HIPPOCAMPAL NEUROGENESIS IN AMYOTROPHIC LATERAL SCLEROSIS LIKE MICE

HIPPOCAMPAL NEUROGENESIS IN AMYOTROPHIC LATERAL SCLEROSIS LIKE MICE

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ABSTRACT

G93A SOD1 mice (G93A mice) are a transgenic model over-expressing a mutant human Cu/Zn-SOD gene, and are a model for amyotrophic lateral sclerosis (ALS), a predominantly motor neurodegenerative disease. Hippocampal neurogenesis in the subgranular zone (SGZ) of dentate gyrus (DG) occurs throughout the life. It is regulated by many pathological and physiological processes. There is controversy with respect to the basal level of hippocampal neurogenesis and its response to exercise in neurodegenerative diseases and their mouse models. Little information regarding hippocampal neurogenesis is available in G93A mice. The present study was designed to study the impact of treadmill exercise and sex differences on hippocampal neurogenesis in this model. In addition, potential molecular mechanisms regulating hippocampal neurogenesis including growth factors (BDNF and IGF1) and oxidative stress (SOD2, catalase, 8-OHdg, and 3-NT) were also addressed in the study. Bromodeoxyuridine (BrdU) was used to label newly generated cells. G93A and wild type (WT) mice were subjected to treadmill exercise (EX) or a sedentary (SED) lifestyle. Immunohistochemistry was used to detect BrdU labeled newly proliferating cells, surviving cells, and their phenotype, as well as for determination of oxidative stress. BDNF and IGF1 mRNA expression was assessed by in situ hybridization. Results showed that (1) G93A mice had an elevated basal level of hippocampal neurogenesis for both cell survival and neuronal differentiation, a growth factor (BDNF mRNA), and an

oxidative stress marker (NT), as compared to wild type sedentary mice. (2) Treadmill running did not show any further effect on hippocampal neurogenesis, growth factors, oxidative stress, and antioxidant enzymes in G93A mice, while treadmill running promoted hippocampal neurogenesis and expression of the growth factor (BDNF mRNA), and lowered oxidative stress (8-OHdg) in WT mice. (3) There also were sex differences in hippocampal neurogenesis in G93A mice, whereby male G93A mice had a significant higher level of cell proliferation but a lower level of survival than female G93A mice. (4) The DG BDNF mRNA was associated with cell survival and neuronal differentiation in sedentary G93A mice, suggesting that BDNF is associated with a higher basal level of hippocampal neurogenesis in G93A mice. We conclude that G93A mice are more permissive in the context of hippocampal neurogenesis, which is associated with elevated DG BDNF mRNA expression. Running did not have impact on hippocampal neurogenesis and BDNF mRNA expression in G93A mice, probably due to a 'ceiling effect' of the already heightened basal levels of hippocampal neurogenesis and BDNF mRNA in this model. In addition, sex differences also affect hippocampal neurogenesis, but the further study is needed to clarify the underlying molecular mechanisms.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AMPA	α-anmino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CNS	central nervous system
CuZnSOD	copper-zinc superoxide dismutase
DAB	diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
ECSOD	extracellular superoxide dismutase
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
FGF2	fibrioblast growth factor 2
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acid protein
GPx	glutathione peroxidase
HD	Huntington's disease
H_2O_2	hydrogen peroxide
i.c.v	intraventricular
IGF1	insulin-like growth factor 1
IHC	immunohistochemistry
i.p	intraperitoneal
i.v	intravenous
JNK	c-Jun amino-terminal kinase
KA	kainic acid
LB	Luria-Bertani
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
NeuN	neuronal nuclear
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NPCs	neural progenitor cells
NSCs	neural stem cells
NSE	neuron-specific enolase
3-NT	nitrotyrosine

O_2^-	superoxide anion
OGG1	8-oxoguanine-DNA glycosylase
OH^-	hydroxyl radical
8-OHdg	8-hydroxy-2-deoxyguanosine
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PCNA	proliferating nuclear antigen
PCR	polymerase chain reaction
PD	Parkinson's disease
PI-3K	phospatidylinositol 3-kinase
ROS	reactive oxygen species
SGZ	subgranular zone
SOD	superoxide dismutase
SVZ	subventricular zone
TBS	Tris-buffered saline
TrkB	tyrosine kinase receptor
VEGF	vascular endothelial growth factor

1. INTRODUCTION

1.1 Neurogenesis in Adult Brain

Neurogenesis, the production of new neurons, was traditionally assumed to occur only during development in the central nervous system (CNS) of mammals. It has become generally accepted that new neurons are indeed added in discrete regions of the adult brain of mammals (Kempermann and Gage 1999; Gross 2000; Lie, Song et al. 2004; Abrous, Koehl et al. 2005; Ming and Song 2005; Suh, Deng et al. 2009). Throughout life, new neurons are generated in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus under normal conditions. Whether adult neurogenesis occurs in areas of mammalian brain other than SVZ and SGZ remains controversial (Gould 2007). The process of adult neurogenesis, including the proliferation of adult neural stem cells (NSCs) or multipotent neural progenitor cells (NPCs), commitment to a neuronal phenotype, differentiation (development of neuronal features), survival, maturation, and integration of newborn neurons, is highly regulated by physiological and pathological factors (Abrous, Koehl et al. 2005; Ming and Song 2005). It has been pointed out that two main lines of research have emerged following the investigation of adult neurogenesis. The first is to understand the fundamental biological properties of neural stem cells, eventually leading to repair and regeneration of neurodegenerative diseases. The second is to understand the functional relevance of neurogenesis, especially in DG, meaning the contribution of new-

born neurons to brain functions. In this chapter, adult hippocampal neurogenesis will be addressed since the present study focused on the effect of exercise and sex difference on hippocampal neurogeneis.

1.1.1 Historic Overview

The belief that neurogenesis did not occur in the adult mammals dominated the neuroscience field for over 100 years (Gross 2000). A breakthrough in neurogenesis came in the 1960s with the new technique of labeling dividing cells using [H^3]-thymidine autoradiography. During the S-phase of cell cycle, [H^3]-thymidine incorporates into the replicating DNA and thus can be used a marker for proliferating cells. Using this method, Joseph Altman did a series of studies and published many papers (Altman 1962; Altman 1963; Altman and Chorover 1963; Altman and Das 1964; Altman and Das 1964; Altman and Das 1965; Altman and Das 1966; Altman 1969) in the most prestigious journals of the time, such as *Science, Nature*, and the *Journal of Comparative Neurololgy.* He reported that new neurons were generated in the young and adult rat, including olfactory bulb (Altman 1969) , hippocampus (Altman 1963; Altman and Das 1965), and cerebral cortex (Altman 1963; Altman and Das 1966). However, his studies were ignored for over ten years. As Gross summarized (Gross 2000), several reasons could account for the neglect of Altman's demonstration of adult neurogenesis. First, no adequate evidence proved that the newly generated cells in adults were neurons not glia due to technical limitations. Second, his studies could not explain the migration of new born cells from

ventricle to the olfactory bulb. Last, and possibly most important is that Altman was a self-taught postdoctoral fellow working on his own.

More than ten years later, Altman's works were confirmed by Michael Kaplan. Using electron microscopy and [^{3}H]-thymidine, Kaplan demonstrated that [^{3}H]-thymidine-labeled cells in the olfactory bulb, hippocampus, and cerebral cortex of adult rats had the ultrastructural morphology of neurons, such as dendrities and synapses, but not of astrocytes or oligodendrocytes (Kaplan and Hinds 1977; Kaplan 1981; Kaplan and Bell 1984; Kaplan 1985). Further evidence showed that newly generated neurons at DG are able to extend their axon projections along the normal mossy fiber pathway (Stanfield and Trice 1988), suggesting that they integrated into the normal hippocampal network. Although all these studies further provided supporting evidence for adult neurogenesis, they had little impact in the neuroscience field at that time. In contrast, the studies of adult neurogenesis in songbird received more acceptance. Nottebohm and his colleagues did a series of experiments using electron microscopy and [^{3}H]-thymidine, showing that thousands of new neurons are added every day to songbird brains, suggesting evidence for functional roles of postnatal neurogenesis in seasonal song learning (Nottebohm 2004). However, these studies were considered to be irrelevant to the mammalian brain.

The introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue as another S-phase marker of the cell cycle (Gratzner 1982), brought an important advance in adult neurogenesis. BrdU labeling has many advantages over previous techniques. BrdU labeled cells can be detected by immunohistochemistry and

quantitatively assessed with stereological counting. In addition, BrdU labeling can be combined with neuronal and/or glial markers to identify the cell fate of BrdU labeled cells by imimmunofluorescent staining and confocal microscopy. Using these techniques, several laboratories confirmed that new neurons are added to the granule cell layer of the DG of adult rodents and other mammals (Cameron, Woolley et al. 1993; Kuhn, Dickinson-Anson et al. 1996; Eriksson, Perfilieva et al. 1998; Gould, Beylin et al. 1999).

Before the end of the twentieth century, adult neurogenesis finally attracted attention in the field and has been demonstrated in mammals and humans (Gage, Coates et al. 1995; Eriksson, Perfilieva et al. 1998; Gould 2007). Most importantly, adult hippocampal neurogenesis has been investigated in human postmortem tissue by using BrdU labeling and Ki-67. The studies further provide strong evidence for the presence of adult hippocampal neurogenesis, by isolating progenitors capable of proliferation and neurogenesis from the hippocampus (Roy, Wang et al. 2000). Very recently, a magnetic resonance spectroscopy-based strategy has been developed to monitor the number of neural stem and progenitor cells in the brain of living humans, confirming the presence of cell proliferation in the hippocampus and demonstrating an age-related decline of stem cells (Manganas, Zhang et al. 2007). Forty years following the original demonstration of adult neurogenesis by Altman, adult mammalian neurogenesis has been firmly established and finally gained acceptance in neuroscience field. Now the question is "Are these new generated neurons functional?"

Hippocampal neurogenesis might contribute to hippocampal-dependent functions, such as learning and memory (van Praag, Schinder et al. 2002; Kempermann, Wiskott et al. 2004). For example, running increases both number of the new neurons at DG and performance of hippocampal-dependent tasks (van Praag, Christie et al. 1999; van Praag, Kempermann et al. 1999). Furthermore, a lower number of new neurons is correlated with impairment on learning and memory (Shors, Miesegaes et al. 2001). The parallel observations on newborn neurons and hippocampal-dependen functions suggest that newborn neurons might be functional. Another evidence for the function of adult neurogenesis is emerging from the fact that the process of adult neurogenesis is highly regulated by physiological and pathological factors (discussed below).

1.1.2 Neurogenic Brain Regions

A brain region that supports neurogenesis is classified as neurogenic (Balu and Lucki 2009). Neurogenic regions contain NSCs or NPCs and have microenviroment permissive for the production of new neurons. In the adult mammalian brain under normal conditions, there are neurogenic regions that are generally accepted, namely the SVZ of the lateral ventricle and the SGZ of the DG in the hippocampus. In the lateral ventricle, new neurons are born in the SVZ and migrate anteriorly into the olfactory bulb where they differentiate into granule neurons and periglomerular neurons. In the hippocampus, new neurons are generated in SGZ, migrate a short distance to granule cell layer, where they differentiate into granule neurons. In addition to these two regions, other adult

brains regions, such as neocortex, striatum, and hypothalamus, have been reported to produce new neurons; however, the results are controversial (Gould 2007).

1.1.3 Hippocampal Neurogenesis: a complex multi-step process

Adult neurogenesis is composed of many steps, including the proliferation of adult NSCs or multipotent NPCs, commitment to a neuronal phenotype, differentiation (development of neuronal features), survival, maturation, and integration of newborn neurons.

1.1.3.1 Cell Proliferation

Most of the neurons in the adult CNS are terminally differentiated and cannot be regenerated. In contrast, NSCs or NPCs have the proliferative capacity to undergo mitosis and generate new neurons. The term "stem, progenitor, and precursor cells" have been used interchangeably in the literature. The NSC is currently defined as an undifferentiated cell that exhibits the ability to self-renew and to differentiate into multiple lineages, including neurons, astrocytes, and oligodendrocytes (Gage 2000; Kempermann, Jessberger et al. 2004). There has been an intense debate regarding the identity of neural stem stems in the adult brain for years (Ming and Song 2005).

The SGZ is located at the interface between the granule cell layer and the hilus of DG in the hippocampus, deep within the parenchyma. Two types of NSCs have been identified on the basis of morphology and cell marker expression (Zhao, Deng et al. 2008; Suh, Deng et al. 2009). These are type 1 and type 2 cells. Type 1 cells are radial glial cells, expressing glial fibrillary acid protein (GFAP), nestin, and the Sry-related HMG

box transcription factor, Sox2. Although these cells express astrocyte cell marker GFAP, they are different from mature astrocytes in morphology and function. Type 2 cells or non-radial cells have short processes and express nestin and Sox2 but not GFAP. The type 1 radial cell has been proposed to be the infrequently dividing cells that have the potential to generate actively dividing NSCs during adult neurogenesis (Seri, Garcia-Verdugo et al. 2001; Seri, Garcia-Verdugo et al. 2004). Seri and colleagues showed that only radial type 1 cells (GFAP+ cells) survived, resumed proliferation, and repopulated granule neurons after the transient cessation of neurogenesis upon the chemical removal of actively dividing cells. These studies indicated that the ablation of selectively targeted non-radial type 2 cells and resulted in the depletion of these cells and the cessation of neurogenesis. However, infrequently dividing radial type cells that escaped this ablation continued neurogenesis and eventually repopulated lost cells, suggesting infrequently dividing radial type 1 cells play the role as NSCs. However, these studies are challenged by Palmer's observation, showing few proliferating cells in the adult DG were GFAP + (Palmer, Willhoite et al. 2000). Consistent with Palmer's observation, a recent study proposed that non-radial type 2 cells positive for Sox2 also have the potential to self-renew and that one single Sox2 positive cell can give rise to neurons (Suh, Consiglio et al. 2007). Together, these studies collectively demonstrated the presence of NSCs responsible for adult neurogenesis in the SGZ. NSCs have been reported to undergo asymmetric division, giving rise to one daughter cell and one neuronal lineage restricted progenitor daughter cell (Kempermann, Jessberger et al. 2004).

The proliferation of NSCs or NPCs is regulated by a complex mechanism, including neural stem niche, growth factors, neurotransmitters, and physiological factors. In addition, pathological conditions can also alter neuronal proliferation as described below.

1.1.3.2 Differentiation and Migration

After proliferation, the next class of cells are the intermediate precursors, called D cells (D2 and D3) (Seri, Garcia-Verdugo et al. 2001). D cells are clearly different from radial type 1 cells. They have a round soma with short cytoplasm extension. These cells no longer express nestin or Sox2, but began to express the polysiliated form of the neural cell adhesion molecule (Seri, Garcia-Verdugo et al. 2004), and microtubule-associated protein, doublecortin (DCX) (Encinas and Enikolopov 2008). DCX was reported to be associated with both of the initiation of neuronal differentiation and migration (Francis, Koulakoff et al. 1999). The evidence suggested that D2 cells may mature through the D3 stage to form new granule cells since the D3 cells have the morphological characteristics of immature neurons, such as prominent, frequently branched, radial processes extending through the granule cell layer and thin processes projecting into the hilus (Seri, Garcia-Verdugo et al. 2004).

Newborn neurons in the SGZ only migrate a short distance into the granule cell layer. The migration may be associated with expression of a schizophrenia susceptibility gene, disrupted-in-schizophrenia 1 (Duan, Chang et al. 2007) and the reelin pathway, a

migration guidance cue that persists in the adult rodent and human hippocampus (Gong, Wang et al. 2007).

1.1.3.3 Neuronal Maturation

After the new born cells become postmitotic and differentiated, characterized by the transit expression of DCX and mature neuronal markers, axon elongation occurs rapidly (Hastings and Gould 1999), and axon connections to the CA3 regions are established within 4-10 days after birth of newborn cells (Markakis and Gage 1999). Mature neuronal markers include calcium binding protein (calretinnin), neuronal nuclear (NeuN), and calbindin. During the early stage of maturation (the first week after birth), newborn neurons start to receive γ-aminobutyric acid (GABA) but not glutamate input (Ge, Goh et al. 2006). During the late stage of maturation (the second and the third week after birth), newborn neurons start to receive functional glutamategic input, along with the development of dendritic spines (Ge, Goh et al. 2006). Seven weeks after division, new granule cells can generate action potentials, exhibit electrophysiological activities similar to mature granule neurons, and are integrated into the hippocampal circuitry (van Praag, Schinder et al. 2002).

