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REST/NRSF is A Master Negative Transcriptional Factor for Neurogenic Program by Maintaining Stem Cell Quiescence in Adult Hippocampal Neurogenesis

A Thesis Submitted to
The Biotechnology Graduate Program
in partial fulfillment of the requirements for the degree of Master’s of Science

By
Mostafa Nashaat Ahmed Abdelhamid

Under the supervision of
Prof. Dr. Hassan M. E. Azzazy
Professor and Associate Dean, School of Graduate Studies and Research, The American University in Cairo

Prof. Dr. Jenny Hsieh
Assistant Professor, Department of Molecular Biology, University of Texas Southwestern Medical Center

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Adult neurogenesis occurs in two distinct regions inside the brain, the subventricular zone (SVZ) in the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in hippocampus. Neural stem cells (NSCs) are controlled by several intrinsic and extrinsic factors that regulate their proliferation, survival and differentiation in the adult hippocampus. REST/NRSF is considered a master transcription factor that regulates hundreds of neuronal genes essential for neuronal fate decision. In this study, we address the role of REST/NRSF (RE1 silencing transcription factor/Neuro-restrictive silencer factor) in regulating adult neurogenesis in hippocampus. REST/NRSF has biphasic expression in neural stem cells and mature neurons. It serves as a critical controller of neural stem cell fate transition and neuronal differentiation. REST/NRSF maintains neural stem cell quiescence preventing its precocious differentiation into neurons. This study shows that deregulation of REST/NRSF function, conditional deletion in REST/NRSF allele, in vivo leads to a transient increase in adult hippocampal neurogenesis and NSCs exit quiescence. Consequently, this reflects a decrease in newborn neurons due to stem cell pool exhaustion. Our work asserts on the critical role of REST/NRSF as a negative master regulator for adult neurogenesis. The results offer a new horizon for the role of NRSF in regenerative medicine and development of new treatment strategies for cancer research.
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DEDICATION

To my father, mother, and my brothers, Ahmed and Mohamed, and their lovely families.
Glossary and Abbreviations

Activated caspase-3 (AC3): A cysteine proteases expressed as inactive pro-enzyme when cell undergo apoptosis.

Ascl1 (achaete-scute complex-like 1): A basic-helix-loop-helix (bLHL) transcription factor essential for neural stem cell fate specification and differentiation (previously known as Mash1).

Astrocytes: Subset of glial cells, star shaped, responsible for supporting endothelial cells and neural networks integration.

Apoptosis: Natural process of programmed cell death.

Bromodeoxyuridine (BrdU): Synthetic thymidine analogue used as a marker for neural stem cell proliferation by incorporation in proliferating neural stem cell, S phase, replacing DNA thymidine.

CA1: Cornu Ammonis 1 of hippocampus.

CA2: Cornu Ammonis 2 of hippocampus.

CA3: Cornu Ammonis 3 of hippocampus.

Conditional knock out mice (cKO): Genetically engineered mice with defined deactivation in one gene or more through inducing mutation or partial deletion in gene sequence. Conditional knockout mice are used when deletion of certain gene at embryonic stage is lethal; therefore, deletion is induced at a late stage after birth using different knockout induction system such as Cre-Lox recombination system.
Confocal microscopy: Optical microscopy that allows image three-dimensional structural reconstruction utilizing pinhole and point illumination to out of focus signals. Confocal microscopy has major advantages over the epifluorescent microscopy such as elimination of out of focus background, controlling field depth, and getting very thin optical sections in case of thick specimen.

Cre-Lox recombination system: Site-specific recombination system that allows specific targeting of DNA sequences and splicing with Cre recombinase. Cre-Lox system is commonly utilized in generating conditional knock out mice.

Dentate gyrus (DG): Region in hippocampus responsible for the formation of new memories. It consists of granular cell layers extending its projections to hippocampus pyramidal cells and interneurons.

4,6-diamidino-2-phenylindole (DAPI): A strong fluorescent stain binds to DNA and used to label all living and fixed cells.

Doublecortin (DCX): Microtubule associated protein expressed in immature cells.

Endothelial cells: A layer of cells lines the blood vessels responsible for supporting neurons with nutrition.

Ependymal cells: Cell layer lines the ventricular zone of mice brain.

GABAergic neurons: Subclass of neurons that receive inhibitory stimuli from the neurotransmitter Glutamate.
**Glutamergic neurons:** Subclass of neurons that receive excitatory stimuli from the neurotransmitter Gama-AminoButyric Acid (GABA).

**Glial fibrillary acidic protein (GFAP):** An intermediate filament protein specifically expressed in radial neural stem cell, and developing and mature astrocytes.

**Green fluorescent protein (GFP):** 238 amino acids protein exhibits a strong green fluorescence when exposed to blue light used as a reporter protein to confirm successful Cre-Lox recombination.

**Glia:** Support cells in central nervous subclassed into oligodendrocytes, astrocytes, and microglia.

**Glutamate:** Excitatory neurotransmitter its increase causes decrease in neurogenesis.

**Granular cells:** Small neurons present in granular zone of hippocampus, and olfactory bulb characterized by diverse functional anatomical structure. Granular neurons in olfactory bulb are axonless and GABAergic while in hippocampus they are glutamatergic extending their axons to the CA3 region.

**Gelsolin (GS):** Actin-binding protein, specifically, expressed in oligodendrocytes in central nervous system.

**Glutathion S-Transferase π (GSTπ):** A member of Gelsolin family, specifically, expressed in oligodendrocytes.
**Hippocampus:** The Greek name of seahorse. It’s located in the medial temporal lobe and consists of proper hippocampus, subiculum and dentate gyrus. Hippocampus is essential for major brain cognitive functions as learning and memory retrieval.

**Immature neurons:** Undifferentiated young neurons haven’t been integrated in the neural network.

**Ki67:** Nuclear protein essential for proliferation in cell cycle interphase used as a proliferation marker for neural stem cells.

**Long-term-potentiation (LTP):** Long lasting increase in neuronal excitability due to high frequency synaptic input. LTP is major molecular mechanism involved in learning and memory formation.

**Mature neurons:** Neurons exited cell cycle and completely integrated in neuronal network expressing mature neuronal factors such as NeuN.

**Microglia:** Non-neuronal cells regulating extracellular environment surrounding neural stem cells and mature neurons.

**Neurogenic differentiation 1 (NeuroD1):** A basic-helix-loop-helix (bLHL) transcription factor essential for neuronal stem cell fate specification and used as a marker for neuronal lineage differentiation marker.

**Neuronal specific nuclear protein (NeuN):** DNA binding nuclear protein expressed in post mitotic neurons used as a marker for mature neurons.
Neuron-specific class III beta-tubulin (TubIII): Also known as Tuj1, essential for microtubules stability in axons and neuron cells bodies, and used as a marker for neuronal cell bodies.

Neural stem cells (NSCs)/Neural progenitor cells (NPCs): Undifferentiated neural cells have the capacity to differentiate into different neural linage, neurons, astrocytes, and oligodendrocytes.

Neurogenesis: The process in which neural stem cell proliferate, differentiate into neuronal linage, migrate, and become fully integrated in neuronal network.

Olfactory bulb: Protrusion located in the vertebral forebrain responsible for olfaction, odor sensation.

Oligodendrocytes: Non-neuronal support cells essential for proper synaptic transmission between neurons.

Proliferating cell nuclear antigen (PCNA): nuclear protein essential for DNA polymerase activity in eukaryotic cells used as a marker for neural stem cell proliferation.

Platelet-derived growth factor alpha receptor (PDGFαR): Cell surface tyrosine kinase receptor, specifically, expressed in differentiating oligodendrocytes.

Prospero homeobox protein 1 (Prox1): Prox1 is used as a marker for neuronal linage differentiation marker.
**Rostral migratory stream (RMS):** A migratory pathway originates in the subventricular zone and ends at the olfactory bulb, through which neural progenitor cells migrate from SVZ until it settles as differentiated neurons and fully integrated in olfactory bulb.

**RE1 silencing transcription factor (REST)/ Neuro-restrictive silencer factor (NRSF):** GLI-Kruppel class C2H2 Zinc finger protein that binds to neuronal genes promoters and negatively regulating their expression.

**SRY (sex determining region Y)-box 2 (Sox2):** Transcription factor essential to maintain stem cell renewal. Sox2 is a neural stem cell marker expressed in quiescence and in the early stages of proliferation.

**Stem cell niche:** The microenvironment where stem cells are found. This microenvironment contains the essential intrinsic factors to facilitate stem cell renewal and maintenance. In adult brain, there are only two neural stem cell niches in subgranular zone in hippocampus and subventricular zone lining the lateral ventricle.

**Subgranular zone (SGZ):** This region located in the dentate gyrus of mouse hippocampus where adult hippocampus neurogenesis takes place. SGZ is a rich microenvironment of intrinsic and extrinsic factors required for adult neural stem cells proliferation and differentiation.

