9 promoted the proliferation of hippocampal neuroblasts, leading to enhanced dendritic complexity of immature neurons and more adult-generated neurons. Isx-9, however, did not change the number of NSCs. This is an important feature, since only an unexpended pool of NSCs ensures continuing adult neurogenesis. Our data suggest that the increased neurogenesis seen after Isx-9 is a direct consequence of increased neuroblast proliferation: more dividing cells leads to more surviving cells. However, the mechanism of how Isx-9 enhances dendritic complexity remains more speculative.

Our search for new molecular pathways that control adult mouse SGZ revealed Isx-9-dependent regulation of gene expression networks critical for cell proliferation, development, and movement. From these networks, MEF2 emerged as a prime candidate involved with Isx-9's effects on adult neurogenesis. We showed that MEF2 was robustly upregulated in nestin-expressing stem and progenitor cells by Isx-9 *in vivo*, similar to prior *in vitro* studies with cultured hippocampal progenitor cells (136). MEF2 family members are expressed in DG and are known for their roles in the developing nervous system in regulating neurite growth of Tuj1-positive neurons *in vitro* (187) and embryonic neural stem cell differentiation *in vivo* (122). However, prior to our data here, Mef2 had never been assessed for a cell-intrinsic role in adult-generated hippocampal neurons. Here we genetically deleted hippocampus-enriched three Mef2 genes (Mef2a/c/d) specifically in NSPCs and their progeny *in vivo* and showed that this deletion leads to three unexpected outcomes. First, iKO mice had relatively normal accumulation of mature YFP+ neurons similar to WT mice. Second, iKO mice exhibited an increase in

the number of YFP+ proliferating progenitors and YFP+ Type-1 stem cells that was not seen in WT mice. Third, Mef2A, C, and D deletion in the dentate gyrus niche led to fewer mature YFP- neurons, but with increased dendritic complexity. In contrast, iKO mice continued to generate YFP+ neurons, but with severely stunted dendritic development.

The accumulation of YFP+ mature neurons in our iKO mice was surprising, given that Mef2 has been shown to be a proneurogenic and prodifferentiation factor (119, 122, 123). However, there are at least four differences between this work and published studies. First, the proneurogenic role for Mef2 emerges from work using conditional transgenic mice to manipulate Mef2 genes *in vivo* (119, 122, 123). Therefore, the previous work targets progenitor or postmitotic cell populations in early life, leading to a broad range of cell types and brain regions ultimately influenced in the adult. This is in contrast to our approach in which we used an inducible transgenic mouse to delete Mef2A, C, and D in nestin-expressing mitotic stem and progenitor cells and their progeny. Our deletion of Mef2A, C, and D at the time of nestin-expressing NSPC generation allows for the unique examination of Mef2 deletion as the cell becomes postmitotic. A second difference is the population size influenced. Studies in which Mef2 was manipulated in the adult brain by viral methods targeted a large population of postmitotic neurons (188-190). This is in contrast to our transgenic mouse in which the lower efficiency of TAM-induced recombination of Mef2 allowed for examination of YFP+ neurons – cells missing Mef2A, C, and D – in the context of YFP- neurons, cells expressing Mef2A, C, and D. These different population dynamics may exert considerable influence on the prodifferentiation function of Mef2 within the neurogenic niche. Third, in the two studies in which Mef2 was knocked down in the hippocampal dentate gyrus or CA1, not all of the hippocampal-enriched Mef2 genes were manipulated (123, 188). This may suggest that individual Mef2 hippocampal-enriched genes may

serve distinct proneurogenic functions that have not yet been explored in depth. Finally, it is possible that the amount of recombination varies among our work and published studies. Published studies range in the efficacy of their Mef2 genetic ablation or knockdown, from reporting 50% knockdown (123) to not quantifying it (122). For our study, we are inducing recombination at 4 loci. Given the challenge of inducing recombination equally at all sites (191, 192), it is reasonable to assume that we are not achieving 100% recombination at all genomic sites flanked by loxP sites, but rather decreasing the amount of Mef2A, C, and D protein in each cell.