It has been reported that the initial depolarization of the cell by GABA plays an important role in the maturation of newborn granule cells (Ge, Goh et al. 2006). DISC1 protein controls dendritic development and physiological maturation (Duan, Chang et al. 2007). Physiological and pathological conditions may affect the process of neuronal maturation.

1.1.3.4 Cell survival

Newborn cells not differentiated die through apoptotic cell death (Biebl, Cooper et al. 2000). After birth, at least 50 % of newborn cells die within three weeks (Dayer, Ford et al. 2003). The survival of newborn cells is subject to regulation by diverse mechanisms. The survival of 1 to 3-week-old newborn neurons is affected by the animal's experience, such as learning and exposure to enriched environment (Kee, Teixeira et al. 2007; Tashiro, Makino et al. 2007). A recent study showed that the survival of newborn cells is competitively regulated by their own NMDA-type glutamate receptor during a short period soon after birth, suggesting that the survival of new neurons are regulated in an input-dependent manner (Tashiro, Sandler et al. 2006). In addition, cell survival is also regulated by other mechanism, such as growth factors. For example, BDNF promotes the survival of newborn cells in the hippocampus.

1.1.4 Evaluation of Adult Neurogenesis

1.1.4.1 BrdU Labelling

BrdU is a halopyrimidine used as an antiviral and antineoplastic agent (Freese, O'Rourke et al. 1994). Later, it was developed as an alternative method to determine the proliferative index of tumors and was introduced for studying cell proliferation in the developing nervous system (Struikmans, Rutgers et al. 1997). It was Gage's laboratory that first used this labelling to examine the adult neurogenesis (Kempermann, Kuhn et al. 1997). BrdU may be delivered by intraventricular (i.c.v), intraperitoneal (i.p.),

intravenous (i.v.) injection, or orally for the study of adult neurogenesis. Oral delivery is less often used because it is difficult to control the amount of BrdU absorbed per animal. The original BrdU labelling protocol used by Gage group was an i.p. injection at a dose of 50 mg/kg for 12 consecutive days. After this work, BrdU labelling has diversified. A single injection of BrdU or repeated injections of BrdU at doses ranging from 50 mg/kg to 100 mg/kg weight has been successfully used in studies of adult hippocampal neurogenesis of rodents. Among these doses, the standard dose is 50-100 mg/kg body weight with multiple injections used in the majority of previous adult neurogenesis studies (Kempermann, Kuhn et al. 1997; van Praag, Kempermann et al. 1999; Palmer, Willhoite et al. 2000; Ziv, Ron et al. 2006; Naylor, Bull et al. 2008). Cameron and McKay reported that a single dose of BrdU at 100, 50, and 25 mg/kg (body weight, i.p.) labels 60%, 45%, and 8% of S-phase cells in adult rat DG and a dose of 300 mg/kg or greater is needed to label most S-phase cells. However, a recent study confirmed that a previously used standard dose of 50 mg/kg is sufficient to label the vast majority of S-phase proliferating cells in mice. As BrdU is a toxic agent, lower doses may be more appropriate for adult neurogenesis studies (Sekerkova, Ilijic et al. 2004).

1.1.4.2 Endogenous Cell Division Markers

Unlike BrdU, an S-phase marker, endogenous protein Ki-67 and proliferating nuclear antigen (PCNA) are expressed during all active phase of the cell cycle, G1, S, G2, and M except G0. Thus, they have been proposed to examine cell proliferation in situ. Although the quantification of Ki-67-positive cells reflects cell proliferation in a manner

consistent with BrdU labelling of DG, the use of Ki-67 for studying adult neurogenesis has been limited by the temporal expression in cell cycle. It is not useful once the newborn cells exit cell cycle and begin their maturation, thus making it useless for the evaluation of cell survival and cell phenotype.

1.1.4.3 Analysis of Proliferation and Survival

Two methods have been used to examine cell proliferation, the process dividing one cell into two daughter cells. One is to treat animals with a compound that labels mitotically active cells and to detect labelled cells with a variety of methods. Another one is to detect an endogenous protein that is only expressed in mitotically active cells (Christie and Cameron 2006). Both [^{3}H]-thymidine and BrdU incorporate into DNA during DNA synthesis, therefore are useful for the evaluation of cell proliferation. However, BrdU labeling has several advantages, including: (1) BrdU labeling can be detected by immnohistochemistry (IHC) using a monoclonal antibody, (2) It allows for rapid detection without handling radioactive material, and (3) It allows for the detection of labelled cells throughout the thick tissue sections required for stereological counting of the adult brain. In addition, BrdU is easier to combine with other cell markers to detect cell phenotype of newborn cells by double or triple immunofluorescent staining. Currently, BrdU labelling is the most often used technique for studying adult neurogenesis in situ (Christie and Cameron 2006; Taupin 2007). By using the BrdU injections, detection of labelled cells by IHC allows for quantitative analysis of proliferation, survival, and differentiation of newborn cells at various time points after

injection (Miller and Nowakowski 1988; Kempermann, Kuhn et al. 1997; Kempermann, Kuhn et al. 1997)

To study proliferation, animals are sacrificed following the last injection of BrdU within a short time delay ranging from a few hours to a few days. Although 2 hours after a single injection has been proposed as the true measurement of proliferation, 24 hours or a few days after multiple injections have been used to avoid the issues of counting BrdU labelled cells when the cell number of proliferating cells is low in some conditions, such in aging animals (making counting every single cell difficult) (Abrous, Koehl et al. 2005). Survival of newborn cells is usually examined several weeks after the last injection of BrdU, when the cell phenotype has been committed (Abrous, Koehl et al. 2005).

1.1.4.4 Determination of Cell Phenotype

During the process of DG neurogenesis, the newborn cells express cell markers progressively and sequentially that reflect neuronal maturation, from NSCs in SGZ to terminally differentiated neuronal cells or glia cells in the granular layer. The analysis of the phenotype of newborn cells (differentiation) requires evaluation of colocalization of BrdU labled cells and the cell-type specific markers. The current standard method is to perform three-dimensional reconstruction with confocal microscopy after immnoflurescent staining (van Praag, Christie et al. 1999).

The identification of neurons can be completed using a number of immature and mature neuronal markers. Common markers for immature neurons include collaspin response-mediated protein 4 (also known as TOAD4) and DCX. In the past, many

studies used TOAD4 to identify immature neurons. However, few suitable antibodies for IHC are available now. Currently, many studies have used DCX expressed in newborn cells between day 2 and day 10 following BrdU injection (Brown, Couillard-Despres et al. 2003). Markers for mature neurons include NeuN, neuron-specific enolase (NSE), and calbindin-D, all of which are expressed at a later stage of neuronal maturation and in the nucleus and /or perinuclear cytoplasm, where their colocalization with BrdU is readily detected (Christie and Cameron 2006).

The commonly used glia markers include GFAP and S100ß (Kempermann, Kuhn et al. 1997; van Praag, Christie et al. 1999). GFAP is widely considered to be as astrocyte marker, but it is also expressed in radial NSC cells. S100ß also labels some neurons in addition to astrocytes and oligodendrocytes (Vives, Alonso et al. 2003; Hachem, Aguirre et al. 2005). Currently, no one marker has been found to be expressed in a specific glia cell type.

It should be noted that BrdU labelling is subject to limitations and pitfalls in spite of it currently being the most used methodology to study adult neurology. As Taupin summarized, the side effects of BrdU include its toxic mutagenic property, the possibility of labelling cells undergoing DNA repair, and uncertain saturating doses (Taupin 2007). The greatest concern is nonspecific labelling caused by the possibility of labelling cells undergoing DNA repair, in addition to those in mitosis (Rakic 2002). However, this concern seems unlikely for two reasons: One is that the standard protocol of BrdU immuohistochemistry for adult neurogenesis is not sensitive enough to detect cells

undergoing DNA repair that replace 3-100 nucleotides at each site (Selden, Dolbeare et al. 1994), whereas the entire genome (2.5 billion nucleotides) is synthesized during the S-phase of mitosis; another one is that adult neurogenesis occurs in two areas of brain, rather than DNA repair which occurs constantly throughout the brain and give a low level of non-specific background (Schmitz, Axmacher et al. 1999). It has been shown that BrdU immunohistochemistry is still specific in the adult brain for neurogenesis even when the level of DNA repair is dramatically increased by irradiation (Palmer, Willhoite et al. 2000; Parent, Valentin et al. 2002).

1.1.5 Magnitude of the Adult hippocampal Neurogenesis

A quantitative study revealed that approximately 9,000 new cells are generated in the adult rat DG each day by determining the length of the cell cycle for dividing cells and the total number of dividing cells (Cameron and McKay 2001). However, another study reported that only 4000 new cells, out of which 3000 were neurons, were added to DG daily in 4-month-old rats (Rao and Shetty 2004). In mice, there is a relatively large strain difference in the size of proliferating population, with over 800 cells in BALB/cByJ mice and 1600 cells in C57BL/6J (Hayes and Nowakowski 2002). These studies were performed under BrdU labelling and by counting the number of BrdU labelled cells by stereology. However, it is difficult to directly compare between different studies since different doses of BrdU were used in different studies and the permeability of the blood brain barrier to BrdU may differ between different strains. In addition, other factors

regulating cell proliferation, such as exercise and age, will affect the total numbers of newborn neurons and should be considered when comparing different studies.

1.1.6 Regulation of Adult Hippocampal Neurogenesis

Adult neurogenesis is regulated by many factors, both of intrinsic and extrinsic. These factors include, but are not limited to, growth factors, neurotransmitters, hormones, stress, and physical activity. Many steps of adult neurogenesis, including the proliferation of NSCs or progenitors, differentiation and fate determination of progenitors, and survival, have been demonstrated to be affected by these factors. Therefore, adult neurogenesis is a consequence of multiple combinations of regulated events.

1.1.6.1 Neurogenic Niche

The factors that regulate proliferation of NSCs at the SGZ have not been fully understood. Many investigations focused on the subject what is the cell origin of adult neurogenesis in the SGZ and SVZ. It has been hypothesized that the microenviroments of the SGZ and SVZ, known as the neurogenic niche, may determine many properties of stem cells, including self renewal, proliferation, differentiation, and fate differentiation (Morrison and Spradling 2008). Investigators have postulated that astrocytes and endothelial cells in the vasculature provide a unique neurogenic niche (Palmer, Willhoite et al. 2000; Doetsch 2003; Lie, Song et al. 2004).

In the SGZ, adult NSCs are closely apposed to granule cell layer. In the microenviroment, there are also astrocytes, ologodendrocytes, and neurons. Among these,

hippocampal astrocytes may play an important role in SGZ neurogenesis. Previous studies indicated that hippocampal astrocytes promote proliferation and neuronal fate specification of cultured adult neuronal progenitors. In contrast, astrocytes from the adult spinal cord, a non-neurogenic region, do not promote neuronal differentiation (Song, Stevens et al. 2002), suggesting a regional specificity of astrocytes. Wnt3 and its receptor are expressed in astrocytes of the hippocampus. Overexpression of Wnt3 is sufficient to increase neurogenesis in vitro and in vivo. By contrast, blockade of Wnt signalling reduces neurogenesis in vitro and abolishes neurogenesis almost completely in vivo, suggesting that hippocampal astrocytes may act through Wnt signalling (Lie, Colamarino et al. 2005). Another cell type providing neurogenic support is endothelial cells. One study reported that in the adult DG, dividing NSCs and endothelial cells are clustered around the capillaries, suggesting that neurogenesis and angiogenesis are active at the same site and they may respond to the same signals from the vasculature (Palmer, Willhoite et al. 2000). Indeed, vascular endothelial growth factor (VEGF) has been found to mediate both hippocampal neurogenesis and angiogenesis (details described in growth factors section below).

1.1.6.2 Growth factors

Growth factors are extracellular signalling molecules that increase cell growth and maintenance. They may regulate proliferation, differentiation, and cell survival. These factors include, but not limited to, brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF-1), fibroblast growth factor 2 (FGF2), and VEGF.

A key positive regulator of adult neurogenesis is BDNF and has been investigated intensely. BDNF, a member of neurotrophin family, is widely expressed in the developing and adult brain, being particularly highly expressed in hippocampus (Hofer, Pagliusi et al. 1990). By binding to its tyrosine kinase receptor (trkB), BDNF activates a number of growth and survival-promoting intracellular signaling pathways, including the mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and the phospatidylinositol 3-kinase (PI3K)/Akt pathways (Barbacid 1995; Yuan and Yankner 2000; Zheng, Kar et al. 2000). BDNF supports the growth and development of immature neurons and enhances the survival and function of adult neurons, including glutamatergic neurons in DG (Barde 1994; Lindvall, Kokaia et al. 1994). In the hippocampus, BDNF controls neurogenesis by cell proliferation, cell survival and cell differentiation. For example, cell proliferation and survival of newborn neurons in the DG are lower in heterozygous BDNF knockout mice (Linnarsson, Willson et al. 2000; Lee, Duan et al. 2002; Sairanen, Lucas et al. 2005), suggesting a role of BDNF in the maintenance of the basal level of hippocampal neurogeneis. Consistent with this finding, Scharfman and his colleagues showed that infusion of BDNF into the DG leads to higher level of hippocampal neurogenesis (Scharfman, Goodman et al. 2005). Chan and his colleagues reported that depletion of central BDNF in mice impeded terminal differentiation of new granule neurons in the adult hippocampus (Chan, Cordeira et al. 2008), suggesting that BDNF is required for the terminal differentiation of new neurons in the adult hippocampus. In addition to the role of maintenance of basal hippocampal

neurogenesis, BDNF regulation by pathological processes or physical exercise is also involved in the regulation of hippocampal neurogenesis. The up-regulation of hippocampal BDNF has been observed in the enhancement of neurogenesis following exercise (Russo-Neustadt, Beard et al. 1999; Berchtold, Kesslak et al. 2001), dietary restriction (Lee, Seroogy et al. 2002), and antidepressant treatment (Sairanen, Lucas et al. 2005). Furthermore, a reduction of BDNF correlates with reduced granule neuron survival in immune-deficient mice (Ziv et al., 2006). Finally, BDNF could work as a feedback loop with other factors, such as serotonin, FGF2, and nitric oxide (NO), to promote the proliferation, differentiation and survival of new neurons. For example, NO has been reported to act in a positive feedback loop with BDNF to promote proliferation and differentiation (Cheng, Wang et al. 2003). Thus, BDNF appears to be a converging point for distinct signalling pathways regulating hippocampal neurogenesis.

IGF1 is a growth promoting peptide hormone produced both centrally in neurons as well as glial cells and peripherally in the liver. In addition to IGF1 locally synthesized in the brain, circulating IGF1 can cross blood-brain barrier and exert an effect in the CNS (Reinhardt and Bondy 1994). By binding to its receptor, type-1 IGF receptor (IGF-1R), IGF1 activates several growth and survival-promoting intracellular signalling pathways, including the MAPK and PI3K/Akt pathways (Zheng, Kar et al. 2000; Bondy and Cheng 2004). It has been reported that IGF1 signalling is essential for the postnatal development of the hippocampus (O'Kusky, Ye et al. 2000; Liu, Ye et al. 2009). IGF-1 mRNA and IGF-1R have been detected in both pyramidal and granule neurons in the

hippocampus (Werther, Abate et al. 1990; Zhang, Moats-Staats et al. 2007). IGF1 is predominately located in neurons and astrocytes and, to a lesser extent, in oligodendrocytes and their precursors (Bondy and Cheng 2004). Previous studies indicated that IGF1 promotes proliferation and differentiation into neuronal fate in the DG (Aberg, Aberg et al. 2000; Trejo, Carro et al. 2001). In addition, IGF1 is also involved in physical activity induced hippocampal neurogenesis for blockage of IGF1 prevented the effect of running induced neurogenesis (Trejo, Carro et al. 2001).