**Subventricular zone (SVZ):** This region located in the mouse lateral ventricle where adult neurogenesis takes place. SVZ is a rich microenvironment of intrinsic and extrinsic factors required for adult neural stem cells maintenance and proliferation.
**Synaptic transmission:** Chemical signals responsible for communication between neurons.

**Tamoxifen (TAM):** Estrogen analogue used to induce conditional deletion in vivo using Cre-Lox recombination.

**Wnt Signaling:** A major pathway characterized by a network of proteins with essential role in embryogenesis and cancer progression. The word Wnt is a combination of “Wn” which means wingless and “Int” which are group of genes usually integrated near tumor viruses.

**Yellow fluorescence protein (YFP):** Genetic mutant of GFP exhibits a strong yellow fluorescence and used as a reporter protein to confirm successful Cre-Lox recombination.
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Chapter 1: Introduction

A. Adult Neurogenesis

A.1. Background and Introduction

A milestone is now well established in developmental neuroscience that our brains are capable of producing new neurons in adulthood (Ming and Song 2005). This understanding is opposing the early dogma that only our embryonic and developing nervous systems are able to generate new neurons; once development is completed, there is no chance to have further new neurons. Indeed, this old dogma might seem strange, particularly as most organs have certain cells, stem cells, which can replace and expand the organ size. In mammals, blood, skin, and gut have stem cells that are present even after adulthood. Furthermore, non-mammalians such as insects and fish have neural stem cells that can replicate throughout their life. The central dogma of neurobiology, which concluded that no new neurons are generated in the adult brain, was deduced from the speculation that only astrocytes, microglia, and oligodendrocytes can normally divide and repair injury, while neurons can't. Currently, we know that this dogma is not completely true; compelling evidence showed that there are two discrete areas that can generate neurons in adult brain. The first region is the subventricular zone and its rostral migratory stream projection to the olfactory bulb and the other one is the dentate gyrus in the hippocampus (Ming and Song 2005).

Although it is considered a very important event that new neurons generate in certain areas in adult brain, it is very crucial to address the reasons why most regions
in brain don't exhibit this property. The first reason is the neural complexity as each neuron contains axon and extremely branched dendrites with many connections. Therefore, scientists considered all neurons of the adult brain are terminally differentiated and "postmitotic". The other reason was the neural network complexity of the adult brain; if a new neuron is formed, how it would be incorporated in the existing circuit without affecting its integrity? (Weissman, Anderson et al. 2001; Gage 2002). In fact, this reason had been refuted by another concept called the structural plasticity. Simply, structural plasticity, which has been supported by many further studies, is the change that occurs in the organization of neural synaptic network as a result of experience (Gage 2002). Synaptic plasticity had been proposed first by Altman in early 1960s (Altman 1962). Afterward, many studies supported structural plasticity by providing evidence of synaptic reorganization after injury. This theory made the acceptance of neurogenesis less problematic. Mainly, neurogenesis occurs in hippocampus where it might be involved in forming new memories (Wittenberg and Tsien 2002). However, these reasons, neural network complexity and the absence of regeneration after injury, made the process of accepting the idea of new neurons generation in adult brain very slow.

The idea of adult neurogenesis was first proposed in 1960s by Joseph Altman who has reported that some dividing cells in adult rat brain can differentiate to cells similar to neurons in morphology using thymidine autoradiography (Altman 1962). Then, through the subsequent years Altman and his team have confirmed their previous results by more focused experiment using light microscopy to observe the birth of neurons during development (Altman and Das 1966). However, the low
resolution of phenotypes by light microscopy made the observation not good enough to judge the process. Basically, the absence of definite phenotypic markers for tracking progenitor and mature neurons limited the progress in this field. Throughout 1970s and early 1980s the initial observations reported by Altman have been reexamined by Michael Kaplan (Kaplan and Hinds 1977). By using the electron microscopy, Kaplan and his team confirmed Altman's results and showed that the newly formed cells have the same ultra-structure, neurogenic structure, of their neighbor cells in dentate gyrus.

In early 1990s, several papers on adult mice and rats had reported identifying neural cells with stem cell properties that are able to divide and expand when isolated and cultured (Richards, Kilpatrick et al. 1992; Lois and Alvarez-Buylla 1993). Using different culture conditions, these cells demonstrated the ability to differentiate into neurons, astrocytes, and oligodendrocytes as in the developing brain (Reynolds and Weiss 1992). These results had refuted the first reason for resisting adult neurogenesis concept as the discovery of neural stem cell in brain provided a mechanism for the origin of new neurons. While mature neurons will appear in postmitotic state, certain population of immature neural stem cells will proliferate and differentiate in a process called adult neurogenesis.

The term “adult neurogenesis” describes the observation that new neurons are formed in discrete regions in adult mammalian brain. These new cells migrate and differentiate in certain locations where they are integrated and become functional. Therefore, adult neurogenesis is considered a process not an event. It is an exceptional process, which occurs in hippocampus and olfactory bulb (Gage 2002).
The literature review will address the major processes of adult neurogenesis, and the major regulatory intrinsic and extrinsic factors. Also, it will discuss the functional significance of adult neurogenesis. The analysis of adult neurogenesis is based, mainly, on tracing neural stem cells at different developmental stages using specific markers for each development stage. Therefore, generating antibodies for different markers at different developmental stages was a milestone that accelerated the field development. In our study, we used different antibodies to trace several developmental markers that allowed us to precisely identify the expression pattern of REST/NRSF expression, as well as the impact of REST/NRSF deletion on adult neurogenesis.

A.2. Short Description of Subventricular zone (SVZ) and Subgranular zone (SGZ) Niches.

Adult neurogenesis occurs only in two discrete regions in adult brain, the hippocampus and subventricular zone. Hippocampal neurogenesis takes place only in the dental gyrus, therefore, the majority of research that deals with adult hippocampal neurogenesis is focused on the dentate gyrus. Cells generated from neurogenesis process in dentate gyrus are granular neurons. Granular neurons are excitatory neurons with their cell bodies present in dentate gyrus. These cells receive input from cortex and their axons are extended to the pyramidal cells of CA3. The niches where precursors originate reside in thin tissue band close to the granular layer called the subgranular zone (SGZ). This niche comprises different patterns of developing cells. On the basis of cell markers and morphology, two types of progenitor cells have been identified (Figure 1).
Type1 hippocampal progenitors stretch across the entire granular layer, extending their processes in the molecular layer. These progenitors express Sry-related HMG box transcription factor (Sox2), glial fibrillary acidic protein (GFAP), and nestin (Fukuda et al., 2003; Suh et al., 2007; Garcia et al., 2004). Although the astrocytic nature of hippocampal progenitors, as they share the GFAP with mature astrocytes, these cells are completely different in morphology and function. On the other hand, type 2 progenitor cells do not express GFAP. Although, many studies have reported that type 2 is the developed form of type 1, evidence linking the pathway of the two types is still lacking. Type 2 progenitors express Sox2, stemness marker, have provided the first evidence for hippocampal neural stem cell in vivo (Figure 2) (Suh et al., 2007). These cells have the ability of self-renewal and differentiation to neurons or astrocytes.

The subventricular zone (SVZ) is located next to a thin layer of cells lining the lateral ventricle, so called ependyma. First, it was supposed that ependymal cells are responsible for neurogenesis in SVZ (Johansson, Momma et al. 1999). However, many studies showed that this ependymal layer is quiescent (Capela and Temple 2002). Interestingly, neural stem cells in the SVZ migrate to the olfactory bulb (OB) and differentiate to mature neurons. Three types of neural stem cells have been identified in SVZ, Type A, B, and C. Type B cells which express GFAP seems quiescent as antimitogens don't affect them. On the other hand, type A and C progenitors demonstrate stemness property that could be identified by BrdU and markers expression of DCX, and PSA-NCAM (Consiglio, Gritti et al. 2004).
Although neural stem cells have been isolated from several regions in adult brain, only SVZ and SGZ demonstrate consistent neurogenesis in vivo. The reason behind this observation is the unique properties of their neurogenic niche, the microenvironment in SGZ and SVZ. This microenvironment supports neural stem cells proliferation, differentiation and integration as well. In SGZ, adult neural stem cells are closely surrounded by different phenotypes such as mature neurons, astrocytes, oligodendrocytes, and endothelial cells in different developmental stages (Zhao, Deng et al. 2008). In particular, astrocytes play major role in neurogenesis process by promoting neural differentiation and integration of adult hippocampal stem cells (Song, Stevens et al. 2002). More importantly, further studies have proposed that astrocytes Wnt signaling pathway is responsible for promoting neurogenesis. Blockage of this pathway will inhibit neurogenesis in vitro and in vivo (Lie, Colamarino et al. 2005).