The second unexpected result in this study is an increase of the number of YFP+ QNPs and TAPs in Mef2 iKO mice, despite the lack of Mef2 expression in this cell population. These results suggest a non-cell autonomous mechanism resulted from immature or mature neurons lacking MEF2. It is possible that neurons lacking Mef2 secrete diffusible growth-inducing factors in the hippocampal niche that influences neighboring QNPs and TAPs. It is also possible Mef2 iKO neurons affect NSPCs through the existing neuronal circuitry, because parvalbumin positive interneurons in the dentate gyrus could send immature GABAergic synaptic inputs to neural progenitor cells for their survival (193).

The third unexpected result in this study is a decrease of dendritic complexity in YFP+ neurons and an increase of dendritic complexity in YFP- neurons. Because MEF2 is required for neuron survival, it is possible that dendritic degeneration we observed after deleting MEF2 is triggered by neuronal apoptosis. To rule out this possibility, close examination of the intactness of their axons and nuclei is necessary. We should introduce anti-apoptotic compounds to cells lacking MEF2 and examine whether the dendritic arborization defects will still be observed.

If MEF2 promotes dendritic arborization independent of cell death, it suggests that MEF2 regulates dendritic morphology through an unidentified cell intrinsic or circuit-level change. Several contributors have been identified to control dendritic morphology, such as transcription factors, local translational machinery, and cytoskeletal elements (194). It would be interesting in future studies to examine whether MEF2 is involved in these mechanisms.

Deficits in dendritic formation may be compensated by maintenance of neuron production. In the case of our iKO mice, we observed fewer DCX+ neuroblasts, but with more complex dendritic morphology. Although this may be a mixed population of YFP+ and YFP- immature neurons, we presumed the vast majority of DCX+ cells to be YFP- immature neurons. Increased dendritic complexity could be an added compensatory response from non-nestin lineage neurons to maintain a healthy level of neural connections. Such increased dendritic complexity may help maintain connections, and be sufficient to sustain hippocampal function and prevent behavioral deficits in the absence of adult neurogenesis.

Dendritic complexity is positively correlated with learning and memory (180). It is possible that Isx-9-enhanced complexity of the dendritic trees contributes to Isx-9-induced improved Morris water maze memory. Of course, the Isx-9-induced increase in absolute number of adult-generated neurons is also likely to contribute to improved hippocampal-dependent memory. It is interesting in future studies to examine hippocampal-dependent tasks in MEF2 iKO mice, such as Morris water maze.

Future directions

There are several future directions that emerge from our discovery that Isx-9 and MEF2 could regulate adult hippocampal neurogenesis. First, as Isx-9 promotes the later

stages of adult proliferation and enhances neurogenesis without exhausting the NSC pool, Isx-9 might be useful in conditions where proliferation is reduced but NSCs are still present, such as during disease or aging. However, since neurogenesis is evident in other brain regions, such as more anterior subventricular zone, and since neurogenesis in these regions could have functional consequences, future studies should clarify if and how Isx-9 alters neurogenesis outside of the SGZ.

Second, as Isx-9 also promotes spatial memory without significantly influencing learning, it joins the growing list of agents or manipulations that can promote memory but not learning, future studies on Isx-9's specific binding sites and cellular targets might help expand our understanding of how memory and learning is regulated. A G_q protein-coupled receptor, GPR68 was identified as a target of Isx-9 in Notch-activated epicardium-derived cells (NECs). It was also detected in the process of quiescent neural progenitor cells in the brain. However, GPR68 may not be a target of Isx-9 in the brain, because treatment of Isx-9 did not affect the number of quiescent neural progenitor and transit amplifying progenitor cells. A new target of Isx-9 in later stages of neurogenesis needs to be identified in the future.

Finally, while our study focused on Isx-9 induced enhancement of hippocampal neurogenesis and function and its reliance on MEF2, out data show that Isx-9 has a broad effect on genes. When combined with the fact that Isx-9 has positive effects on other cell types and systems, additional work is warranted to clarify whether Isx-9 serves as a proliferation and differentiation agent in many stem cells containing tissues.

In summary, we have uncovered the function of pro-neurogenic compound Isx-9 *in vivo* and characterized the role of one Isx-9 target, MEF2 in adult hippocampal neurogenesis. Our work suggests that Isx-9 may become a promising preclinical drug

candidate for situations when neuroblasts in neurodegenerative diseases and/or adult-generated neurons are reduced and traditional physiological stimulation of adult neurogenesis fails (195).

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