Recent studies indicate that FGF2 may also play a permissive role in SGZ proliferation (Jin, Sun et al. 2003; Zhao, Li et al. 2007). Administration of FGF2 promotes hippocampal neurogenesis in rats (Rai, Hattiangady et al. 2007); in contrast, mice lacking FGF-1 receptor, a major receptor for FGF2, exhibits deficits in hippocampal neurogenesis (Zhao, Li et al. 2007). VEGF is a regulator for both of angiogenesis and neurogenesis. A high level of VEGF induces both of hippocampal neurogenesis by increasing cell proliferation in the SGZ and the survival of newborn cells and angiogenesis, and VEGF induced hippocampal neurogenesis is positively associated with hippocampal function (Jin, Zhu et al. 2002; Cao, Jiao et al. 2004). In contrast, blockage of VEGF signalling abolished running and enrichment induced neurogenesis (Fabel, Tam et al. 2003; Cao, Jiao et al. 2004).

1.1.6.3 Neurotransmitters

Neurogenic niches are heavily innervated by neurons. Neurotransmitters released from the nerve terminals play a role in the communication between neurons. Granule neurons,

the principle neurons at DG, receive multiple classes of neurotransmitters, including glutamate, GABA, serotonin, catecholamines, and acetylcholine. Recent studies indicated that neurotransmitters are associated with controlling the proliferation of NSCs and differentiation into neurons. Glutamate plays a major role in regulating hippocampal neurogenesis through three types of receptors, including the N-methyl-D-aspartate (NMDA) receptor, the kainic acid (KA) receptor, and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. Glutamate input into hippocampus limits baseline neurogenesis via the NMDA receptor (Cameron, Tanapat et al. 1998; Bursztajn, Falls et al. 2007). In contrast, stimulation of the KA receptor produced a large increase in hippocampal neurogenesis (Parent, Yu et al. 1997; Jessberger, Zhao et al. 2007). In addition, chronic administration of AMPA receptor agonists increased hippocampal cell proliferation (Bai, Bergeron et al. 2003). Therefore, it appears that glutamate plays a critical role in the regulation of hippocampal neurogenesis, although its regulation is complex and dependent on a network of different receptors.

GABA is the main inhibitory neurotransmitter in the brain. Recent studies have addressed the role GABA in the regulation of hippocampal neurogeneis. GABA input directly depolarizes NSCs or progenitiors isolated from hippocampus and results in expression of neuronal differentiation factor NeuroD, suggesting a direct GABA enhancement of the differentiation of progenitors in the hippocampus (Tozuka, Fukuda et al. 2005). Deletion of γ-2 subunit of GABA receptor, specifically expressed in immature

neurons, reduced the survival of newborn hippocampal neurons without affecting on proliferation (Earnheart, Schweizer et al. 2007).

NO is a free radical molecule synthesized from L-arginine and also works as an atypical neurotransmitter in the brain. One study reported that exogenous administration of NO by a NO donor increased cell proliferation and migration at DG of adult rat hippocampus (Zhang, Zhang et al. 2001). A recent study confirmed the effect of NO on hippocampal neurogenesis, indicated by the fact that NO administration promotes the cell proliferation and cell survival at DG of adult mice (Hua, Huang et al. 2008).

1.1.6.4 Behaviour

Behaviour, such as enriched environment, physical activity, and learning, greatly affect adult hippocampal neurogenesis by affecting cell proliferation, cell survival, and neuronal differentiation. Enriched environment, involving exposure to novel objects and environmental complexity relative to standard housing, and physical activity, such as voluntary running, have consistently been shown to increase adult hippocampal neurogenesis but not that in the olfactory bulb. Several studies demonstrated that enriched environment increase hippocampal progenitor proliferation and enhanced the survival of newborn cells (Kempermann, Kuhn et al. 1997; Kempermann, Kuhn et al. 1998; Pham, Winblad et al. 2002; Brown, Cooper-Kuhn et al. 2003). Voluntary running has been consistently reported to promote hippocampal neurogenesis by increasing cell proliferation and cell survival (van Praag, Christie et al. 1999; van Praag, Kempermann et

al. 1999; van Praag, Shubert et al. 2005; Van der Borght, Havekes et al. 2007). Details of the effect of running on hippocampal neurogenesis will be described below.

1.1.6.5 Hormones

Hormones including corticosteroids and estrogen modulate adult hippocampal neurogenesis. Studies with rats have shown that proliferation is inhibited by administration of corticosterone and glucocorticoid receptor agonist (Kim, Ju et al. 2004); whereas, greater hippocampal neurogenesis was shown in rats lacking corticosteroids (Gould, Cameron et al. 1992; Cameron, Tanapat et al. 1998). These studies suggest that corticosteroids have a negative effect on hippocampal neurogenesis. Estrogen has also been shown to promote hippocampal neurogenesis through greater cell proliferation. In normal female rats, the level of cell proliferation is positively correlated with estrogen level in the phase of the estrous cycle (Tanapat, Hastings et al. 1999). Following ovariectomy, the exogenous administration of estradiol enhanced neural proliferation in female rats (Tanapat, Hastings et al. 1999), and reverses the reduction of hippocampal cell proliferation in older female rats (Perez-Martin, Salazar et al. 2005). Little work has been reported about the neurogenic effect of the male hormone, testosterone.

1.1.7 Alteration of Adult Hippocampal Neurogenesis in Pathological Conditions

Adult hippocampal neurogenesis is affected by various pathological insults including stroke, seizure, inflammation, neurodegenerative diseases, and psychiatric disorders, such as depression. The upregulation of adult neurogenesis has been consistently reported

following stroke or seizure (Parent, Yu et al. 1997; Jin, Minami et al. 2001; Kee, Preston et al. 2001; Tanaka, Yamashiro et al. 2004; Abrous, Koehl et al. 2005; Lu, Mahmood et al. 2005; Ming and Song 2005), whereas reduced hippocampal neurogenesis has been observed in depression, normal aging, radiation, and with chronic alcohol exposure (Babyak, Blumenthal et al. 2000; Crews, Nixon et al. 2004; van Praag, Shubert et al. 2005; Kronenberg, Bick-Sander et al. 2006; Naylor, Bull et al. 2008). However, investigations of hippocampal neurogenesis in neurodegenerative diseases, including Huntington's disease (HD), Parkinson's disease (PD), and Alzheimer's disease (AD), are conflicting in both of patients and animal models, especially in AD. AD is a common dementia disorder that affects more than 30 million people in the world (Kaneko and Sawamoto 2009). It is characterized by progressive and widespread neuronal death and functional impairments in the brain, including the hippocampus. Several mouse models of AD have been found to have lower hippocampal neurogenesis, (Feng, Rampon et al. 2001; Dong, Goico et al. 2004; Donovan, Yazdani et al. 2006). In contrast, greater hippocampal neurogenesis was reported in human post-mortem tissues of AD patients and in one of the animal models (Jin, Galvan et al. 2004; Jin, Peel et al. 2004). The discrepancy might be due to the use of different lines of transgenic mice, their background strain, gender, or age (van Praag 2008).

1.2 Exercise and Hippocampal Neurogenesis

It has been commonly accepted that exercise is beneficial for general health, such prevention of hypertension, cardiovascular diseases, and type II diabetes. However, the benefits of exercise on brain function are not well appreciated. More recently there is greater evidence supporting the concept that exercise can improve learning and memory, postpone age-related cognitive decline, decrease the risk of neurodegenerative diseases, and alleviate depression (Cotman and Berchtold 2002; Cotman, Berchtold et al. 2007; Hillman, Erickson et al. 2008; van Praag 2008; van Praag 2009). Studies exploring mechanisms underlying the beneficial effects of exercise on brain function have focused on neurotransmitters, growth factors, and the vasculature. The hippocampus is an important area in learning and memory. A large body of evidence indicate that exercise promotes hippocampal neurogenesis, which may contribute to the beneficial effects of exercise on cognition seen in pathological conditions (van Praag 2008).

1.2.1 Studies of Exercise on Hippocampalt Neurogenesis

As discussed above, neurogenesis is a dynamic process composed of multiple steps that is highly regulated by many factors. One of the positive factors is physical activity. In 1997, Kempermann and his colleagues first reported that enriched environment is beneficial for neurogenesis (Kempermann, Kuhn et al. 1997). In this study, mice were housed in either enriched (tunnels, toys, running wheels equipped) or standard conditions for 40 days and then injected with BrdU at 50 mg/kg daily over 12 days. A group of

mice were killed 24 hours after last injection to examine cell proliferation, whereas the rest of mice survived 4 weeks more (survival time-point) to allow maturation and differentiation of newborn cells. BrdU labeled cells for both of cell proliferation and cell survival were detected by immunohistochemistry and cell differentiation of surviving cells was evaluated by triple immunofluorescent staining. Results showed that the enriched mice had more surviving cells as compared to controls. Among surviving cells, 61% and 57% were differentiated into neurons in the enriched and control groups, which showed no difference between two groups. In addition, there was no difference between enriched and controls with respect to cell proliferation. In the same study, the Morris water maze, was also tested to explore the spatial memory, a hippocampal dependent function, in which mice were trained to find a platform hidden under the surface of water made opaque by non-toxic white paint. Over time, mice learned to find the hidden platform based on the cues on the testing room walls. The enriched mice learned faster than control, suggesting the possibility that new neurons contribute to enhanced cognition. Following this study, van Praag and colleagues did further investigations to elucidate the neurogenic factor responsible for environmental enrichment. Mice were assigned to five groups, including water-maze learning (learner), swim-time-yoked control (swimmer), voluntary wheel running (runner), and enriched (enriched) and standard housing (control) groups. Mice were injected with BrdU at 50 mg/kg daily for 12 days to study cell proliferation and cell survival. Results showed that neither maze training nor yoked swimming had any effect on BrdU-positive cell number. However, running doubled the

number of surviving newborn cells, in amounts similar to enrichment conditions. In addition, cell proliferation in the runner group was higher compared to all other groups (van Praag, Kempermann et al. 1999). This study suggests that running is the factor that promoted hippocampal neurogenesis in the environmental enrichment situation.

In subsequent studies, researchers evaluated whether there was an association between the amount of running and the number of new cells produced. In one study, no obvious correlation was found in housed C57BL/6 mice, when there was little variation in distance run between animals (van Praag, Christie et al. 1999). Another mice stain, 129SvEv, which showed a wide range between individual wheel running activities, there was a significant positive correlation between cell proliferation/survival and running distance (Allen, van Praag et al. 2001). The correlation of running distance and neurogenesis was also studied in mice bred for high level of voluntary exercise over 26 generations (Rhodes, van Praag et al. 2003). In this study, there were four groups, including hyperactive runners (selectively bred mice with high levels of wheel running), hyperactive sedentary mice, control runners, and outbred control sedentary animals. After BrdU administration, cell proliferation and cell survival were evaluated. There was no difference between hyperactive sedentary and control sedentary mice in the number of BrdU labeled cells. Hyperactive runners ran 12 km/day had a 5 fold increase in cell survival compared to hyperactive sedentary animals. Control runners ran at 5 km/day had a 4 fold increase compared to control sedentary animals. In addition, there was a significant positive correlation between running distance and cell genesis in outbreed

control mice. However no such a correlation was found in hyperactive runners, likely due to a plateau effect. Furthermore, exercise promoted spatial learning in the water maze in control runners but not in hyperactive runners, suggesting selective breeding for hyperactivity is associated with neurological deficits that affect brain function (Rhodes, Hosack et al. 2001). Thus, a positive correlation between running distance and the number of newborn cells may reflect normal brain function.

Some studies have investigated the kinetics of the effect of exercise on cell proliferation and neurogenesis. It has been reported that 10 days of wheel running increases the number of newborn cells in individually housed rodents (Allen, van Praag et al. 2001; Persson, Naylor et al. 2004; van der Borght, Ferrari et al. 2006). However, the onset of the effect on cell genesis could be earlier. One group found that 5 days of wheel running induced a 30% increase in cell proliferation (van Praag 2009). A recent study reported that cell proliferation reached a peak after 3 days of running and remained enhanced at 10 days. After 32 days of running, the proliferative effect returned to baseline (Kronenberg, Bick-Sander et al. 2006). In addition, a study showed that the greatest amount of cell genesis is the middle of the dark cycle (Holmes, Galea et al. 2004), suggesting the effect of exercise on neurogenesis was modulated by circadian phase in addition to amount of exercise. However, this data is not consistent with another study, which showed the onset of the active cycle is optimal for the effect of physical activity on cell genesis by using Ki67 labelling (Kronenberg, Bick-Sander et al. 2006).

Most studies of exercising-induced neurogenesis were performed with voluntary wheel running. Mice were housed with a free access to running wheel and run as much as 3-8 km a night, which was thought to reflect a natural activity. They had a dramatic increase in the number of newborn cells at the DG, which could be detected as soon as 24 hours after the start of exercise and seen at 3 days of exercise with the most profound effect (van Praag, Christie et al. 1999; Kronenberg, Reuter et al. 2003).

Recently, studies have investigated the effect of forced treadmill exercise on hippocampal neurogenesis in normal rodents. As expected, treadmill exercise also up-regulates hippocampal neurogenesis. In one study, rats were trained in a treadmill 30 minutes/day for 7 days to examine the cell proliferation by BrdU IHC. This study found that 30 minutes treadmill running for 7 days enhanced the number of newborn cells in the DG (Kim, Kim et al. 2002). Another study reported that up-regulation of cell proliferation could be detected with treadmill running after 3 days (Ra, Kim et al. 2002). In addition, treadmill running also enhanced cell survival of newborn cells, their maturation, and neuronal differentiation. Uda and colleagues found that chronic treadmill running at a speed of 22 m/min, 30 min/day for 7 days significantly increased the number of proliferating cell (PCNA+/GFAP+ cells) and the number of cells double-labelled with DCX+/NeuN+ in a transit stage during the neuronal maturation process in mice, indicating that chronic treadmill running stimulates the proliferation of neural progenitors in the SGZ and promotes neuronal maturation process (Uda, Ishido et al. 2006). In middle-aged mice, 5 weeks of treadmill running not only increased NSC

proliferation and the number of immature neurons but also promoted the maturation and survival of immature neurons. Significantly, treadmill running for 5 weeks restored the age-dependent decline of BDNF and its receptor, TrkB, which are known to promote neuronal differentiation and survival (Wu, Chang et al. 2008).

In addition to up-regulation of hippocampal neurogenesis, the positive effect of treadmill running on hippocampal dependent function has also been reported. One study showed that treadmill running enhanced expression of long-term potentiation in dentate gyrus and enhanced object recognition learning. These changes were associated with an increase in expression of BDNF in the dentate gyrus. Spatial learning in the Morris water maze was unaffected by exercise (O'Callaghan, Ohle et al. 2007). In a later study, one week of treadmill running significantly enhanced both object displacement (spatial) and object substitution (nonspatial) learning. These behavioural changes were accompanied by higher BDNF protein content in the DG. The effects of exercise on object substitution were mimicked by i.c.v. injection of BDNF protein. These data suggest that exercise has the potential to enhance cognitive function, possibly via a mechanism involving increased BDNF expression in DG (Griffin, Bechara et al. 2009).

The positive effect of treadmill on hippocampal neurogenesis has been reported to be modulated by the intensity and duration of treadmill running, where light or moderate, but not high intensity running, enhances hippocampal neurogenesis (Ra, Kim et al. 2002; Kim, Kim et al. 2003; Lou, Liu et al. 2008). To investigate the dependence of this effect on the magnitude of exercise, animals were divided into the control, light-exercise,

moderate-exercise, and severe-exercise groups and ran for 1, 3, 7, 14, and 28 days, respectively. It was found that cell proliferation was most prominent in the light exercise group and reached a maximum level for 7 days of exercise, suggesting that cell proliferation is modulated by the intensity and duration of treadmill exercise (Kim, Kim et al. 2003).

Taken together, enhancement of hippocampal neurogenesis by running is a robust and reproducible phenomenon. However, such is not the case in SVZ/olfactory bulb. Some investigated whether exercise increases the production of neurons or glial in other regions. It was found that there was no effect of physical exercise on neurogenesis in SVZ (Brown, Cooper-Kuhn et al. 2003).

1.2.2 Mechanisms Underlying Effects of Exercise on Neurogenesis

Three factors appear to be involved in the mechanisms underlying the effects of exercise on neurogenesis, including growth factors, neurotransmitters, and vasculature changes.