In SVZ, the ependymal cell layer is adjacent to neural progenitors. Ependymal cells promote SVZ neurogenesis process by two ways. First, ependymal cells express Noggin protein that will antagonize bone morphogenetic protein (BMP) signaling, a group of cytokines and growth factors. A Second way by which ependymal cells promote neurogenesis is by expressing pigment epithelium-derived factor. Furthermore, dopaminergic neurons adjacent to progenitor cells release dopamine that supports SVZ adult neurogenesis (Lim, Tramontin et al. 2000). Neural stem cells in SGZ and SVZ are present in close proximity to vasculature. Vascular endothelial growth factor (VEGF) has great impact on promoting stem cell proliferation in SVZ and SGZ (Palmer, Willhoite et al. 2000; Cao, Jiao et al. 2004). Many factors can
influence neurogenesis process in niche microenvironment. Progenitors might be affected directly by local cell-cell interaction or indirectly by cells out of the microenvironment through synaptic contact in neural circuit (Alvarez-Buylla and Lim 2004).

A.3. Intrinsic and Extrinsic Factors Affecting Adult Neurogenesis.

SGZ stem cells microenvironment is surrounded by complex neuronal circuitry. Consequently, neurons in dental gyrus receive several inputs through numerous neurotransmitters from different parts in the brain (figure 3). Mainly, neurons of dental gyrus receive excitatory glutamatergic and inhibitory GABAergic inputs from entorhinal cortex and dental gyrus, respectively (Hagg 2005). Glutamate excitatory input can be detected by neural progenitors through NMDA receptor. NMDA expression is closely related to neural progenitors' proliferation and differentiation. Evidence has shown that NMDA receptor is down regulated in proliferating progenitors, while its expression will drive the differentiation of neural stem cells by the action of glutamate (Nacher and McEwen 2006). On the other hand, inhibitory GABAergic input depolarizes hippocampal type 2 progenitors by increasing calcium ion influx, which results in expressing NeuroD1, neuronal differentiation factor. Also, calcium antagonists have shown a great impact on decreasing hippocampal progenitor differentiation (Deisseroth, Singla et al. 2004; Tozuka, Fukuda et al. 2005; Zhao, Deng et al. 2008). Through a number of neuronal markers, neural stem cells can be tracked at different stages of neuronal differentiation by tracing neuronal gene expression. NeuroD1 and Ascl1 are well known markers for early neuronal differentiation, while DCX and Prox1 are usually
expressed in immature neurons. As for postmitotic neurons, the expression of NeuN and morphological characterization are considered to describe mature neurons.

As previously mentioned, potent mitogens as EGF and FGF2 effectively promote the proliferation of neural stem cells in vitro. In vivo, the effect of each factor depends mainly on its receptor expression. While both mitogens increase the proliferation of SVZ neural stem cells, only FGF2 demonstrates the same action in olfactory bulb (Kuhn, Winkler et al. 1997). Basically, EGF suppresses the differentiation of type c progenitors by acting on the epidermal growth factor receptor (ErbB2) (Doetsch, Petreanu et al. 2002). In SGZ, FGF2 will promote neurogenesis through acting on fibroblast growth factor receptor (fgfr1). In addition, there are several external signals that regulate neurogenesis. In SVZ, brain derived neurotrophic factor (BDNF) affect adult neurogenesis process positively (Zhao, Deng et al. 2008). In SGZ, BDNF play an important role in survival and integration of new neurons (Olson, Eadie et al. 2006). Furthermore, extrinsic factor may affect neurogenesis indirectly by affecting neighbor cells within the neurogenic niche. Therefore, more studies are required to reveal the perplexity of extrinsic pathways in neurogenesis.

Beside the action of intercellular signaling of the mentioned mitogens and neurotrophic factors, many other intracellular factors still affect adult neurogenesis. Transcription factors such as TLX, nuclear receptor family of transcription factors, and BMI-1, polycomb finger oncogene, promote proliferations in forebrain, while Pax6 promote differentiation in SVZ. Also, epigenetic factors affect adult neurogenesis; methyl-CpG binding protein deficiency will decrease neuronal

A.4. Contribution of Newborn Neurons to Hippocampal Circuitry and Function

Developing newborn neurons in dentate gyrus pass through different morphological and neurophysiologic stages. First, GABA input induces depolarization by increasing intracellular chloride (figure 4). After 2 weeks from birth date, GABA action will switch to polarization that will induce glutamate input and the growth of dendritic spine (Overstreet Wadiche, Bromberg et al. 2005). At this stage, new neurons will form synapses with hilar and CA3. Then, Disrupted-in-Schizophrenia-1 protein (DISC) will control further dendritic growth and maturation (Duan, Chang et al. 2007). More importantly, several regulatory mechanisms regulate the survival of newborn neurons in the first 3 weeks. These include NMDA signaling, animals' experience, and exposure to enriched environment that all regulate survival rate in the first 3 weeks (Kee, Teixeira et al. 2007).

After 4 weeks, new neuron will have almost the same morphological and physiological features of mature neurons, as they will demonstrate the same glutamatergic, and GABAergic response as the existing mature neurons in dentate gyrus (Laplagne, Kamienkowski et al. 2007). Neural integration within the existing network has been observed by measuring the threshold for long-term potentiation, the degree of synaptic connectivity. While 2 weeks old neurons have demonstrated low threshold, 4 weeks old neurons demonstrated a higher one. In hippocampal neurogenesis, the critical time in neurogenesis process is the first three weeks from
birth date. After integration, new neurons start their function normally (Ge, Yang et al. 2007).

Hippocampal region is involved in major cognitive processes such as learning and memory. The relationship between SGZ neurogenesis and spatial memory, memory required to identify animal's environment, was observed in several strains in mice (Kempermann and Gage 2002). Basically, neurogenesis was induced by environmental methods such as physical exercise or exposure to enriched environment. In contrast, aging and stress has been used as negative regulators for neurogenesis. Mice with lower rate of neurogenesis demonstrated impaired hippocampal-learning ability. Moreover, mice with induced neurogenesis showed higher recognition memory than normal (Snyder, Hong et al. 2005; Olson, Eadie et al. 2006). Further studies have developed more direct way to demonstrate the relation between neurogenesis and cognitive functions. Studies used several experimental methods to ablate neurogenesis such as irradiation, antimitotic treatment, and genetically engineered animal models. Although these methods were not selective in ablating SGZ neurogenesis, it provided us with very interesting results. Methylazoxymethanol acetate (MAM)-treated rat (antimitotic treatment) showed defect in forming hippocampal dependant fear conditioning response, learning assay uses conditioned fear. Similar results have been found in X-ray irradiated mice (Shors, Miesegaes et al. 2001; Saxe, Battaglia et al. 2006).

Pervious results provide a primary insight on the role of SGZ neurogenesis in cognitive functions. However, extensive future studies are required with more selection ablation methods, and specific behavioral tests.
B. REST/NRSF

B.1. Discovery

The transcriptional repressor Neuro-restrictive silencer factor (NRSF), also, known as REST (RE1 silencing transcription factor), was first discovered in 1995 as a key regulator of neuronal genes expression in developing nervous system (Chong, Tapia-Ramirez et al. 1995; Schoenherr and Anderson 1995). NRSF is GLI-Kruppel class C2H2 Zinc finger protein that binds to neuronal genes promoters that contain 21-23 bp highly conserved DNA sequence, known as RE1 (repressor element 1, also called NRSE) (Chong, Tapia-Ramirez et al. 1995; Schoenherr and Anderson 1995) (Figure 5). NRSF has been shown to be an essential transcriptional factor for vertebrates’ development. It orchestrates epigenetic regulation in non-neuronal tissues by harboring three functional domains. NRSF has a DNA binding domain having eight zinc fingers that allows it to bind to RE1 motif. The other two binding domains reside on the amino and the carboxyl terminals of the transcription factor controlling its transcriptional activity (Andres, Burger et al. 1999; Ballas, Battaglioli et al. 2001; Ballas and Mandel 2005). Straight homozygous deletion of NRSF in mouse exhibits embryonic lethality at early stage (E 9.5), with malformation in telencephalon accompanied with enormous cell death. Dysregulation of REST expression or function throughout embryogenesis causes growth retardation such as ectopic neuronal genes expression and defects in heart development leading to embryonic death (Chen, Paquette et al. 1998). Downregulation of NRSF in chick mitome by introducing dominant negative NRSF construct leads to neuronal gene expression. On the other hand, downregulation of NRSF in chick embryos leads to neuronal
pathfinding errors (Chen, Paquette et al. 1998; Paquette, Perez et al. 2000). Several studies have shown that NRSF regulates neural genes expression by activating several epigenetic mechanisms to mediate a controlled repression (Ballas, Grunseich et al. 2005).