1.2.2.1 Growth factors

As discussed above, growth factors play an important role in basal level of neurogenesis. Physical exercise increases the expression of growth factors and thus regulates hippocampal neurogenesis.

BDNF is considered to be the most important factor up-regulated by exercise because it is a key molecule shown to regulate baseline hippocampal neurogenesis as discussed above, and it has emerged as a key mediator of learning and depression

(Cotman, Berchtold et al. 2007). Many studies reported that both the mRNA and protein content of BDNF increased in the hippocampus rapidly with exercise. A study reported that hippocampal BDNF mRNA expression was rapidly influenced by physical activity, showing significantly higher expression levels as soon as 6 hours of voluntary wheel running. Moreover, there was a strong positive correlation between running distance and BDNF mRNA expression (Oliff, Berchtold et al. 1998). A subsequent study showed that BDNF protein levels increased with 7 nights of wheel running and were positively correlated to the previous nights distance run (Johnson and Mitchell 2003). Using in situ hybridization, one study reported that effect of running on the elevated BDNF mRNA expression at DG was sustainable to 20 days (Garza, Ha et al. 2004). Quantitative polymerase chain reaction (PCR) analysis also revealed that BDNF mRNA levels were significantly higher in the DG of wheel runners, but not in other areas of the hippocampus (Farmer, Zhao et al. 2004). In addition to an effect of exercise on BDNF expression under normal conditions, exercise can also counteract the reduction in hippocampal BDNF protein caused by acute immobilization stress (Adlard and Cotman 2004). Furthermore, exercise can combine with dietary restriction to up-regulate BDNF expression (Stranahan, Lee et al. 2009). A role for BDNF in exercise-induced neurogenesis is further supported by a trkB knockout study. Li and colleagues showed that selective knockout of trkB in hippocampal neural progenitor cells prevented the exercise-induced increase in neurogenesis (Li, Luikart et al. 2008). Furthermore, inracerebral infusion of BDNF increased nrurogenesis in the SGZ (Scharfman, Goodman

et al. 2005), but not in the SVZ (Galvao, Garcia-Verdugo et al. 2008), consistent with data showing that the meurogenic effect of exercise is limited to the hippocampus (Brown, Cooper-Kuhn et al. 2003) .

IGF1 is another molecule shown to be involved in exercise-induced neurogenesis. With exercise, IGF1 level increases in the serum and crosses the blood brain barrier to enter the brain (Carro, Nunez et al. 2000; Trejo, Carro et al. 2001). Following exercise, it has also been reported that both level of IGF1 mRNA expression and protein increased in the hippocampus (Carro, Nunez et al. 2000). In addition, peripheral blockade of IGF1 prevented exercise-induced neurogenesis. Trejo and colleagues blocked the entrance of circulating IGF-I into the brain by subcutaneous infusion of blocking IGF-I antiserum to rats undergoing exercise training. The results showed that inhibition of brain uptake of IGF-I was associated with complete inhibition of exercise-induced increases in the number of new neurons in the hippocampus, showing that IGF1 is a necessary component of physical activity-induced neurogenesis. Moreover, mutant mice with low levels of serum IGF1 do not respond to physical exercise; whereas administration of exogenous IGF-I restored neurogenesis in such mice (Trejo, Llorens-Martin et al. 2008). Taken together, these findings discussed above clearly show a role for IGF1 as a mediator of exercise-mediated hippocampal neurogenesis.

1.2.2.2 Angiogenesis and vascular growth factors

As discussed above, neurogenesis is associated with a vascular niche and neurogenesis and angiogenesis are closely correlated. With exercise, brain vasculature is involved in

the regulation of neurogenesis. In particular, exercise increased the proliferation of brain endothelial cells (Lopez-Lopez, LeRoith et al. 2004) and angiogenesis (Kleim, Cooper et al. 2002; Swain, Harris et al. 2003) throughout the brain. These effects may be mediated by IGF1 and VEGF. Similar to IGF1 discussed above, VEGF were also increased in the serum by exercise and crossed the blood brain barrier to enter the brain (Fabel, Tam et al. 2003). With exercise, IGF1 and VEGF mediate up-regulation of neurogenesis and angiogenesis. In addition to the role in neurogenesis, IGF1 is also required for exercise-induced vessel remodeling in the brain (Lopez-Lopez, LeRoith et al. 2004). Moreover, VEGF is associated with exercise-induced angiogenesis (Ferrara and Davis-Smyth 1997).

1.2.2.3 Neurotransmitters

Physical activity can change the function of neurotransmitter system, such as glutamatergic system, in the brain. As discussed above, the glutamatergic system plays a role in hippocampal neurogenesis, and thus, exercise-induce changes in the glutamatergic system will influence hippocampal neurogenesis. It has been found that exercise increased gene expression of the NMDA receptor subtypes (NR2A and NR2B) in the hippocampus (Molteni, Ying et al. 2002). Another study reported exercise induced upregulation of NR2B mRNA only in the dentate gyrus but not in other non-neurogenic hippocampal subfields (Farmer, Zhao et al. 2004). In addition, the increase in hippocampal neurogenesis and BDNF protein level following exercise were suppressed in mice lacking NR2A (Kitamura, Mishina et al. 2003) . The above evidence suggested that exercise induced changes in glutamategic function may affect hippocampal

neurogenesis. Another transmitters system, serotonin, may also be involved in exercise induced neurogenesis (van Praag 2008), but it is not addressed here.

1.2.3 Potential Benefits of Exercise on Hippocampal Neurogenesis in Normal Aging and Pathological Conditions

As described earlier, reduced hippocampal neurogenesis has been reported in depression, normal aging, radiation, and chronic alcohol exposure (Babyak, Blumenthal et al. 2000; Crews, Nixon et al. 2004; van Praag, Shubert et al. 2005; Kronenberg, Bick-Sander et al. 2006; Naylor, Bull et al. 2008). In addition, it has been proposed that neurodegenerative disease may be partly attributed to a failure of regeneration in the brain regions which containing neural stem cells/neurogenesis, such as the hippocampus (Armstrong and Barker 2001). In the presence of mounting evidence showing the benefits of exercise on hippocampal neurogenesis under normal conditions, exercise may be one of the strategies to enhance hippocampal neurogenesis, thus at least in part alleviating the progress of diseases described above. At present, most of consistent and positive data on the benefits of exercise on neurogenesis have come from studies on normal aging (Fabel and Kempermann 2008). It has been known that neurogenesis declines to low levels with normal aging in rodents (Kuhn, Dickinson-Anson et al. 1996). With 45 day voluntary running, newborn cell survival was increased in the DG in normal aging mice. The phenotype analysis indicated that there were more newborn cells differentiated into neurons (25.6%) in aging running mice compared to aging sedentary mice (9.5%) (van

Praag, Shubert et al. 2005). In addition, the age-dependent reduction in cell genesis was partially prevented by 6 months of wheel running (Kronenberg, Bick-Sander et al. 2006). Kronenberg and colleagues examined adult hippocampal neurogenesis at the age of 6 weeks, 9 months, 12 month, and 24 months in mice and showed that cell proliferation was lower with increasing age. Increasing age reduced adult neurogenesis at 9 months to 50 % of the value at 6 weeks and to 17 % at the age of 2 years. Three month old mice with sustained exercise for 6 months significantly reduced the age-dependent decline in cell proliferation (Kronenberg, Bick-Sander et al. 2006). Recently, Wu and colleagues performed a study to test the effect of forced treadmill exercise on hippocampal neurogenesis on aging mice. They found an age-dependent decrease in cell proliferation and the number of immature neurons in 7, 9, 13, and 24 -month-old mice compared to 3-month-old mice. Five weeks of treadmill running not only increased cell proliferation and the number of immature neurons but also promoted the maturation and survival of immature neurons in middle-aged (9 and 13-month-old) mice (Wu, Chang et al. 2008).

At present, there are some reports showing that exercise can rescue hippocampal neurogenesis after radiation (Clark, Brzezinska et al. 2008; Naylor, Bull et al. 2008) or alcohol exposure (Yu, Yoon et al. 2003; Crews, Nixon et al. 2004; Redila, Olson et al. 2006). However, the investigation in neurodegenerative diseases is still conflicting (van Praag 2008). In AD transgenic mouse models, long term wheel running (8 months duration) did not change hippocampal neurogenesis and did not improve spatial learning

(Wolf, Kronenberg et al. 2006). Thus, the benefits of exercise on deficits in hippocanpal neurogenesis still need to be further investigated.

1.3 Oxidative Stress

1.3.1 Reactive Oxygen Species, Oxidative Stress, and Antioxidants

Free radicals are highly reactive molecules that have unpaired electron in their outer orbital. The term "reactive oxygen species" (ROS) is often used instead, in that nonradical compounds having high oxidative properties can be included. The most important ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), peroxynitrite ($ONOO^-$), and nitric oxide (NO). Peroxynitrite is formed by NO and $ONOO^-$. NO is produced from L-arginine by a class of enzymes called nitric oxide synthase (NOS). ROS are generated in many physiological processes during the course of aerobic metabolism. The main endogenous source of ROS is mitochondria, which produces ATP by mitochondrial electron transport chain. During the energy transduction in mitochondria, a small number of electrons "leak" to oxygen prematurely, forming O_2^-. The primary ROS, i.e. O_2^- , can further interact with other molecules to generate secondary ROS, through enzyme- or metal-catalysed process. Under normal conditions, superoxide is dismutated by superoxide dismutase (SOD) into H_2O_2. Hydrogen peroxide is further converted to H_2O by the enzyme glutathione peroxidase (GPx) or catalase. Alternatively, H_2O_2 can be broken into highly reactive radical OH^- by iron-dependent Fenton reaction.

ROS plays a dual role, both beneficial and deleterious (Valko, Leibfritz et al. 2007). Beneficial effects of ROS occur at low/moderate concentrations and involve many physiological roles. These physiological roles may include the regulation of vascular tone and platelet aggression, a sensor of ventilation, regulation of erythropoietin, and regulation of cell adhesion (Droge 2002). In particular, ROS can induce a number of signalling pathways through activation of extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), MAPK, and phosphoinositide 3-kinase (PI(3)K)/Akt pathway. In addition, ROS can activate transcription factors, such as AP-1 and NF-kB. Finally, ROS themselves can act as second messengers downstream of receptors for TGF-β1, FGF-2, TNF-α, IFN-γ, angiotensin II, endothelin and more (Droge 2002).

The deleterious effect of ROS causing potential biological damage is termed oxidative stress (Valko, Leibfritz et al. 2007). It occurs when there is an imbalance in ROS production and antioxidant deficiency. In another words, oxidative stress results from a relative overproduction of ROS related to the enzymatic and non-enzymatic antioxidants. The excess ROS can react with lipids, proteins, and nucleic acids, and other biomolecules, altering their structure and function. Oxidative stress can lead to an accumulation of oxidized products such as aldehydes and isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation and base adducts from DNA oxidation, all of which can be used as oxidative stress markers (Markesbery 1999). DNA oxidation leads to single or double-strand breaks, cross-links, and base modification and can have far reaching consequences. The best studied marker of oxidative damage to

DNA is the formation of the 8-hydroxy-2-deoxyguanosine (8-OHdg). Protein modifications resulting from oxidative stress include fragmentation, cross-linking, unfolding and conformational changes. A typical marker of protein oxidation is the formation of carbonyl compounds. Lipids are also targets of oxidation and are damaged as a consequence of a self-propagating chain reaction of peroxidation. The most often studied products of lipid oxidation are aldehydes, with malondialdehyde (MDA), being the most popular. Oxidative damage is widely involved in many diseases, including muscle wasting, malignant diseases, diabetes, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, HIV infection, ischemia and reperfusion injury (Droge 2002).

Cells mitigate ROS by antioxidants, including antioxidant enzymes and non-enzymatic antioxidants, such as the lipophilic free radical scavenger tocopherol (vitamin E) and the hydrophilic compound ascorbate (vitamin C). The major antioxidant enzymes include SOD, catalase and GPx. Each of these enzymes is capable of neutralizing or transforming particular ROS species and altogether they create a powerful detoxification system. SOD has three isoforms, including SOD1, SOD2 and SOD3. Copper zinc superoxide dismutase, also known as SOD1 or cytosolic SOD, dismutates superoxide radicals to hydrogen peroxide and oxygen. It is localized primarily in the cytosol but was also detected in peroxisomes, lysosomes, nucleus and intermembrane space of mitochondria (Geller and Winge 1982; Chang, Slot et al. 1988; Keller, Warner et al. 1991; Okado-Matsumoto and Fridovich 2001; Sturtz, Diekert et al. 2001). This enzyme is present in all tissues with the highest abundance in the liver and brain (Okado-Matsumoto

and Fridovich 2001). Manganese superoxide dismutase, MnSOD or SOD2, is one of the most important intracellular antioxidant enzymes because it provides the first line of defense against ROS by dismutation of superoxide at the site of its synthesis, the mitochondrial matrix. Extracellular superoxide dismutase (ECSOD), known as SOD3, also dismutates superoxide radicals to hydrogen peroxide and oxygen. SOD3 is the predominant extracellular antioxidant enzyme and has been found in the uterus, umbilical cord, placenta, arteries, serum as well as cerebrospinal, ascitic and synovial fluids (Marklund, Holme et al. 1982). Catalase degrades hydrogen peroxide to water. It is most abundant in erythrocytes, hepatocytes, kidney and highly oxidative muscles (Powers, Criswell et al. 1994; Forsberg, Lyrenas et al. 2001). Subcellular localization of catalase include cytoplasm, peroxisomes and, to lesser extent, mitochondria (Radi, Turrens et al. 1991).

1.3.2 Oxidative Stress, Exercise and Brain

Brain is particularly vulnerable to the free radical damage. A number of reasons may account for this particular vulnerability as Rao and Balachandran have reviewed previously (Rao and Balachandran 2002). First, brain has been shown to contain low antioxidant enzymes, such as catalase and GPx. Second, the membrane lipids in brain contain high levels of polyunsaturated fatty acid susceptible to free radicals. Third, brain consumes a large amount of oxygen relative to its small weight, thus producing more free

radicals. Finally, brain has high amount of iron, which is associated with increased free radical production through Fenton reaction.

Studying the role of oxidative stress has led to the development of a variety of measurements to evaluate the degree of oxidative stress (Kontos 1989). However, none of them are fully satisfactory because ROS are highly reactive and are short-lived, making them difficult to measure directly. Therefore, most methodologies involve indirect determinations of antioxidant enzymes and the extent of oxidative stress. The evaluation of the activity of antioxidant enzymes can offer an estimation of the antioxidant defence system that may be regulated by oxidative stress (Rao and Balachandran 2002). For example, increased levels of antioxidant enzymes, such as SOD, catalase, and GPx activities has been documented in the brains of AD patients (Subbarao, Richardson et al. 1990). However, there is a remarkable heterogeneity in the degree of response in different brain regions (Balazs and Leon 1994). For example, an higher level of catalase activity was found in the cerebellum, frontal cortex, and temporal cortex; whereas, the level of catalase activity was not different in other brain regions, such as hippocampus, parietal cortex and entorhinal cortex in AD patients as compared to controls (Chen, Richardson et al. 1994). Thus, additional evaluation of oxidative markers will be needed to study the role of oxidative stress in neurodegenerative disease.

Exercise requires higher ATP and endurance exercise enhances aerobic metabolism, which results in an increased formation of ROS. However, regular moderated exercise has been shown to be beneficial in many oxidative stress related

diseases, such as heart disease, type II diabetes, some neurodegenearative diseases, and certain cancers (Radak, Chung et al. 2008). Although the mechanism of exercise benefits is complex, it has been proposed that it is, at least in part, associated with oxidative stress-induced adaption, that is activation of the antioxidant system, interferes with the oxidative damage repair/eliminating systems, and influences redox-sensitive signalling pathways (Radak, Chung et al. 2008). It has been suggested that the oxidative challenge-associated adaptive response after exercise training is most significant in skeletal muscle. Our lab showed that exercise resulted in a significant increase in the activity of SOD1 and catalase in the vastus lateralis muscle of older adult humans (Parise, Phillips et al. 2005). In the brain, the oxygen flow is relatively constant during exercise as compared to muscle, and despite this relative stability of energy metabolism and oxygen supply it seems that oxidative challenge-associated adaptation occurs in the brain, although there is some paradoxical data described below.