**B.2. REST/NRSF Action as Transcriptional Repressor**

The major role of NRSF is to prevent neural gene expression in embryonic stem cells and non-neuronal tissues (Jorgensen and Fisher 2010; Yamada, Aoki et al. 2010). However, evidence has shown that NRSF is considered a negative key regulator of neuronal genes expression in developing nervous system (Sun, Greenway et al. 2005). NRSF and its co-repressors control the transition of pluripotent stem cells to neural stem/progenitor cells and their further differentiation into mature neurons suggesting that NRSF supports stem cell phase and prevents precocious neuronal expression (Ballas, Grunseich et al. 2005; Sun, Greenway et al. 2005) (Figure 6). NRSF orchestrates several epigenetic mechanisms at the RE1 site to inactivate neural genes. NRSF binds to its co-repressors mSin3 A and B through its N-terminal, then recruits Co-REST on its C-terminal activating a set of chromatin modifying enzymes, such as histone deacetylases (HDACs), histone demethylases, and histone methyltransferases, and heterochromatin protein 1 (HP1), and consequently mediate chromatin condensation that silence gene expression (Figure 6) (Ballas, Battaglioli et al. 2001; Ballas, Grunseich et al. 2005; Ballas and Mandel 2005). During neuronal development, downregulation and degradation of NRSF complex has been noticed along the differentiation of cortical progenitor to mature neurons. Although NRSF complex degradation leaves the RE1 site of neuronal genes,
only partial activation of a subset of neuronal genes happens as Co-REST and MeCP2 maintain the repression the adjacent sites of neuronal genes promoters (Figure 6). Surprisingly, other studies detected high expression level of NRSF in adult granular and pyramidal neurons in hippocampus. Also, brain insults such as ischemia and seizure lead to upregulation of NRSF in mature neurons (Palm, Belluardo et al. 1998; Consiglio, Gritti et al. 2004; Kuwabara, Hsieh et al. 2004; Jessberger, Nakashima et al. 2007).

Studies have shown that NRSF degradation promotes the transition of the pluripotent stem cells to neural progenitors making neural genes poised for expression; however, it maintains the chromatin inactive. As neural stem cells differentiate to mature neurons, NRSF dissociates from the RE1 site provoking neural gene expression (Ballas, Grunseich et al. 2005; Sun, Greenway et al. 2005). The implications of NRSF dysfunction in central nervous system was reported in the pathogenesis of Down’s syndrome, Huntington, and Alzheimer diseases (Okazaki, Wang et al. 1995; Bahn, Mimmack et al. 2002; Zuccato, Tartari et al. 2003). These studies emphasize on the importance of NRSF for central nervous system development and adult neurogenesis. Although several research groups have elucidated the role of NRSF in neuronal development, data that elucidate NRSF expression profile in developing and adult nervous system is not detailed. In this research work, we, experimentally, support the role of NRSF as a master negative regulator of neuronal gene expression.
Chapter 2: Materials and Methods

2.A. Generation of Conditional Induced NRSF Knockout Mice.

In this study, we utilized the Cre/LoxP system to generate NRSF$^{loxP/loxP}$ conditional alleles based on the original method of Chen and colleagues. This work was done in collaboration with Dr. Robert Hammer, the head of transgenic facility at UT Southwestern who designed and performed this experiment, and the animal facility at University of Texas Southwestern Medical Center. REST/NRSF targeting vector was constructed using the pGKNEO-F2L2DTA vector, which contains two loxP and FRT sites flanking a neomycin resistance gene, and a diphtheria toxin gene cassette at the 3’ end. The 5’ long arm, KO arm, and 3’ short arm of the targeting construct were generated with high-fidelity PCR amplification (LA and Primestar from Takara Bio Inc) of 129SvEv genomic DNA and correlate to a 6.2-kb fragment containing the promoter region and the first three non-coding exons, a 1.8-kb fragment harboring the first coding exon IV, and a 2.3-kb fragment in intron 4, respectively.

The targeting vector linearized by Bcg I was electroporated into 129SvEv embryonic stem cells. Two hundred ES cell clones were screened for homologous recombination first by PCR and then confirmed by Southern blotting. 5’ loxP incorporation was confirmed using a 5’ probe following digestion with Xba I and 3’ loxP incorporation was confirmed with a 3’ probe following digestion with Hpa I. Five clones with properly targeted alleles were injected into 3.5 day old C57BL/6 blastocysts. Four of the clones generated high percentage chimeras and achieved germline transmission when crossed to C57BL/6 females. Heterozygous
REST/NRSF<sub>neo-loxP/+</sub> mice were crossed with hACTB:FLPe transgenic mice to remove the neomycin resistance cassette. Global deletion of the first coding exon (IV) was then achieved by breeding REST/NRSF<sup>+</sup>/fl mice to CAG-Cre transgenic mice, the offspring of which recapitulated the embryonic lethal phenotype as observed with REST/NRSF conventional knockout mice. Mouse genotypes were determined by using PCR tail DNA and primers specific for the REST locus (figure 7). All the in vitro experiments were done by Zhingliang Gao, a postdoctoral fellow at Jenny Hsieh laboratory at UT Southwestern Medical Center.

2.B. Characterization of NRSF Conditional Knockout Phenotype

2.B.1. Tamoxifen Treatment Scheme.

Mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility at UT Southwestern on 12 hours light-dark cycle. NRSF<sup>loxP/loxP</sup> mice were crossed with nestin-CreER<sup>T2</sup>/R26R-YFP to generate the NRSF cKO mice. At the age of 4-6 weeks, mice were injected with intra peritoneal (i.p.) tamoxifen at 150 mg/kg/day for 6 days. Tamoxifen was prepared in 10% ethyl alcohol and 90% sunflower oil. Mice were sacrificed at different time points after the 5<sup>th</sup> day, last injection of TAM. For YFP+ cells counts and morphological analysis of the phenotype, mice (n=8-10 per each group) were sacrificed at 6, 10, 20, 30, 70, and 120 days post-TAM injection (Figure 8). All mice were injected with BrdU, at concentration 10 µg/gm/ml dissolved in 0.9% NaCl, 2 hours before sacrifice.
2.B.2 Sacrifice of Animals, Perfusion and Tissue Processing.

2.B.2.a. Perfusion:

Mice were injected with chloral hydrate, approximately 10 µg/15 gm/mL in mouse ascetic membrane and returned back to the cage. Mice were placed on their back and notes were taken for their twists around, whickering, tail pinching. Using fine scissors, the sternum was cut up to the neck. Mice were held on either side of sternum, and the muscles were cut on both sides until the diaphragm was seen clearly. The diaphragm was cut and the sternum was pulled back to uncover the heart. The needle was inserted horizontally into the left ventricle and the right atrium of the heart was cut. After 6 minutes of pumping 0.1M Phosphate Buffer Saline (PBS) in mouse left ventricle, the pump was stopped and the pumping tube was switched from 0.1M PBS to 4% paraformaldehyde (PFA). Afterward, the pump was reopened for about 15 minutes to assure proper fixation of the brain with 4% PFA. After perfusion, the head of the mouse was detached from the body and brain removed. The brains were placed in 4% PFA overnight at 4°C. Next day, the 4% PFA were replaced by 30% sucrose for cryoprotection and brains stored at 4°C until sinking in sucrose before cutting with microtome.

2.B.2.b. Fractional Sectioning:

After washing with TBS, the brains were cut sagittally into halves, where the right hemisphere was sectioned and the left one stored at 4°C. Using a vibratome, 30 µm think coronal sections were prepared and coronal slices were collected in small wells containing TBS.
Slices were collected in a fractional manner where the 1\textsuperscript{st} slice was placed into the 1\textsuperscript{st} well, 2\textsuperscript{nd} slice into the 2\textsuperscript{nd} well, and so on. The 13\textsuperscript{th} slice was placed into the 1\textsuperscript{st} well, and the 14\textsuperscript{th} into the 2\textsuperscript{nd} well and so on until the 25\textsuperscript{th} slice placed into the 1\textsuperscript{st} well again. This process was repeated until the brain hemisphere was completely sliced. Each slice was separated from the next slice in the same well by 360 µm. A typical adult mouse brain has 120 slices, each slice was 30 µm thick, therefore, every well had included 11 slices per well. Thus, each well had included a set of sections that represents different coronal regions of mouse brain to allow reliable quantification.

2.C. Immunohistochemistry and Co-localization.

2.C.1. Mounted Tissue Immunohistochemistry Fluorescence

On the 1\textsuperscript{st} day of the protocol, sections were washed twice with 0.3% Triton X-100 and TBS. Afterwards, sections were mounted on slides and let to dry for 30 minutes until they stuck onto the glass. Slides were immersed in antigen unmasking solution (0.01M citric acid in MilliQ-water at pH 7.0; freshly prepared) for 15 min at 95°C. Slides were rinsed to cool with TBS for 15 minutes.

Quenching endogenous peroxidases was achieved by covering slides with 0.3% H\textsubscript{2}O\textsubscript{2} (30% V/V) in TBS solution for 30 minutes at room temperature (RT); this step was cancelled if signal amplification were planned. Slides were rinsed twice with TBS, circled with wax pen, and left in TBS. Sections were blocked by 3% normal donkey serum (NDS) and 0.3 % TritonX100 in TBS for 60 minutes at RT. After blocking, primary antibody was added in TBS, 3% NDS and 0.3% Tween 20, and left overnight at
RT. On the second day, primary antibody was washed off with TBS for three times. Fluorescent-conjugated secondary antibody (1:200) was added and left overnight at RT.

In case of signal amplification, biotinylated secondary antibody (1:200) was used and left overnight at RT. After washing twice with TBS, sections were immersed in ABC kit solution, Vector Elite avidine-biotin complex, for 60 minutes at RT. Slides were washed 6 times with TBS before adding tyramide (1:50 in TSA buffer) for 10 minutes at RT. Slides were washed with TBS for 6 times and fluorescence signal observed. Before slides dehydration, DAPI (1:5000) was added for 5 minutes then the slides were dehydrated and covered.

In case of BrdU staining, permeabilization was achieved with 0.1 trypsin in Tris solution and 0.1 CaCl₂ for 8 minutes at RT. After rinsing twice with TBS solution, denaturation step was done using 2N HCl in TBS for 25 minutes at RT. Then, blockage and BrdU staining was done using primary and secondary antibodies. Afterward, DAPI was added and dehydration took place. Dehydration was achieved through 2 steps; first, slides were immersed in ethyl alcohol with different concentration 70%, 95%, and 100% for 20 minutes. Second, the slides were immersed in Citro-solv solution for 20 second, 1 minute, and 5 minutes; respectively.

Reagents used in this study: mouse anti-Ascl1 (1:750 RDI Fitzgerald), rabbit anti-AC3 (1:500, Cell Signaling), mouse anti-PCNA (1:500, XX), mouse anti-GS (1:500, XX), mouse anti-GSTπ (1:1300, BD Transduction), rat anti-PDGFαR (1:300, XX), rabbit anti-TubIII (Tuj1) (1:5000, Covance), rat anti-BrdU (1:500, Accurate), goat anti-DCX (1:5000, Santa Cruz Biotechnology), mouse, rabbit, chicken anti-GFAP (1:4000,
Advanced Immunochemical), chicken or rabbit anti-GFP (used for GFP or YFP detection; rabbit 1:500, Invitrogen; chicken 1:8000, Aves Lab), rabbit anti-Ki67 (1:500, neomarkers), goat anti-NeuroD1 (1:1000, Santa Cruz Biotechnology), mouse anti-NeuN (1:50, Santa Cruz Biotechnology), rabbit anti-Prox1 (1:500, Chemicon), and rabbit or goat anti-Sox2 (1:500, Chemicon or Santa Cruz Biotechnology). For double or triple labeling, primary antibodies were simultaneously incubated (e.g., AC3/YFP, BrdU/YFP, NeuroD1/YFP, Ki67/YFP, Ascl1/NeuroD1) and processed for each antibody separately.

Whenever amplification was performed, the staining for the first antigen, such as for YFP and DCX, was done individually on the first day, followed by a second (or third) antibody the next day (e.g., Sox2/GFAP, NeuroD1/Ki67, Prox1/NeuN, NeuroD1/NeuN). For AC3, BrdU, NeuN, Ki67, NeuroD1, Sox2, GFAP, Prox1, and GFP, a fluorescent-tagged secondary Ab was used (1:200, Jackson ImmunoResearch). For YFP, DCX, and Ascl1, primary antibody incubation was followed with an appropriate biotin-tagged secondary Ab (1:200, Jackson ImmunoResearch) followed by ABC (Vector Laboratories) and Tyramide-Plus signal amplification (1:50, PerkinElmer). Slides were counterstained with DAPI (4,6-diamidino-2-phenylindole, 1:5000; Roche). REST/NRSF antibody was used at 1:100-200 (Abcam, Cat-26375) using free-floating protocol.

2.C.2. NRSF Free Floating Tissue Immunohistochemistry

Mice brains coronal sections were incubated in 24 wells plate in TBS enough to cover the section (500 µL). Sections were frozen and thawed twice for 15 minutes each. After “freeze-thaw” TBS was removed and cold methanol was added and left at -20°C for 10 min. Then, plates were washed for 4 times with TBS carefully, and sections were incubated with blocking solution for 30 minutes to 2 hrs using 3% NDS, TBS, and 0.5%
Triton X100. Primary NRSF rabbit antibody was added (Abcam; 1:200) in blocking solution and left for 1.5 to 2 days (1\textsuperscript{st} use cycle), 2.5 to 3 days (2\textsuperscript{nd} use cycle), or 3.5 to 4 days (3\textsuperscript{rd} use cycle). NRSF primary antibody was removed and sections washed twice. Then, secondary fluorescent antibody (1:1000 in TBS, 1% TritonX, 0.1% Na azide) was added and left for 4-6 hours. After antibody incubation, slides were washed and signal checked. The concentrations of all antibodies used are detailed in the Appendix.

2.D. Microscopic Analysis and Quantification

Quantification of cell number within the hippocampus was done using a Nikon TE2000-U inverted microscopy (Nikon, Inc.) using 400X and 80X lenses by a researcher blind to experimental groups, where immunohistochemistry slides codes were not revealed until the end of the experimental analysis. YFP+ cells were quantified throughout the SGZ, and the outer part of the GCL in every eleventh coronal section, each 30 \( \mu \)m thick section, and the average number of YFP+ cells in DG was reported. For YFP+ cells, phenotypical analysis and co-localization between YFP+ cells and different DG markers, was done using confocal microscopy (Leica confocal; emission wavelengths 488, 543, 633, 40X oil objective). A three-dimension (3D) reconstruction program for 3D rendering has been utilized to get optical sectioning on the Z plane.

2.E. Statistics and Analysis

In vivo experiments were analyzed for statistical significance using a Student's t test, with all error bars expressed as \( \pm \) standard error of mean (s.e.m.). Values of \( p < 0.05 \) were considered significant. All statistical analyses were carried out using Graphpad Prism5.
Chapter 3: Results

1. NRSF Expression Pattern in Adult Brain

1.A. REST/NRSF has Biphasic Expression in Adult Hippocampal NSCs and Mature Granular Neurons

REST/NRSF has shown high expression in embryonic stem cells and specific neural progenitors (Gupta, Gressens et al. 2009); however, down-regulation in NRSF expression has been reported in cortical neurons (Ballas, Grunseich et al. 2005). In contrast, NRSF maintains its expression in mature hippocampal neurons where it plays a dynamic role in regulating its target genes in response to ischemia and epileptic seizures (Kuwabara, Hsieh et al. 2004; Sun, Greenway et al. 2005).

Moreover, the REST/NRSF expression pattern in the adult brain, using immunohistochemistry, was determined by colocalizing REST/NRSF with NeuN, a marker for postmitotic mature neurons. Colocalization was confirmed in adult hippocampus along the dentate gyrus (DG), CA1, CA3, and cortical neurons (Figure 9A). Zhingliang Gao, a postdoctoral fellow at Hsieh lab in UT Southwestern Medical Center, has confirmed the immunohistochemistry data by in situ hybridization analysis showing REST/NRSF RNA expression in DG, CA1, and CA3 of adult hippocampus (Figure 9B). In further investigation, the expression pattern of REST/NRSF was identified by staining and colocalizing with nestin promoter-driven GFP transgenic reporter mice (Yamaguchi, Saito et al. 2000). NRSF is expressed in radial quiescent stem cells (Type 1), which are GFAP and nestin-GFP double positive cells (Figure 10A). Similarly, NRSF was colocalized with Sox2+ radial and non-radial cell type confirming REST/NRSF
expression in type 1 and transit amplifying cells (type 2a) (Figure 10B). Along the type 2a stem cells transition to neuroblasts, upregulation of the basic-helix-loop-helix (bLHL) transcriptional factor NeuroD1 takes place (Steiner, Klempin et al. 2006; Gao, Ure et al. 2009).

Therefore our observation of REST/NRSF expression pattern in NeuroD1 positive cells has demonstrated partial expression of REST/NRSF in a subset NeuroD1+ cells (Figure10C, D and E). This suggests that downregulation of REST/NRSF expression occurs along the transition of type2 NSCs to neuroblasts. Then, along further transition of neuroblasts to immature neurons, DCX+ cells, an upregulation in REST/NRSF expression occurs as showed in figure 11A. This suggests that REST/NRSF has a biphasic expression pattern during adult neurogenesis in hippocampus.

1.B. REST/NRSF Expression in Glia

We reported REST/NRSF expression in mature astorcytes, GFAP+ and GS+ cells, and mature oligodendrocytes, GSTπ+ and PDGFαR+ cells, in adult hippocampus (Figure 11B, C). Our postulation of NRSF expression in the adult brain is depicted in figure 12.
2. NRSF Regulation of Basal Adult Neurogenesis in NRSF Conditional Induced Knockout Mice.

2.A. NRSF^loxP/loxP Mice have Normal Brain Morphology and Basal Adult Neurogenesis.

Staining with Nissl stain, BrdU, and DCX for newborn neurons has shown normal anatomical feature, hippocampal integrity, and equivalent ratio of neurogenesis between loxP/loxP and wt/wt mice. Also, Western blotting and RT-PCR analyses on hippocampal cell lysate have shown comparable levels of NRSF protein and mRNA, respectively (Figure 13). Homozygous NRSF^loxP/wt and NRSF^wt/wt mice; CAG-Cre are fertile viable and follow the Mendelian segregation ratio, while NRSF^loxP/loxP; CAG-Cre mice are embryonic lethal as homozygous straight NRSF knockout mice (Chen, Paquette et al. 1998) (Figure 13).