There is a discrepancy regarding the effect of exercise on the activities of antioxidant enzymes in the CNS. In an early study, voluntary moderate exercise for 2 weeks was shown to result in oxidative damage in low vitamin fed animals (Suzuki, Katamine et al. 1983). Swimming for 60 minutes caused elevations of both lipid peroxidation markers (MDA and 4-hydroxy-2-nonenal) and the antioxidant enzyme glutathione peroxidise (Hara, Iigo et al. 1997). In contrast to these studies, Romani reported that a treadmill for 7.5 weeks led to higher activities of SOD and GPx in brainstem and corpus striatum of rats (Somani, Ravi et al. 1995). Meanwhile, Romani

observed that the activities of antioxidant enzymes were dependent on brain regions, which was consistently reported in Chen's study (Chen, Richardson et al. 1994). In addition, Romani also reported that higher activities was not found in the hippocampus and cerebral cortex although they were in the brainstem and corpus striatum, suggesting that the effect of exercise on antioxidant enzymes was also dependent on brain region (Somani, Ravi et al. 1995).

Radak reported that a single bout of exercise, which caused oxidative damage to kidney and liver (Radak, Asano et al. 1996), eliminated the immobilization-induced oxidative damage to lipids, proteins and nuclear DNA in rat brain (Radak, Sasvari et al. 2001). A recent study reported that treadmill exercise did not affect antioxidant enzyme activity of SOD1, catalase, and GPx in normal rats, yet it increased the activity of GPx in the brain of diabetic rats (Ozkaya, Agar et al. 2002), suggesting the effect of exercise on antioxidant enzymes was also dependent on animal conditions. Taken together, these suggest that the response of activities of antioxidant enzymes is determined by brain regions, by animal physiological/pathological status, and by exercise, including type, intensity, and duration (Radak, Kumagai et al. 2007).

1.3.3 Oxidative Stress and Hippocampal Neurogenesis

As discussed earlier, the role of oxidative stress has been widely investigated in both physiological and pathological conditions. However, the role of oxidative stress in hippocampal neurogeneis is still not well defined. So far, it has been demonstrated that

oxidative stress caused by irradiation, chronic alcohol exposure, and vitamin E deficiency may be involved in regulation of hippocampal neurogenesis. A relationship between irradiation and impaired hippocampal neurogenesis has been well established (Limoli, Giedzinski et al. 2004; Limoli, Giedzinski et al. 2006; Limoli, Giedzinski et al. 2007). Oxidative stress has been proposed to be one of candidates leading to impaired hippocampal neurogenesis. In an *in vitro* study, increased oxidative stress is associated with an acute and chronic depletion of neural precursor cells after irradiation (Rola, Otsuka et al. 2004). Furthermore, in the irradiated brain, increased oxidative stress in the hippocampus occur at a time when the number of proliferating precursors and their progeny are significantly reduced (Mizumatsu, Monje et al. 2003; Limoli, Giedzinski et al. 2004). A recent study showed that both endogenous and exogenous oxidative stress inhibited the cell proliferation in the hippocampus (Limoli, Giedzinski et al. 2006). While these studies discussed above clearly suggested a negative role of oxidative stress in hippocampal neural progenitor cells, one study reported that irradiation, which resulted in an 86% reduction of newly born cells in the dentate gyrus in wild type mice, had no apparent effect in EC-SOD (SOD3) knock-out mice (Rola, Zou et al. 2007). It suggests that a microenvironment characterized by persistent oxidative stress, such as in EC-SOD KO mice is much more permissive in the context of hippocampal neurogenesis. The mechanism behind this observation still needs clarification.

Another model for studying oxidative stress and hippocampal neurogenesis is vitamin E deficiency. In one study, neurogenesis was evaluated in the dentate gyrus by

BrdU labelling of vitamin E-deficient rats. It was found that BrdU labelled cells were more numerous in vitamin E-deficient rats in comparison to age-matched controls, consuming a standard diet (Ciaroni, Cuppini et al. 1999). Later, another study further examined hippocampal neurogenesis by evaluation of neural precursor proliferation and newborn cell survival in the vitamin E-deficient model. In this study, BrdU-labelled cells were counted at 1 and 30 days after BrdU administration in order to evaluate proliferation and newborn cell survival, respectively. In addition, the TUNEL technique was also used to determine apoptotic cell death. The results showed a higher number of proliferating cells but a low number of surviving cells in vitamin E-deficient rats than in age-matched controls, suggesting vitamin E-deficiency enhanced neural progenitor proliferation but inhibits newborn cell survival (Ciaroni, Cecchini et al. 2002). Vitamin E deficiency is a complex condition and involves many systems, such as alternations in the adrenal glands and ovary. To narrow down the direct role of vitamin E, hippocampal neurogenesis was investigated in rats with supplementation of α-tocopherol, the most important compound of vitamin E. It demonstrated inhibited cell proliferation, reducing dying cells, and increased numbers of immature neurons in α-tocopherol supplemented rats, suggesting α-tocopherol inhibits cell proliferation but has a protective role with respect to cell death (Cecchini, Ciaroni et al. 2003).

1.4 Oxidative Stress, ALS and G93A Mice

Oxidative stress has been implicated in many diseases involving cardiovascular disease, cancer, neurological disorders, diabetes and aging. In particular, oxidative stress is associated with neurodegenerative disease, such as AD, PD, HD and ALS. The association of oxidative stress and neurodegenerative disease might be due to the fact that brain and nervous system are particularly vulnerable to free radical damage as described earlier.

1.4.1 Oxidative Stress in Motor Neuron Area in ALS and G93A Mice

ALS, commonly known as Lou Gehrig's disease, is a progressive and a fatal neurodegenerative disease characterized by selective loss of motor neurons in the spinal cord, brain stem, and cerebral cortex, which leads to skeletal muscle atrophy, paralysis and eventual death. Generally, the onset of ALS occurs in the fourth or fifth decade of life, although it can be at any age (Rowland 1995). Its incidence is about 2 to 3 per 100,000 people (Yoshida, Mulder et al. 1986), making it the most common paralytic disease in adults. Although the etiology accounting for selective motor neuron death is not completely identified, increased oxidative stress may contributed to the pathology in ALS (Simpson, Yen et al. 2003) since 15-25% of familial ALS (FALS) is associated with mutations in SOD1 gene (Rosen, Siddique et al. 1993).

The development of ALS murine model, the G93A transgenic mouse, highlights the role of oxidative stress in motor neuron degeneration. The generation of a transgenic

model was based on the finding of the autosomal-dominant mutation of the SOD1 gene that encodes the enzyme copper-zinc superoxide dismutase (CuZnSOD) in patients with FALS. Several transgenic mouse models with human mutant SOD1 have been generated, including G93A, G37R, and G85R (Shibata 2001). The G93A mouse has a transgenic overexpression of a transition mutation (glycine substitution to alanine at amino acid 93, G93A) found in CuZnSOD from some FALS patients. Overexpression of mutant SOD1 in the G93A mouse causes motor neuron death and progressive paralytic disease, which resembles human ALS in clinical and pathological features (Gurney 1994). The pathological changes in G93A mice mainly involve the grey matter areas of the motor neuron system, such as spinal cord anterior horns and brainstem motor nuclei, both of which are usually involved in human ALS (Shibata 2001). At 3 months of age, G93A mice display motor neuron loss with clinical signs, such as a subtle shaking in their limbs, and corticospinal tract findings, such as hyperreflexia and crossed spread of spinal reflexes. By 4 months of age, progressive paralysis appears in the rear legs. Depending on the transgene copy number, animals can survive from 130 to 210 day of age when they become completely paralysed.

Extensive evidence shows higher oxidative damage in ALS patients and in G93A mice, both in motor neuron areas and in non-motor neuron areas. Higher level of protein oxidation has been found in the spinal cord and motor cortex of sporadic ALS patients (Shaw, Ince et al. 1995). A higher level of 3-nitrotyrosine (3-NT), an oxidative damage marker mediated by peroxynitrite, was observed both in the sporadic and familial form of

ALS (Beal, Ferrante et al. 1997), and was found to be prominent in motor neurons in ventral horn of the spinal cord by immunohistochemistry (Abe, Pan et al. 1995; Abe, Pan et al. 1997). In addition, 8-OHdG, a DNA oxidative stress marker, has also been found to be most prominent in the ventral horn of spinal cord in ALS patients (Ferrante, Browne et al. 1997). Furthermore, a marker of lipid peroxidation, 4-hydroxynonenal, was shown to be elevated in both cerebrospinal fluid (CSF) and serum in ALS patients (Smith, Henry et al. 1998; Simpson, Henry et al. 2004). Taken together, the above evidence showed the presence of oxidative damage to protein, DNA, and lipid in motor neuron areas in ALS patients. Consistently, oxidative damage to DNA, protein and lipid has been reported in the spinal cord of G93A mice (Hall, Andrus et al. 1998; Liu, Althaus et al. 1998; Warita, Hayashi et al. 2001; Aguirre, Beal et al. 2005).

1.4.2 Exercise in ALS and G93A mice

The current studies are controversial regarding the benefits of exercise in ALS patients (Francis, Bach et al. 1999). In humans, epidemiological studies showed an increased incidence of ALS in marathon runners and some sport professionals (Scarmeas, Shih et al. 2002; Chio, Benzi et al. 2005). In addition, it has been proposed that lifelong and habitual heavy exercise may be a risk factor for developing ALS (Kurtzke 1991; Longstreth, Nelson et al. 1991; Strickland, Smith et al. 1996). However, other epidemiological studies reported that exercise is not likely a risk factor for developing ALS (Armon, Kurland et al. 1991; Longstreth, McGuire et al. 1998). Several clinical

trials have showed the benefits of certain types of exercise including treadmill running in ALS patients (Pinto, Alves et al. 1999; Drory, Goltsman et al. 2001; Bello-Haas, Florence et al. 2007). In G93A mice, Gage's group found that daily non-exhaustive wheel running results in significant increase of 83 days in life span compared to sedentary mice (Kaspar, Frost et al. 2005). Another also reported consistent benefits of treadmill running at 13 m/min for 30 minutes, 5 days per week (Kirkinezos, Hernandez et al. 2003). In addition, a study reported that a lifetime of vigorous exercise, wheel running at 3.4 m/min for 400 minutes per day, 7 days per week, does not promote onset or progression of motor degeneration (Liebetanz, Hagemann et al. 2004). Our lab showed that high-intensity endurance exercise training (treadmill running at 45 min/day, 5 times per week, with a progressive increase from 9 to 22 m/min) did not affect onset of clinical symptoms in female G93A mice but hastened the decrease in motor performance, death, and onset of clinical symptoms in male mice (Mahoney, Rodriguez et al. 2004). In these animal studies, exercise intensity, duration, and frequency varied. Lui and colleagues summarized these investigations and pointed out that the most favourable results in terms of improved life span were associated with a self-paced running wheel exercise programs for 2 to 12 hours per day or a moderate treadmill exercise at 3.4-13 m/min for 0.5 to 6.7 hours, while the negative effects on life span were associated with high-intensity treadmill exercise at 9-22 m/min for 20-45 minutes per day (Lui and Byl 2009).

1.4.3 Sex Difference in ALS and G93A mice

The exact aetiology and risk factors for the development of ALS are unknown, but sex has been proposed as one of the possible modifying factors (Chio, Meineri et al. 1991). Epidemiological studies reported that there is a higher incidence of ALS in men than women. The ratio of men to women is 4:1, when age at onset is the second decade, followed by a steady decline leading to a 1:1 ratio at ages above 60 years (Haverkamp, Appel et al. 1995). In addition, women who develop ALS have often had a later menarche and earlier menopause than healthy controls (Chio, Meineri et al. 1991). Similarly, sex differences in onset of disease have been shown in ALS murine models. In G93A mice, Veldink and colleagues found that the onset of disease was significantly earlier in male than in female G93A mice. Treadmill running (at 16 m/min for 45 min, 5 days per week) delayed the onset of disease in female but not in male mice. Also, running delayed the total survival time in female mice (Veldink, Bar et al. 2003). Later, our lab found that male G93A mice had a trend for earlier onset of disease than female G93A mice ($p = 0.062$). Treadmill running (at 45 min/day, 5 times per week, progressive increase from 9 to 22 m/min) did not affect onset; however, it hastened death in male but not female mice. Furthermore, female G93A mice survive longer than male, irrespective of exercise training (Mahoney, Rodriguez et al. 2004). In an ALS model of rat, overexpressing a transgenic gene of human mutant SOD1, a recent study showed the onset of disease in males was significantly earlier than in females, although the progression was not different between the sexes (Suzuki, Tork et al. 2007). These

observations suggested the involvement of sex hormones in the onset or disease progression of ALS and its models.

1.4.4 Hippocampal Changes in ALS and G93A mice

Although ALS is traditionally considered a motor neuron disease, the involvement in non-motor areas of the CNS has been demonstrated in ALS patients and G93A mice. For example, both clinical and neuropathological aspects suggest the presence of abnormal hippocampal function and fronto-temporal dementia in ALS patients (Waldemar et al, 1992; Hanagasi et al, 2002; Strong et al, 2006). Oxidative injury has been shown in the parietal cortex and cerebellum, regions that are typically clinically unaffected in ALS, suggesting widespread oxidative stress (Kim et al, 2004). In G93A mice, elevated oxidative stress has been reported in the brain, (Cha, Chung et al. 2000; Chung, Hong et al. 2003; Chung, Joo et al. 2004). Immuohistochemical studies found that nitrotyrosine-immunoreactive neurons were observed in many areas of brain in G93A mice, including the hippocampus (Cha, Chung et al. 2000). In the hippocampus of G93A mice, nitrotyrosine-immunoreactive neurons in the CA1 region showed intense staining, and the immunoreactivity was localized mainly in the pyramidal cell layer (Cha, Chung et al. 2000). In addition, one study investigated MnSOD protein content and found significantly higher immunoreactivity for MnSOD in several brains areas of G93A mice, primarily in the brain stem. However, they did not report on the MnSOD content in the hippocampus (Chung, Joo et al. 2003). In addition to oxidative stress, other changes,

such as increased growth factor expression and extracellular pathways, have also been observed in the hippocampus of G93A mice. For example, higher expression of the IGF1 receptor, EPO, and pERK were observed in the hippocampus of G93A mice (Chung, Joo et al. 2003; Chung, Joo et al. 2003; Chung, Joo et al. 2004), suggesting the activation of PI3K/MAPK signaling cascades. In addition, a higher expression of AMPA subunit GluR1 has been shown in the hippocampus with enhanced hippocampal-dependent spatial capability of G93A mice (Spalloni, 2006). All these lines of evidence, including the changes in oxidative stress, growth factors, and related extracellular pathway, as well as hippocampal-dependent function, have been reported to be involved in the regulation of hippocampal neurogenesis. Together, these suggest that all these factors may further modify hippocampal neurogenesis in this ALS model, particularly in an exercise context.

1.5 Hypothesis

1. Based on the hippocampal changes in G93A mice described above, including higher oxidative stress, higher growth factor content, activation of ERK pathway and higher hippocampal dependent function, we hypothesized that G93A mice would have a higher basal level of hippocampal neurogenesis compared to wild type mice.

2. Due to extensive evidence that exercise promotes hippocampal neurogenesis under normal wild-type conditions and possibly in neurodegenerative disease, we hypothesized that exercise would promote neurogenesis both in wild type mice and in G93A mice.

3. As discussed earlier, estrogen can up-regulate hippocampal neurogenesis and there is a sex difference in clinical aspects of ALS demographics and G93A mice. We hypothesized that female G93A mice would show greater hippocampal neurogenesis versus male G93A mice.

4. Based on the evidence that BDNF and IGF1 play a role in basal hippocampal neurogenesis and up-regulation of hippocampal neurogenesis following exercise, we hypothesized that BDNF and IGF1 would be involved in basal levels of hippocampal neurogenesis in G93A mice with exercise increasing hippocampal neurogenesis in association with higher levels of BDNF and IGF1 in wild type mice and G93A mice.

5. Based on the evidence that higher oxidative stress was found in motor neuron areas and non-motor neuron areas in G93A mice, we hypothesized that antioxidant enzymes would increase to compensate for elevated oxidative stress in the hippocampus of G93A mice.