2.B. Demonstration of REST/NRSF Knockout in YFP+ Cells.

To confirm NRSF knockout, we used different techniques to demonstrate knockout efficiency. First, free-floating REST/NRSF staining technique was used where NRSF has been stained with YFP to detect co-localization with Cre-targeted cells. Results show a significant decrease in co-localization between REST/NRSF and YFP in REST/NRSF mutant mice. This decrease confirms the efficiency of the knockout strategy (Figure 14A), however, the REST/NRSF antibody used in immunohistochemistry staining binds to the C-terminal of the protein, which is not the targeted region in our knockout model. Unfortunately, NRSF antibodies, which bind to the N-terminal of the protein, have been adjusted to work only in vitro. Thus,
assessment of the knockout efficiency using immunohistochemistry becomes inappropriate. Zhingliang Gao has confirmed the knockout with a group of techniques, such as; Southern blot analysis, genotyping with genomic PCR, recombination with global deletion CAG-Cre, RT-PCR, and Western blot. These results confirmed efficient deletion of NRSF in conditional knockout mice post-TAM injection (Figure 14B, C, D, E, F). Moreover, and to confirm recombination strategy, neural stem cells from TAM treated mice were cultured. NSCs culture with more than 95% YFP were assessed for recombination using REST/NRSF genomic PCR and RT-PCR. (Figure 14 G, H).
3. REST/NRSF Conditional Deletion Transiently Increase Adult Hippocampal Neurogenesis

3.A. REST/NRST Deletion Has No Effect on Total YFP at 10, 20, and 30 Days After TAM Injection.

Precise quantification of total YFP+ cells in REST/NRSF null mice in comparison with their wild type littermates has shown no significant difference in total YFP+ cells in both groups after 10, 20 and 30 days after TAM injection (Figure 16).

3.B. REST/NRSF Conditional Deletion Increase Neural Stem Cells (NSCs) Proliferation and Neuronal Differentiation.

Since NRSF is expressed in type 1 and type 2 NSCs, a more detailed examination of NRSF role in NSCs was done by sacrificing mice at 10, 20, and 30 days after TAM injection. Although quantification of totally YFP+ cells has shown no significant difference between knockout and wild type mice, yet a morphological quantification for each cell type has been done to investigate the major phenotype (Figure 17A). Type1 cells have shown a significant increase in the percentage of type1 stem cells ($P=0.0254$) after 30 days post-TAM injection in knockout mice in comparison with their wild type littermates (Figure 17B).

To define whether an increase in type1 cells due to NRSF deletion is accompanied by an increase in neuronal differentiation, we checked for Ascl1 expression, a marker for neuronal differentiation, in YFP+ cells at early time points
post-TAM Injection (Figure 18, a & b). Surprisingly, we detected a significant increase in the percentage of Ascl1 expression in YFP+ cells in NRSF knockout mice (P=0.0450) at 20 days, and not in 10 and 30 days post-TAM, when compared with wild type mice (Figure 18, c). Such increase confirmed the derepression of neuronal genes by the loss of NRSF and, consequently, an increase in neurogenesis.

Using a set of BrdU-injected animals, we used BrdU injection protocol by which we managed to assess the fate of YFP+ proliferating cells, type 1 and type 2 stem cells (Imayoshi, Sakamoto et al. 2010). We injected 10 μg/kg/day BrdU for 14 days, and then mice were left without BrdU injection for 18 days (Figure 19A). By combining BrdU staining with morphological analysis, we confirmed the proportional increase of BrdU in knockout compared to wild type mice (Figure 19B, C, and D), however, the increase reported in BrdU/YFP colocalization was insignificant (p=0.0511) (Figure 19B). Additionally, BrdU+/DCX+/YFP+ neuroblasts colocalization confirmed the increase in neurogenesis, while, the data showed somewhat increase, but, with no statistical significance (p=0.324) (Figure 19D).
4. REST/NRSF is Required to Maintain Quiescence in adult NSCs.

4.A. REST/NRSF Conditional Deletion Causes Depletion of Neural Stem Cells Pool

The generation of new neurons in adult hippocampus is based on the transition of quiescent stem cell population to transient amplifying progenitor cells. For further investigation of the role of NRSF in the transition of stem cells from quiescence to proliferation, we quantified the YFP-type1+ cells that express Ki67, a proliferation marker. Interestingly, cKO mice at 10 days post-TAM have shown a significant increase in the percentage of YFP+/Ki67+/type1 cells in comparison to their wild type littermates (p=0.0446) (Figure 20A, B). This might account for more type 1 cells found in cKO mice 30 days post-TAM. We detected a significant decrease in proliferating type 1 cells in mutant REST/NRSF mice at 30 days post-TAM (p=0.0270) (Figure 20C). Moreover, we confirmed NRSF expression in proliferating stem cells by colocalizing NRSF with PCNA, a proliferation marker (Figure 21). Conclusively, the deletion of REST/NRSF causes transient increase in proliferating type1 stem cells followed by decrease in proliferating type 1 progenitor cells (Figure 22).


Morphological analysis of YFP+ Type 1 cells, has exhibited more abnormalities in YFP+/type1 population in cKO mice in comparison with wild type littermates. Generally, more than 60% of the YFP+/type1 populations in wild type
mice demonstrate stereotypic morphology, in which neural stem cell has a thick central stack ends with a tuft, extensively branched dendrite. On the other hand, the other 40% of YFP+ type1 population exhibits some abnormal morphologies such as; hair-like appendage from the single thick process, absence of the central stack, and central stack in an opposite orientation. In cKO mice, the percentage of abnormal morphologies was much more than that of wild type littermates (Figure 23A, B).


Previous studies on SGZ adult neurogenesis in mice have reported that 2 to 6 months are required for maximum contributions of NSCs to new neurons in the granular layer of dentate gyrus (Ahn and Joyner 2005; Lagace, Whitman et al. 2007; Ninkovic, Mori et al. 2007). Therefore, we decided to do morphological analysis of YFP+ cells phenotype at 70 and 120 days post-TAM. Interestingly, a significant decrease in newborn mature neurons has been detected in cKO mice at 120 days (p=0.0009), however, such decrease was not significant at 70 days post-TAM (p=0.280) (Figure 24A, B). Moreover, we confirmed these results using mature neurons specific markers, such as Prox1 and NeuN, which conformed to the morphological analysis data exhibiting a significant decrease in mature neurons in the cKO mice (p=0.0472) (Figure 24C). To test whether such a decrease in mature neurons was due to cell death, caspase 3 (AC3+), an apoptotic marker that detects cell death, was stained at 30 and 70 days post-TAM. Results showed no increase in AC3+ cells in cKO in comparison to wild type (Figure 25). These results supported our
early findings that REST/NRSF drive stem cells to exit quiescence, which will eventually result in depletion of stem cell pool.
Chapter 4: Discussion

Our results elaborated the role of NRSF as a master intrinsic factor in adult neurogenesis regulation. REST/NRSF mediates the transition of NSCs from quiescence to the proliferative stage, preventing precocious differentiation. In addition, NRSF controls neuronal gene expression that drives the differentiation and generation of new mature neurons. In conclusion, NRSF is a master negative regulator controlling adult neurogenesis in mouse hippocampus.

4.A. REST/NRSF Regulation of Adult Neurogenesis and Quiescence.

Our results suggested that the early complication of NRSF deletion is precocious neuronal differentiation. In mice, conditional deletion of NRSF at early stage cause transient acceleration of the generation of new neurons. NSCs exit quiescence, prematurely, and shift to the proliferating stage to produce new neurons, accelerating neurogenesis. Our lab has confirmed these results in vitro, where knockdown of REST/NRSF in HCN cells using REST/NRSF specific short hairpin RNAs (shRNA) has reflected an increase in pro-neuronal genes expression, NeuroD1, Tuj1, and DCX. In another experiment, REST/NRSF shRNA was inserted into a lentivector with GFP to visualize the effect of knockdown on HCN cells. In growth condition, no difference in proliferation and differentiation has been noticed, which showed that REST/NRSF knockdown is insufficient to induce differentiation. However, culturing HCN cells with differentiation factors such as; retinoic acid and 1% Fetal Bovine Serum, for 24 hours, cells appeared to have neuronal morphology and expressed neuronal markers in shRNA2-GFP expressing cells.
Consequently, this precocious differentiation of NSCs was reflected on the ability to generate new neurons for an extended period of time. Hence, the decrease in new neurons production in REST/NRSF cKO mice is a result of quiescent stem cell depletion in niche due to the exhaustion of the NSCs pool by early-accelerated neurogenesis. On the other hand, the type1 YFP+ cells remaining in cKO mice at later stages suggested that those cells might be inert as they may have exit cell cycle.