1.6 Specific Aims

1. To investigate the basal level of hippocampal neurogenesis in G93A mice.

Rationale. Many studies have investigated hippocampal neurogenesis in degenerative disease and showed conflicting results, such as in AD patients and AD animal models (van Praag 2008). To date, little information regarding hippocampal neurogenesis is available in G93A mice although one reported lower cell proliferation in DG and no change of net hippocampal neurogenesis in 16-week-old symptomatic G93A mice (Liu

and Martin 2006). However, this study did not investigate any molecular changes that might affect neurogenesis, such as growth factors and it was contrasting with two other studies regarding the data on cell proliferation in the spinal cord (Chi, Ke et al. 2006; Guan, Wang et al. 2007). In addition, few studies regarding growth factors and oxidative stress in the hippocampus and their role in hippocampal neurogenesis are available. Furthermore, no study has reported whether exercise would have impact on neurogenesis, growth factors, and oxidative stress in the hipppocamus in this model.

2. To investigate whether treadmill exercise would affect hippocampal neurogenesis in G93A mice.

Rationale. It is well established that both voluntary wheel and force treadmill running promote hippocampal neurogenesis. However, whether this is the case in neurodegenerative diseases is unclear. In ALS patients and G93A mice, there is conflicting data as to whether exercise is beneficial. Therefore, it is necessary to determine the impact of exercise on hippocampal neurogenesis in G93A mice.

3. To investigate whether a sex difference impacts basal level of hippocampal neurogenesis and the response of neurogenesis to exercise in G93A mice.

Rationale. The concept that there is a sex difference in hippocampal neurogenesis is beginning to be established (Galea, Spritzer et al. 2006; Galea 2008). In addition, it has also been reported that there is sex difference in the progression of neurodegenerative diseases including ALS (Czlonkowska, Ciesielska et al. 2006). To date, no study has

reported the role of sex differences in the basal level of hippocampal neurogenesis and the alternation following exercise in G93A mice.

4. To investigate whether growth factors (BDNF and IGF1) are involved in the regulation of basal level of hippocampal neurogenesis and the response to exercise in G93A mice.

Rationale. BDNF and IGF1, especially BDNF, have been demonstrated to have multiple functions, including maintaining basal levels of neurogenesis, upregulation of hippocampal neurogenesis following exercise, involvement of learning and memory, antidepressant action. In addition, BDNF may be regulated by oxidative stress, such as NO. Thus, our investigation of BDNF and IGF1 mRNA expression in hippocampal neurogenesis will help to elucidate the molecular mechanism of basal level of hippocampal neurogenesis and its response to exercise. It will also help to clarify the connection between hippocampal neurogenesis and oxidative stress.

5. To investigate whether oxidative stress in G93A mice is elevated in the dentate gyrus and whether exercise would decrease oxidative stress in the dentate gyrus.

Rationale. It has been suggested that oxidative stress is involved in impaired hippocampal neurogenesis mediated by irradiation and chronic alcohol exposure. Exercise can restore the impaired hippocampal neurogenesis. Although the G93A mouse model is associated with excessive oxidative stress in the spinal cord, it is not certain whether it would affect the dentate gyrus and whether it further influences hippocampal

neurogenesis. In addition, it is not known the effect of exercise on oxidative stress and hippocampal neurogenesis at the level of the dentate gyrus.

2. MATERIALS AND METHODS

2.1 Schematic Representation of Study

The present project was divided into three parts: a neurogenesis study, a growth factor study, and an oxidative stress study. The neurogenesis study was assessed by cell proliferation, cell survival, and cell differentiation of newly generated BrdU labeled cells in the SGZ of DG of the hippocampus. The growth factor study was investigated by examination of mRNA expression of BDNF and IGF1 at the DG in the hippocampus using in situ hybridization. Oxidative stress was evaluated by mRNA expression and protein content of antioxidant enzymes (SOD2 and catalase) and the level of oxidative stress markers at DG and CA3 region of hippocampus, including 8-OHdg as a DNA damage marker and 3-NT as a peroxynitrite of amino acid.

2.2 Animals and Experimental Design

Male transgenic G93A mice (B6SJL-TgN[SOD1-G93A]1Gur) were purchased from the Jackson Laboratory (Bar Harbour, ME), and harem bred with female wild-type B6SJL mice (Jackson Laboratory), and a colony was established. Offspring were genotyped for the G93A transgene using polymerase chain reaction (PCR) of DNA extracted from tail samples as outlined by Jackson Laboratories. Animals were housed one to five per cage with a 12-h light/dark cycle. All mice were fed standard murine chow and water ad libitum, and food intake was recorded weekly for each cage. The experimental protocol

was approved by the McMaster University Animal Research Ethics Board and was carried out in accordance with guidelines of the National Institutes of Health and the Canadian Council on Animal Care. At the 40 days of age, G93A mice and wild-type mice (B6SJL) were randomly divided into cell proliferation study group (N=46, 5-6/group for 8 groups), and cell survival study group (N=92, 9-13/group for 8 groups) based on training status and sex (Figure 2.1).

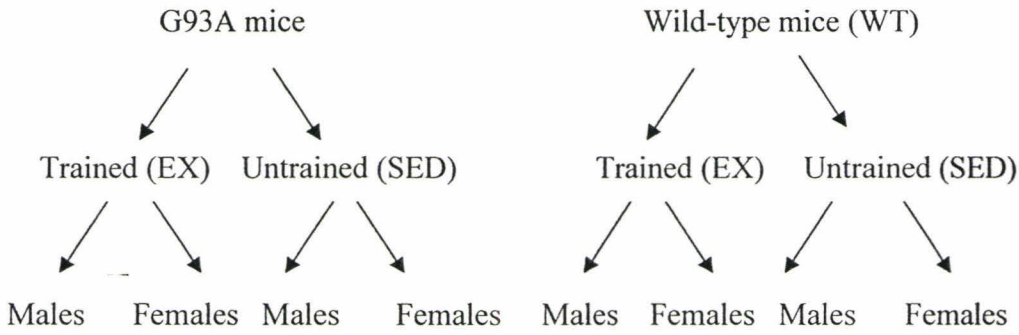

Figure 2.1 Outline of animal groups

In the cell proliferation group, mice at 90 days of age were injected for seven consecutive days with BrdU and were subjected to treadmill running for one week (see below) or to a sedentary lifestyle. Twenty-four hours after last BrdU administration, mice were sacrificed and brains were collected to quantity BrdU-labelled cells in the hippocampus by immunohistochemistry (IHC) for cell proliferation. In the cell survival group, mice at 80 days of age were injected for seven consecutive days with BrdU and were subjected to treadmill exercise for four weeks or to a sedentary lifestyle. Three weeks after last administration of BrdU, mice were sacrificed to examine the BrdU

labelled cells by IHC, the cell fate of the BrdU labelled cells by imimmunofluorescence staining, mRNA expression of growth factors (BDNF and IGF-1) and antioxidant enzymes (SOD2 and catalase) by in situ hybridization, protein level of antioxidant enzymes (SOD2 and catalase) and oxidative stress markers (8-OHdg and 3-NT) by IHC.

Cell proliferation Cell proliferation is the mitotic activity of the neuron progenitor cells. The cell proliferation rate can be measured hours to days after injection of the mitosis marker, usually BrdU or ^{3}H thymidine. In this study, at approximately 90 days of age, mice were injected with BrdU and trained for one week on a customized treadmill using a training protocol (describe bellow). Twenty-four hours after the last BrdU administration, mice were sacrifice and brains were collected (Figure 2.2) to analyze BrdU-labelled cells in the hippocampus by IHC. This time point was chosen over a shorter time point to minimize possible effects of inter-animal differences in survival time after BrdU injections (Cameron and McKay 2001).

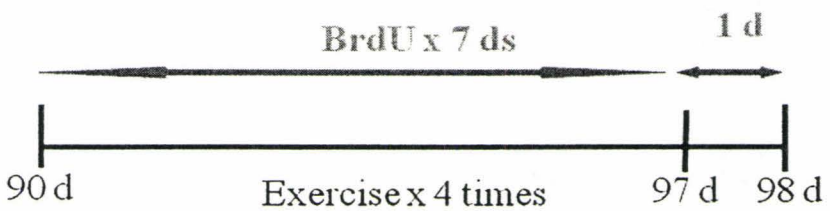

Figure 2.2 Timeline for the cell proliferation study

Cell survival and Cell Differentiation At 80 days of age, mice were trained and injected with BrdU for 7 days. Three weeks after the last administration, mice were sacrificed to examine the cell numbers of BrdU labelled cells by IHC and cell fate of the BrdU

labelled cells by immunofluorescence staining (Figure 2.3). At this time, labelled cells have extended axons (Hastings and Gould 1999), survived a period of cell death, and begin to express mature neuronal markers (Cameron and McKay 2001). Previous studies indicated that the proliferative cells of the SGZ gave rise to neurons, astrocytes, and oligodendrocyte progenitors in mice (van Praag, Schinder et al. 2002). Thus, NeuN a mature neuron marker, and GFAP, a glial cell marker, were used to determine the phenotype of BrdU-labelled cells by immunofluorescent labeling for cell survival. Meanwhile, a growth factor study and oxidative stress study were performed in the animals for cell survival and cell differentiation study (determined by cell phenotype)..

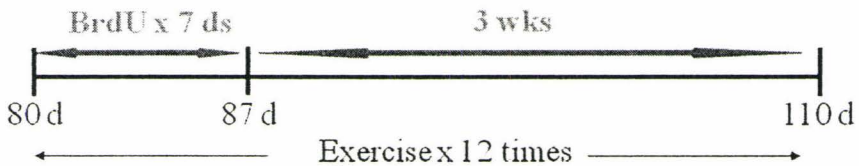

Figure 2.3 Timeline for cell survival, cell differentiation, growth factor, and oxidative stress

2.3 BrdU Injection

BrdU (Sigma, St. Louis, MO) was dissolved in fresh 0.9 % NaCl and sterile-filtered through a 0.2 μm filter. Each mouse received one single dose (50 mg/kg) of 1 μl/g body weight at a concentration of 1 mg/ml, one i.p. injection per day for seven consecutive days.

2.4 Exercise Training

Cell proliferation exercise training. Exercise training consisted of four sessions over a one week period. In the first and second training session, the mice were acclimatized to the treadmill, running at 15 m/min for 30 min. In the third and fourth training session, the exercise duration was 45 min at 15 m/min.

Cell survival and cell differentiation exercise training. Exercise training lasted for four weeks, 3 times a week. In the first and second weeks, the mice were acclimatized to the treadmill, running at 15 m/min for 30 min. In the third and forth weeks, the duration of training reached 45 min at 15 m/min.

2.5 TissuePreparation

Mice were anesthetized with isoflurane inhalation and perfused transcardially with 50 ml of phosphate buffered saline (PBS), followed by 50 ml of 4% paraformaldehyde (PFA). Brains were removed and fixed at 4°C overnight, transferred into a 30 % sucrose solution until saturated, and embedded in OCT and stored at -80°C until sectioning. The cryostat was used to cut sections. In the cell proliferation group, brains were cut to coronal sections (40 μm /section) throughout the entire rostral-caudal extent of the hippocampus (Bregma -0.94 ~ -3.88 mm) (Paxinos and Franklin 2001) for BrdU IHC. In the cell survival study group, half atmosphere of brains was cut into coronal sections (40 μm/section) throughout the entire rostral-caudal extent of the hippocampus (Bregma -0.94 ~ -3.88 mm) (Paxinos and Franklin 2001) for BrdU IHC and immunofluorescence

staining. Another half of brains were cut to sagittal sections (16 μm/section) throughout the extent of the hippocampus (Lateral 0.72 ~ 2.28 mm) (Paxinos and Franklin 2001), collected in gelatine-coated slides, and kept at -80 °C for *in situ* hybridization and IHC. Coronal sections were stored at –20 °C in cryoprotectant containing 25 % glycerin, 25 % ethylene glycol, and 0.05 M phosphate buffer.

2.6 IHC for Detection of BrdU-labeled Cells

A one in six series of sections throughout the entire rostral-caudal extent of the hippocampus was subjected to assess the number of BrdU-labeled cells. Staining was carried out on free-floating sections as previously described (van Praag, Kempermann et al. 1999). Briefly, free-floating sections were washed with Tris-buffered saline (TBS) and treated with 0.6% H_2O_2 in TBS for 30 min to block endogenous peroxide activity. Sections were then incubated for 2 h in deionized 50% formamide/50% 2 X SSC buffer (0.3 M NaCl/0.03 M sodium citrate) at 65 °C, rinsed in 2 X SSC (5 min), incubated in 2 N HCl at 37 °C (30 min), and then placed in 0.1M boric acid (pH 8.5, 10 min). Sections were then rinsed in TBS (6×10 min), incubated in TBS++ (TBS, 0.1% Triton X-100 and 3% normal donkey serum) for 30 min and then incubated in mouse anti-BrdU monoclonal antibody (1:200, Chemicon, Temecula,USA) for 12 h at 4°C. After being rinsed with TBS, sections were immersed in biotinylated donkey anti-mouse antibody (1:500, Chemicon, Temecula, USA) for 2 hours at 4°C. Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA) and diaminobenzidine (DAB) kit (Vector Laboratories)

were used to visualize BrdU-positive cells. Finally, sections were mounted on gelatine-coated slides, air dried overnight, counterstained with cresyl violet staining, dehydrated by graded ethanol and xylene, and coverslipped using permount. The number of BrdU-labeled cells in the DG was examined using light microscopy.

Cell counting of BrdU-labeled cells BrdU-labeled cells were counted in every section of a one-in-six series (240 μm apart) throughout the rostral-caudal extent of the subgranule cell layer (defined as the area ~20 μm between granule cell layer and the hilus). Because we were interested in relative differences and not necessarily an absolute value of BrdU-labeled cells in the DG, BrdU positive cells in the entire DG were quantified by profile-sampling methods (Nixon and Crews 2002). In the x-y plane, BrdU positive cells limited to the subgranule cell layer were manually counted in a blind fashion at 40 X magnification (Olympus, BX60, Center Valley, PA). For the z-plane, a modified optical dissector method was employed that excluded immune-labeled cells on the uppermost surface of the section. BrdU labeled cells were counted in the DG of both sides (for the cell proliferation group) or one side (the cell survival group) for each section.

The corresponding sample area of DG granule cell layer was outlined and determined by using image analysis (Image-pro 6.0, Media Cybernetics, Bethesda, MD). Data were expressed as cell density (cells/mm^2) per DG of BrdU-labeled cells.

2.7 Immunofluorescence for Cell Phenotypes

Immunofluorescent triple labeling for BrdU, NeuN, and GFAP was done on free floating sections as previously described (van Praag, Kempermann et al. 1999). Briefly, after pretreatment with deionized formamide for 2 h at 65 °C, 2 N HCl for 30 min at 37 °C, and 3% normal goat serum (Vector Laboratories) for 30 min at room temperature, sections were incubated in a cocktail of rat anti-BrdU (1:100, Serotec, Martinsried, Germany), mouse anti-NeuN monoclonal antibody (1:500, Chemicon, Temecula, USA), and chicken anti-GFAP polyclonal antibody (1:200, Chemicon, Temecula,USA) overnight at 4 °C. The next day, sections were rinsed in TBS, blocked in 3% normal goat serum and then incubated in a cocktail of Alexa Fluor 488 goat anti-rat antibodies (1:500, Molecular Probes, Carlsbad, USA), Alexa Fluor 568 goat anti-mouse highly cross-adsorbed antibody (1:500, Molecular Probes, Carlsbad, USA), and Alexa Fluor 647 goat anti-chicken antibody (1:500, Molecular Probes, Carlsbad, USA) for 4 h at 4°C. Then sections were rinsed several times and mounted on gelatin-coated slides and coverslipped with SlowFade Gold antifade reagent (Molecular Probes, Carlsbad, USA) and examined by confocal microscopy.

Analysis of cell phenotype A one-in-six series of sections from mice surviving 3 weeks after the last injection of BrdU was triple labeled as described above and analyzed by confocal microscopy (Leica TCS SP5, Germany). Fifty BrdU positive cells per animal (n=5~6 per group) were analyzed for co-expression of BrdU and NeuN for neuronal phenotype and GFAP for glial phenotype. Co-labeling was verified throughout the z-axis

of focus. The percentage of BrdU+ cells co-labeled with NeuN, with GFAP, or without NeuN or GFAP was determined.