Since our results have shown that NRSF has biphasic expression in stem cells and mature neurons, therefore this suggests the role of NRSF in regulating the timing of neuronal gene expression. Our finding reflects that NRSF is downregulated when stem cell exit quiescence and through the initiation of neuronal differentiation. Results showed that NRSF is less expressed with NeuroD1 in cKO mice in comparison with wild type littermates. In vitro results, obtained by Zhingliang Gao, supports the in vivo data, as molecular investigation has shown that NRSF is physically associated with mSin3A and CoREST in NSCs repressing neuronal gene expression. In addition, ChIP assay detected NRSF and p300, a histone acetyltransferase, on NeuroD1 RE/NRSE site. We postulated that NRSF-mSin3A-CoREST complex controls transcriptional regulation of neuronal genes during the transition of adult NCS to neuroblasts and further on to mature neurons. These results are consistent with a previous study reporting that NRSF occupies distinct group of transcriptional active genes in neural stem cells and mature neurons (Ballas, Grunseich et al. 2005).

In order to identify the role of NRSF in late stages of neuronal differentiation, a similar conditional knockout experiment could be designed, however, Cre
expression should be linked to a late neuronal differentiation gene promoter such as; DCX or NeuN, instead of nestin promoter which was originally used in this experiment. This suggested experiment will guarantee successful NRSF knockout, only after neuronal expression in immature and mature neurons. In addition, further identification of new markers will strongly support better tracing of neural stem cells along the neurogenesis pathway. Identifying more markers for neural stem cells is considered one of the major challenges in the field of adult neurogenesis that can facilitate precise differentiation between quiescent, proliferating and trans-amplifying stem cells.

4.B. Implications of REST/NRSF Dysregulation on Different Diseases.

Many central nervous system pathological conditions demonstrate deregulation in REST/NRSF such as; Huntington’s Disease (Zuccato, Tartari et al. 2003), ischemia (Calderone, Jover et al. 2003), and epileptic seizures (Jessberger, Nakashima et al. 2007). In addition, REST/NRSF defect is correlated to other diseases such as; cardiomyopathy (Kuwahara, Saito et al. 2001), and cancer (Westbrook, Hu et al. 2008). However, the role that REST/NRSF plays in the development of these diseases is almost unknown. REST/NRSF involvement in tumorigenesis was first reported a decade ago (Lawinger, Venugopal et al. 2000). Currently, NRSF is suggested to correlate with different types of cancers such as; medulloblastoma, neuroblastoma, and small cell lung carcinoma (Palm, Metsis et al. 1999; Coulson, Edgson et al. 2000; Lawinger, Venugopal et al. 2000). The role of NRSF in inducing cancer is now widely recognized. However, considering REST/NRSF as a tumor suppressor gene adds more complexity and ambiguity to its
role in cancer development (Westbrook, Martin et al. 2005). Currently, an open question is left to scientists to address; how overexpressing a tumor suppressor gene could induce carcinogenesis? In this context, our study elucidated the role that REST/NRSF might play in different types of cancer and, particularly, tumors with neural origin, as we postulated that REST/NRSF is required for proper transition of neural stem cells to mature neurons. Brain cancers such as; neuroblastoma, glioma and medulloblastoma, which might have evolved from quiescent stem cells in the brain, could be more understood only if more detailed investigations were considered. Thus, elucidating the molecular mechanisms by which REST/NRSF regulates NSCs will support developing better treatment strategies for cancer diseases.
Chapter 5: Figures

Figure 1. Adult neurogenesis occurs across the granular layer in dentate gyrus of mice adult hippocampus.
Neural stem cells in subgranular zone pass by a number of developmental stages where stem cells proliferate in the neurogenic niche, before determining their fate. Then, the new neurons migrate and become fully differentiated and integrated in the granular layer.
Figure 2. Markers indicating different stages of adult neurogenesis.
In Adult neurogenesis, neural stem cells are involved in step-wise stages of development till differentiation into mature neurons. This complex process could be identified by their respective cell type morphology, characteristic for each developmental stage, and cell type-specific markers (Gao, Ure et al. 2009).
Figure 3. Adult neurogenesis in subventricular zone (SVZ).
(A) The SVZ-OB system is represented in the schematic diagram of sagital brain section, the olfactory bulb (OB) to the right and the cerebellum (CB) to the left. Neural progenitors migrate from SVZ, which resides along the lateral wall of the lateral ventricle (LV). Stem cells migrate across the rostral migratory stream (RMS), where young neurons are interconnected in a path to disperse radially, dotted line, at the olfactory bulb. NC, Neocortex; cc, corpus callosum. (B) Lineage organization in the SVZ is diagramed where anterior cross section, left, in rodent brain showing SVZ orientation lining the lateral ventricle. The right figure represents a typical SVZ niche, where a chain of young neurons is surrounded with tube-like forming cells type B (blue). Highly proliferating cells Type C are attached to the young neurons chain. Ependymal cells (E) are lining the connection between the niche and lateral ventricle. At the bottom left, some intrinsic factors that affect SVZ neurogenesis where BMP, bone morphogenetic factor, inhibits neurogenesis and promotes astrogenesis. Also, ependymal cells release noggin protein which inhibits BMP, activating some B cells to produce C and A cells (Alvarez-Buylla and Garcia-Verdugo 2002).
Figure 4. The time frame and major regulatory factors required for the transition of neural stem cells, green cells, to mature granular neurons in the dentate gyrus of adult hippocampus. (Zhao, Deng et al. 2008)
Figure 5. RE1-silencing transcription factor (REST) or Neuron-restrictive silencer factor (NRSF).

REST/NRSF is a negative regulator of neuron-specific genes in nonneuronal cells. It binds conserved 23 bp DNA element (NRSE/RE1) in neuron-specific genes. Its DNA binding domain is consisted of 8 zinc fingers. REST/NRSF recruits HDACs and corepressors mSin3 (N-term) and CoREST (C-term).
Figure 6. REST/NRSF negative regulation of neuronal gene in stem cells.
REST/NRSF recruits corepressors mSin3 to bind to the N-terminal, then recruits CoREST on its C-terminal activating a set of chromatin modifying enzymes, such as; histone deacetylases (HDACs), histone demethylases, and histone methyltransferases, which, in turn, mediate chromatin condensation silencing gene expression. HDAC, histone deacetylases; HMTases K4, histone H3–K4 methyltransferases; mk, methylated lysine residues; SCP, small carboxyl terminal domain phosphatase; Pol II, RNA polymerase II; RE1, repressor element 1; MeCP2, methyl CpG binding protein 2; REST, neuro-restrictive silencer factor (Ballas and Mandel 2005).
Figure 7. Generation of REST/NRSF conditional knockout allele. Strategy used to generate REST/NRSF knock alleles using corresponding exonic structure, targeting vector, and targeted conditional alleles is portrayed.
Figure 8. Schematic diagram of TAM injection strategy and different sets of animals perfused at different time points post-TAM. REST/NRSF$^{loxP/loxP}$ represents conditional knockout mice, and REST/NRSF$^{+/+}$ represents wild type mice. TAM, tamoxifen; YFP, yellow fluorescent protein; NRSF, neuro-restrictive silencing factor.
Figure 9. REST/NRSF expression in postmitotic neurons.
(A) Immunostaining of NRSF with NeuN in 6-weeks-old adult mouse hippocampus. NRSF is expressed and colocalized with NeuN in DG, CA1, CA3, and cortex. (B) In situ hybridization experiment for Rest/Nrsf RNA to confirm the Immunohistochemistry results. For NeuN, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. As for NRSF, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by green channel.
Figure 10. REST/NRSF has biphasic expression in adult hippocampus and mature granule neurons.

(A-B) Immunohistochemistry of the dentate gyrus showing NRSF expression in Nestin-GFP mice. NRSF is colocalized with Nestin-GFP, Sox2 (markers for neural stem and progenitor cells) and GFAP (marker for neural stem cells). (C-D) REST/NRSF immunostaining with NeuroD1 and NeuN (markers for immature and mature cells, respectively). REST/NRSF is extensively expressed in NeuN+ cells, while less expressed in NeuroD1 positive cells. (E) Quantification of NRSF/NeuroD1 double positive cells has shown inverse relation between them, reflecting a repressive role of REST/NRSF in NeuroD1 expression. Dotted line indicates the borders of granular cell layer. For NRSF, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. For Nestin-GFP, chicken primary antibody was used and tagged with a
secondary fluorescent antibody viewed by green channel. For Sox-2, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by green channel. For NeuroD1, mice primary antibody was used and tagged with a secondary fluorescent antibody viewed by green channel. For GFAP, chicken primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. As for NeuN, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5.
Figure 11. REST/NRSF expression in immature neurons and glial cells.