2.8 In Situ Hybridization for mRNA Expression

Construction of cRNA probes. The cDNAs from ATCC clones (SOD2: MGC-6144, catalase: MGC-18464, and IGF1: clone ID: 4194295) were used as templates for PCR reactions. PCR was performed to amplify cDNA fragments. For mouse IGF1, forward 5'–TGG ATG CTC TTC AGT TCG TG-3' and reverse 5'-TCC TGC ACT TCC TCT ACT TGT-3' primers were used to amplify a 318 bp cDNA fragment. For mouse SOD2, forward 5'-CGC CAC CGA GGA AAG TA-3' and reverse 5'-CAG TCA TAG TGC TGC AAT GC-3' primers generated a 559 bp cDNA fragment. For mouse catalase, forward 5'-GCT ATG GAT CAC ACA CCT T-3' and reverse: primer 5' -GTT CAC AGG TAT CTG CAG-3' primers generated a 488 bp cDNA fragment. After running gel and purified, PCR products were cloned into pGEM-T easy vector (Promega Biotech, Madison, WI) and sequenced to verify their identities. The vector containing rat full-length BDNF cDNA insert (gift of Drs. J. Lauterborn and C. Gall, University of California Irvine, Irvine, CA) or 318 bp cDNA fragment of mouse IGF1 or 559 bp cDNA fragment of mouse SOD2 or 488 bp cDNA fragment of mouse catalase was used as templates for riboprobe synthesis. The antisense and sense riboprobes were synthesized using the Riboprobe System (Promega Biotech, Madison, WI) with α-35S-UTP (specific activity >1,000 Ci/mmol; Perkin Elmer, Boston, MA) and T3 or T7 RNA polymerase

(Promega Biotech, Madison, Wisconsin). The transcribed products were purified using ProbeQuant G-50 Micro Columns (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and probe labeling was determined by scintillation counting.

In situ hybridization was performed as previously described (Foster, Puchowicz et al. 2004). Briefly, brain sections were fixed with 4% formaldehyde, acetylated with acetic anhydride, dehydrated in graded ethanol, delipidated with chloroform, and air dried. Radioactive-labeled probes were diluted in a hybridization buffer and applied to brain sections (approximately 500,000 CPM/section). Hybridization was carried out overnight at 55° C in a humidified chamber. After hybridization, RNase treatment, high-stringency post hybridization washes, and dehydration were performed. Slides and ^{14}C plastic standards were placed in X-ray cassettes, opposed to film (BioMax MR; Eastman Kodak, Rochester, NY) for certain days (7 days for BDNF, 7 days for IGF1, 2 days for SOD2, and 10 days for catalse), and developed in an automatic processor.

Quantitative analysis of autoradiograms was done using a Macintosh computer-based image-analysis system with NIH Image software (http://rsb.info.nih.gov/nih-image). Autoradiographic film images were captured during one session with constant settings of camera (Q Capture v1.2.0, Q Imaging Corporation, Surrey, Canada) and light (Northern Light, Model-R95, Imaging Research Inc, St Catharines, Canada). NIH Image software was used to construct the calibration curve of the ^{14}C standards and to quantify signal at the DG and CA3 region of hippocampus. Then, contours were drawn over the

DG and CA3 and optical densities were automatically measured from the corresponding regions of the autoradiographic images.

2.9 IHC for Detection of SOD2, catalase, 8-OHdg, and 3-NT

Previous studies consistently reported that oxidative stress was elevated in the spinal cord and in the brain of G93A mice. However, the information whether oxidative stress is altered in the DG of hippocampus of G93A mice is unclear. Thus, antioxidant enzymes, including manganese superoxide dismutase (MnSOD or SOD2) and catalase as well as oxidative stress markers (8-OHdG for DNA oxidative damage marker and 3-NT for peroxynitrited amino acid) were examined to evaluate the oxidative stress.

Sagittal brain sections were fixed with 4% formalin for 10 minutes, blocking with normal goat serum, avidin and biotin, 0.03% hydrogen peroxide. The primary antibodies, including rabbit anti-mouse SOD2 (1:600 dilution, abcam, Cambridge, UK) rabbit anti-mouse catalase (1:1000 dilution, abcam, Cambridge, UK), rabbit anti-mouse 8-OHdG (1:400, Secrotec, Martinsried, Germany), and rabbit anti-mouse 3-NT (1:200, Upstate, Billerica, USA) were then applied and incubated overnight at 4 °C. The following day, the secondary antibodies, including biotinylated goat anti-rabbit IgG (1:500, Jackson lab, Bar Harbor, USA) were applied and incubated for one hour at room temperature followed by one hour incubation with streptravidin-horseradish peroxidase. Slides were developed for 5-10 minutes in DAB, dehydrated, and mounted with permount.

Quantitative analysis of optical density was done using a Macintosh computer-based image-analysis system with NIH Image software. Black-and-white images were captured during one session with constant settings of camera. NIH Image software was used to draw contours over the DG and optical densities were automatically measured from the corresponding regions of images.

2.10 Statistical Analysis

Data were analyzed based on our *planned comparisons* to answer the following questions: a) Are there any differences in the outcome measures at the basal sedentary levels between the G93A and WT mice? b) Are there any effects of activity and sex within each genotype variant? To address these main questions, we used a two-way analysis of variance (ANOVA) (Statistica, version 6.0, StatSoft, Tulsa, OK) to determine significant differences a) in the sedentary mice, with the two factors being genotype (G93A vs. WT) and sex (male vs. female), b) in the WT mice, with the two factors being activity (EX vs. SED) and sex (male vs. female), and c) in the G93A mice, with the two factors being activity (EX vs. SED) and sex (male vs. female). When there was significant difference, Tukey's honest significant difference test was used post hoc to determine the source of difference. Based on the hippocampal changes in G93A mice described above, including higher oxidative stress (Cha, Chung et al. 2000; Chung, Joo et al. 2003), higher growth factor content (Chung, Joo et al. 2003; Chung, Joo et al. 2004), activation of ERK pathway (Chung, Joo et al. 2005), higher hippocampal dependent function (Spalloni,

Geracitano et al. 2006), and increased cell proliferation and neurogenesis in the spinal cord of G93A_mice (Chi, Ke et al. 2006; Guan, Wang et al. 2007), we *a priori* hypothesized that G93A mice would have a higher basal level of hippocampal neurogenesis compared to WT mice. In addition, due to extensive evidence showing that exercise promotes hippocampal neurogenesis under normal wild-type conditions (van Praag, Kempermann et al. 1999; Holmes, Galea et al. 2004; Uda, Ishido et al. 2006) and possibly in neurodegenerative disease, we *a priori* hypothesized that exercise would promote neurogenesis both in WT and G93A mice. Furthermore, due to the evidence that estrogen up-regulates hippocampal neurogenesis (Galea 2008) and that there is a sex difference in clinical aspects of ALS demographics and G93A mice (Mahoney, Rodriguez et al. 2004), we *a priori* hypothesized that female mice would show greater hippocampal neurogenesis versus male mice. And based on the evidence that BDNF and IGF1 play a role in basal hippocampal neurogenesis (Linnarsson, Willson et al. 2000) and up-regulation of hippocampal neurogenesis following exercise (Oliff, Berchtold et al. 1998; Carro, Nunez et al. 2000; Johnson and Mitchell 2003), we *a priori* hypothesized that BDNF and IGF1 would be involved in basal level of hippocampal neurogenesis in G93A mice with exercise increasing hippocampal neurogenesis in association with higher levels of BDNF and IGF1 in WT and G93A mice. Finally, based on evidence that higher oxidative stress was observed in motor neuron areas (Abe, Pan et al. 1997; Beal, Ferrante et al. 1997; Warita, Hayashi et al. 2001) and non-motor neuron areas in G93A mice (Cha, Chung et al. 2000; Chung, Joo et al. 2003), we *a priori* hypothesized that markers of

oxidative stress and antioxidant enzymes would increase to compensate for elevated oxidative stress in the hippocampus. Hence, for these specific analyses, a 1-tailed test was used. For all other analyses, a 2-tailed test was used. Unless otherwise noted, all data are presented as means ± standard error of the mean (SEM). Significant differences were defined as $P \leq 0.05$.

3. RESULTS

3.1 Hippocampal Neurogenesis

3.1.1 Cell Proliferation

Twenty-four hours after the last injection of BrdU, BrdU-labeled proliferating cells could be detected in the SGZ throughout the hippocampus in all mice. The majority of the BrdU-labeled cells were located in the SGZ and less frequently in the hilus **(Figure 3.1.A)**, typically appearing in clusters and showing an irregular shape with dense and homogenous staining of the nuclei (**Figure 3.1.A insert**). The appearance and general distribution of BrdU-labeled cells did not differ between WT mice (**Figure 3.1.B**) and G93A mice (**Figure 3.1.C**).

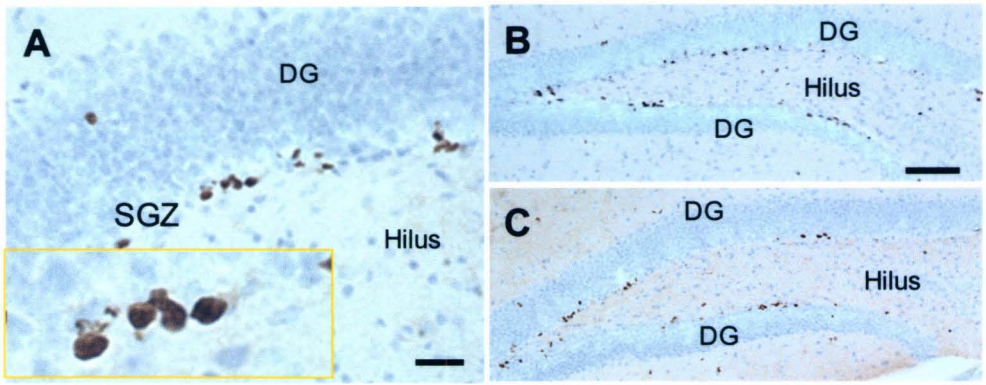

Figure 3.1 Representative images of BrdU labeled proliferating cells in the dentate gyrus (DG) of wild type (WT) and G93A mice. (A) A representative image showed the majority of the BrdU-labeled cells were located in the subgranular zone (SGZ), typically appearing in clusters and having an irregular shape with dense and homogenous staining of the nuclei (insert). Representative images showed proliferating cells in WT mice (B) and in G93A mice (C). Scale bar = 25 μm in A, 100 μm in B,C.

In sedentary mice, G93A mice had 18.5% more proliferating cells than WT mice collapsed across sex (179 ± 23/mm^2 vs 151 ± 19/mm^2; p=0.159), due to 68.7% greater number of proliferating cells in G93A males vs G93A females (226 ± 32/mm^2 vs 134 ± 17/mm^2; p=0.085) (**Figure 3.2**). This suggests that G93A sedentary males, but not females, have higher basal levels of cell proliferation.

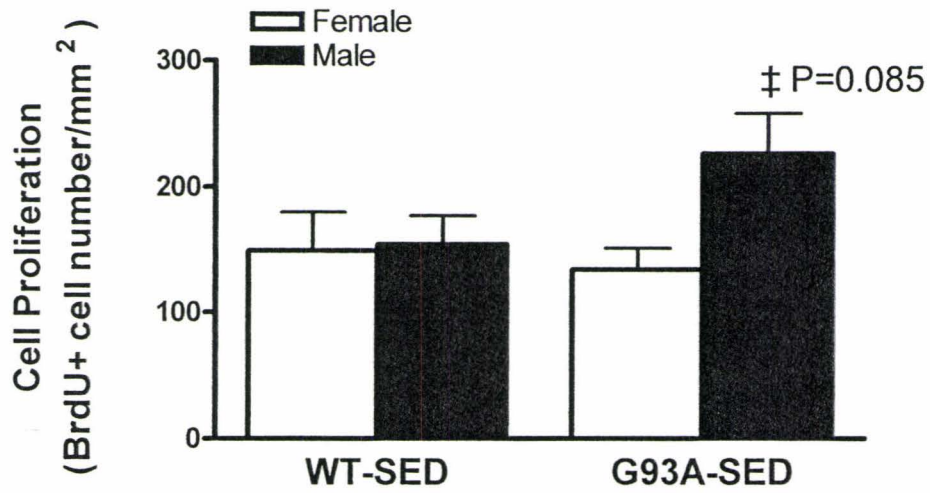

Figure 3.2 Cell proliferation in the dentate gyrus (DG) of wild type (WT-SED) and G93A (G93A-SED) sedentary mice. G93A mice had 18.5% more proliferating cells than WT mice collapsed across sex, due to 68.7% greater number of proliferating cells in G93A males vs G93A females (‡ a trend, G93A-M-SED > G93A-F-SED, p=0.085, n = 6 per group). Data are means ± SEM.

For WT mice following treadmill running, EX mice collapsed across sex had 42.4% more proliferating cells than SED mice (215 ± 25/mm^2 vs 151 ± 19/mm^2, p=0.036) (**Figure 3.3**), showing that EX increases cell proliferation in the DG in WT mice. There was also no significant difference in cell proliferation between WT male and WT female mice.

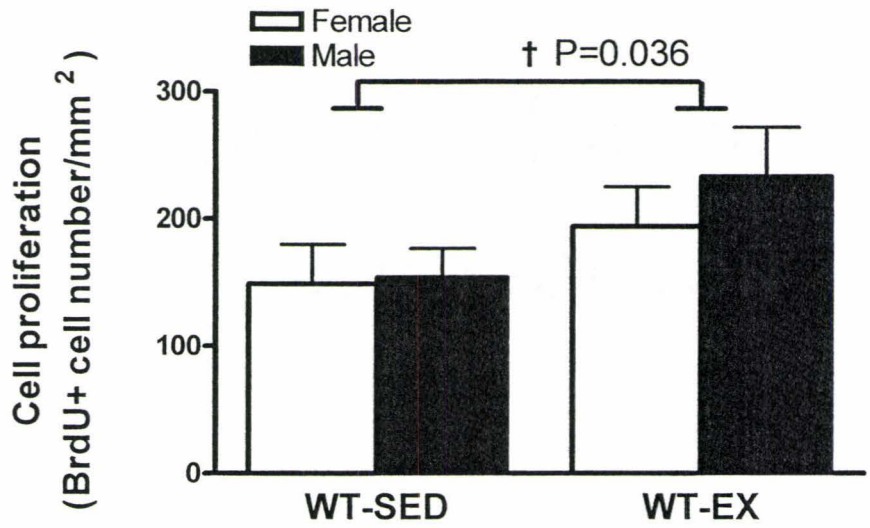

Figure 3.3 Cell proliferation in the dentate gyrus (DG) of wild type sedentary (WT-SED) and wild type exercising mice (WT-EX). WT-EX mice had 42.4% more proliferating cells than WT-SED mice collapsed across sex. † WT-EX>WT-SED, p=0.036, n= 5-6 per group. Data are means ± SEM.

For G93A mice, EX mice had a trend to have 24.4% fewer proliferating cells vs SED mice (136 ± 10/mm^2 vs 180 ± 22/mm^2; p=0.056) (**Figure 3.4**), due to a 32.7% fewer proliferating cells in EX males vs SED males (152 ± 7/mm^2 vs 226 ± 32/mm^2). Meanwhile, G93A male mice had 50.0 % more proliferating cells than G93A female mice (192 ± 21/mm^2 vs 128 ± 11/mm^2; p=0.009).