(A) Immunostaining of NRSF shows colocalization with DCX, immature neuronal marker (arrows) and NeuN, mature neuronal marker. A subset of DCX+ cells (arrowhead) exhibits low NRSF expression. (B-C) Immunostaining of NRSF show colocalization with GS+/GFAP+ cells, mature astrocytes, and GSTπ+/PDGFαR+, mature oligodendrocytes. Dotted line represents the borders between the DG and hilus. For NRSF, rabbit primary antibody was used and tagged with a secondary fluorescent
antibody viewed by cy3. For GS, rat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. For GSTπ, rat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. For NeuN, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. For GFAP, chicken primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. For PDGFa, rat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. As for DCX, goat primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification.
Figure 12. Summary of REST/NRSF expression with different markers during adult hippocampal neurogenesis.

REST/NRSF immunostaining shows biphasic expression pattern of REST/NRSF along the adult neurogenesis axis. REST/NRSF colocalizes with GFAP, Nestin-GFP, and SOX2 NSC/progenitor markers at early phase of neurogenesis. Then, a gradual down-regulation of REST/NRSF expression occurs when NSCs exit quiescence to proliferative stages (Sox2+/PCNA+/Ascl1+ cells). After stem cell exit cell cycle and differentiation starts, REST/NRSF becomes reduced in high NeuroD1+ or DCX+ cells. In the late phase of adult neurogenesis, REST/NRSF shows a high-level expression in mature granule neurons (Prox+/NeuN+ cells).
Figure 13. REST/NRSF floxed allele recapitulates the null allele.
(A-B) No difference by gross brain morphology (Nissil staining) between wild type and REST/NRSF$^{loxP/loxP}$ mice. (C-D) No difference in hippocampal integrity. (E-F) No difference in NSC proliferation, BrdU+, and immature neurons, DCX+. (G) No difference in level of NRSF expression, mRNA (top panel), or protein (bottom panel). (H) REST/NRSF$^{loxP/loxP}$, REST/NRSF$^{wt/wt}$; CAG-Cre mice follow Mendelian segregation. GADPH is used as a loading control in Western blot experiment. For DCX, goat primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. As for BrdU, rabbit primary antibody was
used and tagged with a secondary fluorescent antibody viewed by cy5 after several treatments.
Figure 14.A. Confirmation of successful deletion of REST/NRSF.
(A) Immunostaining of REST/NRSF in YFP+ cells in cKO mice at 70 days post-TAM injection (B) Quantification of NRSF/YFP double positive cells showed a decrease trend in cKO in comparison with their wild type littermates. For YFP, chicken primary antibody was used and with a secondary fluorescent antibody viewed by cy3. As for REST/NRSF, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by green channel.
Figure 14.B. Confirmation of successful deletion of REST/NRSF.

(B) Southern blot analysis of WT, REST/NRSF neo-loxP/+ and REST/NRSF Neo-loxP/Neo-loxP. Tail DNA was digested with Xba I (5’ probe) and Hpa I (3’ probe), and the corresponding WT (∼16.5/7.5 kb) and targeted bands (∼10.5/9.5 kb) are indicated for the 5’/3’ probe. (C) Genotyping of REST/NRSF loxP mice by genomic PCR. The primer set flanks the 3’ loxP site, resulting in one ∼550-bp product for the WT allele and one ∼650 bp band for the targeted allele. (D) Successful recombination with global deletion by CAG-Cre. The primer set flanks both the 5’ and 3’ loxP sites, resulting in one ∼2.8 kb product for the WT allele and ∼1.1 kb band for the targeted allele. REST/NRSF<sup>loxP/loxP</sup>; CAG-Cre mutant is embryonic lethal, re-capitulating the previously reported global KO phenotype. (E, F) RT-PCR and Western blot analyses to confirm successful deletion of the REST/NRSF conditional allele by Ad-Cre-mediated recombination in vitro in REST/NRSF<sup>loxP/loxP</sup> NSCs (not shown). Data presented in figure 14.B has been generated by Zhingliang Gao, a postdoctoral fellow at Hsieh lab.
Figure 15. REST/NRSF inducible conditional knockout strategy. 
(A) Generation of tamoxifen inducible REST/NRSF knockout mice, NRSF$^{loxP}$ mice were crossed with Nestin CreER$^{T2}$ and Rosa26R-YFP mice.
Figure 16. Schematic diagram shows no significant difference in total YFP counts at different time points, 10, 20, and 30 days, post-TAM, tamoxifen, injection between wild type (WT) and conditional knockout (cKO) mice.
Figure 17. REST/NRSF Ablation causes a transient increase in type 1 cells. (A) Image represents morphology of YFP+ cells, type 1, type 2, and mature granular neurons. Dotted line indicates the borders of granular cell layer. (B) Morphological quantification of type 1 cells showed an increase in type 1 cells in cKO mice at 30 days post-TAM. For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification.
Figure 18. Deletion of REST in vivo causes a precocious neuronal differentiation of neuronal progenitors.
(A) A schematic diagram shows TAM treatment strategy and days of sacrifice. (B) Representative images showing Ascl1+/YFP+/DCX+ cells in WT (left panel) and cKO mice (right panel). (C) Quantification has shown that RESR/NRSF deletion leads to transient significant increase in Ascl1+/YFP+ neuronal progenitors at 20 days post-TAM causing increase in neurogenesis. For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. For DCX, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. As for Ascl1, mouse primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3.
Figure 19. REST/NRSF ablation accelerates the transition of neural stem cells to immature and mature neurons.

(A) Schematic diagram represents TAM and BrdU treatment and birth date. (B) Representative images of YFP+/BrdU+/DCX+ cells in WT (left panel) and cKO (right panel). Dotted line presents the borders between hilus and SGZ. (C-D) REST/NRSF deletion increase neuronal differentiation and the transition form neural stem cells to immature and mature neurons as morphological quantification has shown in BrdU+ cells (C), which has been confirmed by colocalization with immature neuronal marker DCX (D). For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. For DCX, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. As for BrdU, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3 after several treatments.
Figure 20. REST/NRSF Deletion causes impaired maintenance of type 1 quiescent stem cells in REST/NRSF cKO mice.

(A) Representative image of YFP+Ki67+ cells in WT (left panel) and cKO (right panel). Sox2 is a stemness marker (blue) for type 1 and type 2 neural stem cells and dotted line indicates the borders between SGZ and hilus. (B) NRSF deletion causes transient significant increase in proliferating neural stem cell (YFP+/Ki67+) type I cells in cKO mice at 10 days post-TAM injection. (C) At 30 days post-TAM injection, NRSF deletion reflects a significant decrease in proliferating neural stem cells (YFP+/Ki67+) type 1 cells in cKO mice. For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. For Sox2, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. As for Ki67, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. 10 animals were used to generate the statistical data for each group.
Figure 21. PCNA staining in REST/NRSF+ type 1 cell.
REST/ NRSF was colocalized with PCNA, proliferation marker, in radial (GFAP-) and non-radial (GFAP+) progenitor cells in DG. GFAP is a marker for radial stem cells and the dotted line indicates the distinction between hilus and SGZ. For GFAP, chicken primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. For PCNA, mouse primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. As for NRSF, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by green channel.
Figure 22. REST/NRST Deletion causes a transient increase followed by decrease in Type 1+ YFP cells due to depletion of neural stem cells pool. WT, wild type; F/F, floxed mice with successful knockout.
Figure 23. Variable Morphology of REST/NRSF cKO Type 1 cells.
(A) Representative images of YFP+ cells in WT and REST/NRSF cKO mice where arrows refer to different type 1 variable morphology. YFP+ Type 1 cells were colocalized with GFAP and Sox2. (B) Quantification of YFP variable morphology type 1 in WT (n=7) and cKO mice (n=9). Dotted line indicates the GCL. For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. For Sox2, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3. As for GFAP, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5.
Figure 24. Decreased immature and mature newborn granular neurons in REST/NRSF cKO.

(A) Representative image of YFP+/Prox1+/NeuN+ in WT (left panel) and cKO (right panel). (B) Quantification of Prox1+/YFP+ cells in SGZ shows a significant decrease in cKO at 120 days post-TAM. (C) Morphological quantification of immature and mature newborn granular YFP+ cells exhibits a significant decrease of newborn granular neurons in cKO mice at 120 post-TAM, but not at 70 days. For YFP, chicken primary antibody was used and tagged with a biotin-tagged secondary antibody followed by ABC kit and Tyramide-Plus signal amplification. As for NeuN, goat primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy5. For Prox1, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3.
Figure 25. No change in AC3+ cells in REST/NRSF cKO
Representative images of AC3 staining in WT mice, top two panels, and cKO mice bottom panel, dotted line shows the distinction between the hilus and SGZ region. (B) Quantification of AC3 positive cells across the SGZ region exhibits no significant change between WT and cKO mice. For AC3, rabbit primary antibody was used and tagged with a secondary fluorescent antibody viewed by cy3.
**Appendix**

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*Primary incubation for all antibodies was done overnight at 4°C.

**Secondary incubation for all antibodies was done from 4 to 6 hours, except for GSTπ and PDGFR where the incubation were done over night at 4°C.
Chapter 6: References