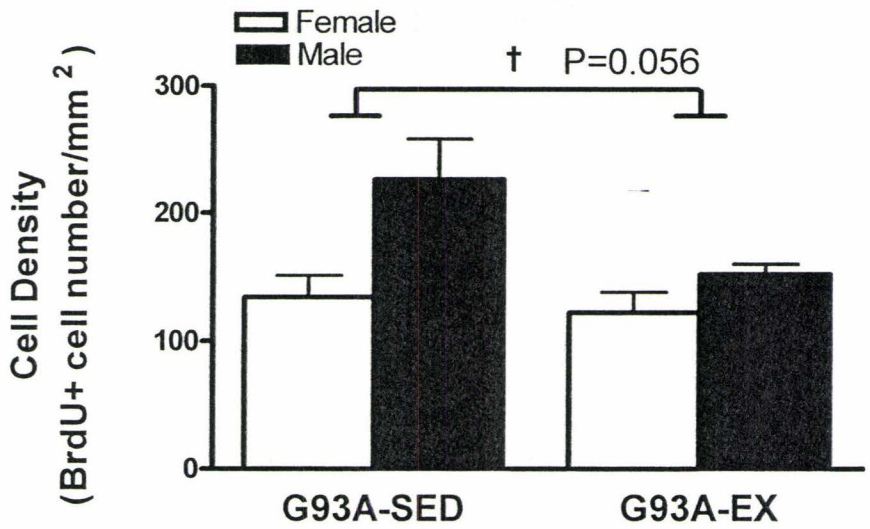

Figure 3.4 Cell proliferation in the dentate gyrus (DG) of G93A sedentary (G93A-SED) and G93A exercise mice (G93A-EX). EX mice had 24.4% fewer proliferating cells vs SED mice, due to 32.7 % fewer proliferating cells in EX males vs. SED males. † G93A-EX < G93A-SED, p=0.056, n = 6 per group except for G93A EX males = 5. Data are means ± SEM.

3.1.2 Cell Survival

Three weeks after the last injection of BrdU, survival of BrdU-labelled newborn cells was observed in all mice. Most surviving cells were located in the DG with much less in the hilus (**Figure 3.5.A**). These cells had a more rounded nuclei, sometimes with the typical chromation structure of granular cells (**Figure 3.5.A** insert). Figure 3 showed representative images of surviving cells in G93A (**Figure 3.5.B**) vs WT (**Figure 3.5.C**) mice.

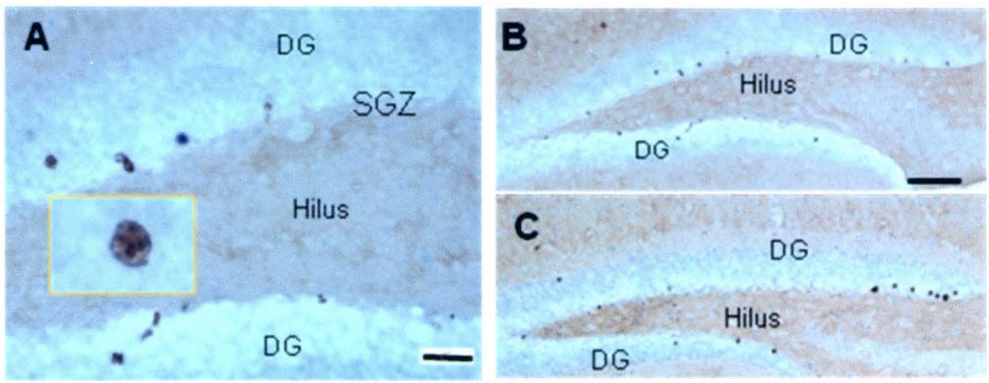

Figure 3.5 Representative images of BrdU labeled surviving cells in the dentate gyrus (DG) of wild type (WT) and G93A mice. (A) A representative image showed that most surviving cells were located in the DG, with more rounded nuclei, sometimes with the typical chromation structure of granular cells (insert). Representative images showed surviving cells in G93A mice (B) than in WT mice (C). Scale bar = 25 μm in A, 100 μm in B,C. G93A mice, n=21; WT mice, n=23.

In the sedentary group, there was a significant difference between G93A-SED mice and WT-SED mice collapsed across sex, where G93A mice had 30.1 % more surviving cells than WT mice (134 ±12/mm^2 vs 103 ± 8/mm^2; p=0.017) (**Figure 3.6**), suggesting that the basal level of cell survival of newborn cells in G93A mice is higher than that in WT mice.

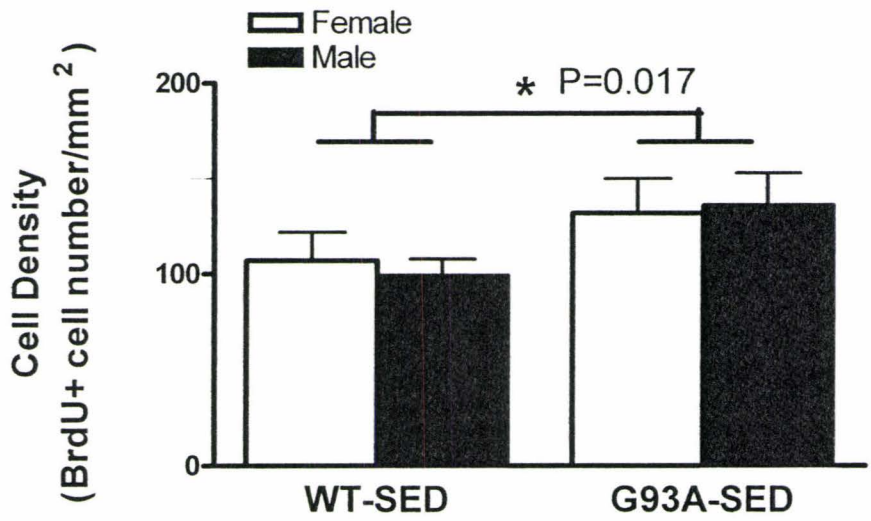

Figure 3.6 Cell survival in the dentate gyrus (DG) of wild type sedentary (WT-SED) and G93A sedentary mice (G93A-SED). G93A mice had 30.1 % more surviving cells than WT mice collapsed across sex. *G93A-SED>WT-SED, p=0.017, n=10-12 per group. Data are means ± SEM.

For the WT group, in response to treadmill running, EX mice collapsed across sex had 29.1 % more surviving cells than SED mice (133 ± 14/mm^2 vs 103 ± 8/mm^2, p=0.028) (**Figure 3.7**). There was no significant difference between WT males and WT females for cell survival.

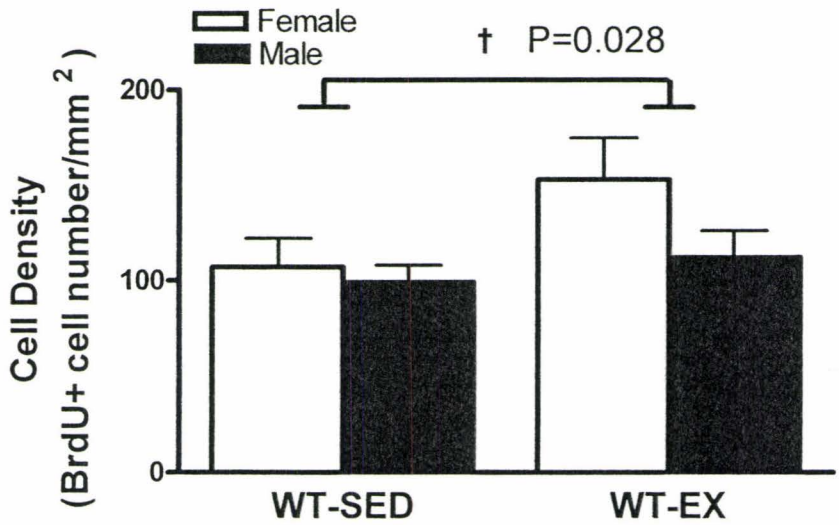

Figure 3.7 Cell survival in the dentate gyrus (DG) of wild type sedentary (WT-SED) and wild type exercising mice (WT-EX). EX mice collapsed across sex had 29.1 % more surviving cells than SED mice. † WT-EX>WT-SED, p=0.028., n=10-12 per group. Data are means ± SEM.

Within the G93A exercised group, male EX mice had significantly 41.5% fewer surviving cells than female EX mice (113 ± 7/mm^2 vs. 193 ± 27/mm^2, p=0.017) (Figure **3.8**) and G93A female EX mice had a trend to have 31.6% more surviving cells than G93A female SED mice (193 ± 27/mm^2 vs. 132 ± 18/mm^2, p=0.057). In addition, male G93A mice had 22.4% fewer surviving cells than female G93A mice (125 ± 10/mm^2 vs 161±17/mm^2, p=0.028), due mainly to the G93A-EX males having 41.5 % fewer surviving cells than G93A-EX females.

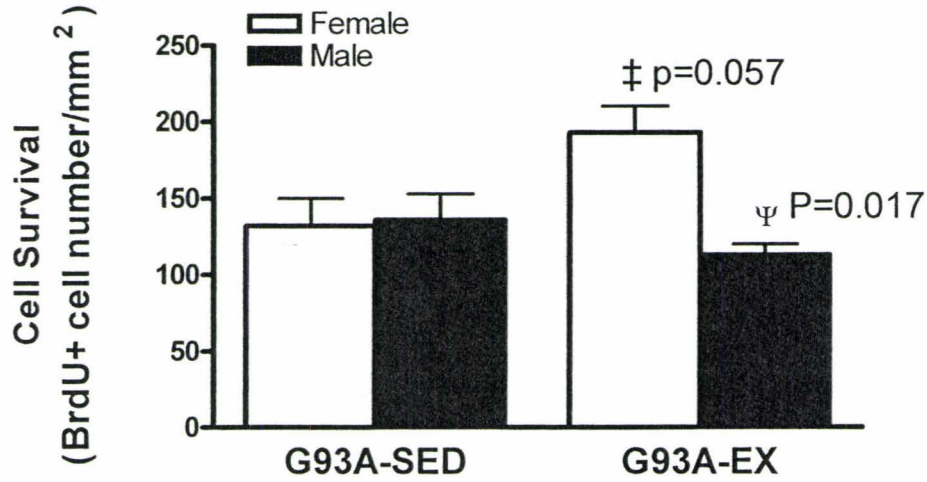

Figure 3.8 Cell survival in the dentate gyrus (DG) of G93A sedentary (G93A-SED) and G93A exercising mice (G93A-EX). Male EX mice had significantly (41.5%) fewer surviving cells than female EX mice G93A female EX mice had a trend to have 31.6% more surviving cells than G93A female SED mice. $^{\Psi}$ G93A-M-EX< G93A-F-EX, p=0.017; ‡ a trend, G93A-EX females > G93A-SED females, n= 10 per group except for G93A-SED females =11. Data are means ± SEM.

3.1.3 Cell Differentiation

Colocalization of BrdU positive staining (green color) with neuronal marker NeuN (red color) and astrocytic marker GFAP (blue color) was investigated to determine the phenotype of newborn cells in the DG 3 weeks after the last injection of BrdU via triple immnuofluorescent staining. Figure 3.9.A, B, and C show representative confocal microscopic images of triple staining in the DG of the hippocampus. Figure 3.9.A shows red granule cells (neurons) stained with NeuN in the DG and blue cells (astrocytes) stained with GFAP in the hilus and molecular layer. Meanwhile, there were several orange cells (merged green and red colors) double stained with BrdU and NeuN in SGZ (**Figure 3.9.A**). Newly generated neuronal cells were double stained with green (BrdU positive) and red (NeuN positive) (**Figure 3.9.B**). Newly generated astrocytes were double stained with green (BrdU positive) and blue (GFAP positive) (**Figure 3.9.C**). Some of BrdU+ cells in green color only did not show either neural or astrocytic cell phenotype, indicating that they either were not differentiated progenitor cells or that they differentiated into other cell phenotypes not investigated here.

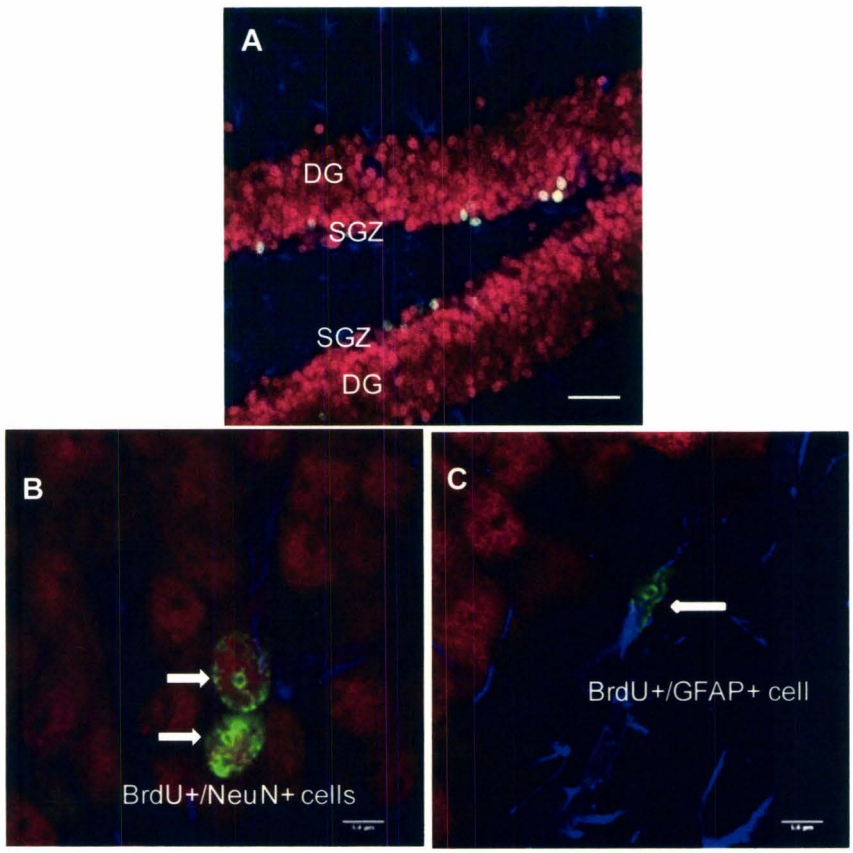

Figure 3.9 Representative confocal microscopic images of triple fluorescent staining in the DG of the hippocampus. Green: BrdU, red: NeuN, Blue: GFAP. (A) A representative confocal microscopic image showed BrdU labeled cells in the dentate gyrus (DG). (B) A representative confocal microscopic image showed two newly generated neurons (white arrows) colocalized with BrdU and NeuN. (C) A representative confocal microscopic image showed one newly generated astrocyte (white arrow) colocalized with BrdU and GFAP. Scale bar = 100 μm in A; 5 μm in B and C.

Neuronal differentiation The percentage of double labelled BrdU and NeuN cells was 22.4% higher in G93A-SED than in WT-SED collapsed across sex (80.3% ± 3.1 % vs. 65.6 % ± 4.3 %; p=0.007) (**Figure 3.10**), suggesting that G93A-SED mice have a higher basal neuronal differentiation as compared to WT-SED mice. Within the WT exercised group or the G93A exercised group, there was no significant difference in the percentage of BrdU and NeuN double labelled cells (**Figure 3.11**, **Figure 3.12**), indicating that there was no effect of exercise or sex on neuronal differentiation in G93A mice or WT mice.

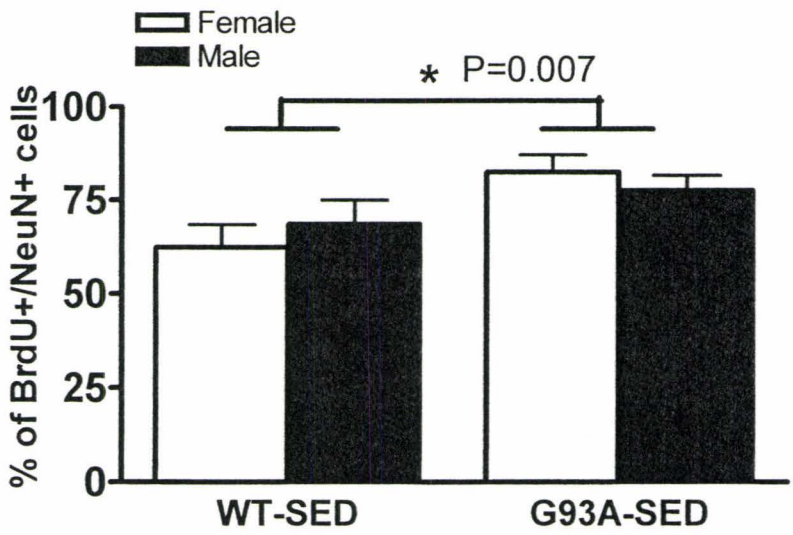

Figure 3.10 The neuronal differentiation of BrdU labeled surviving cells in the DG of WT and G93A mice. The percentage of double labelled BrdU and NeuN cells was 22.4% higher in G93A-SED than in WT-SED collapsed across sex. *G93A-SED>WT-SED, p = 0.007, n=5-6 per group. Data are means ± SEM.

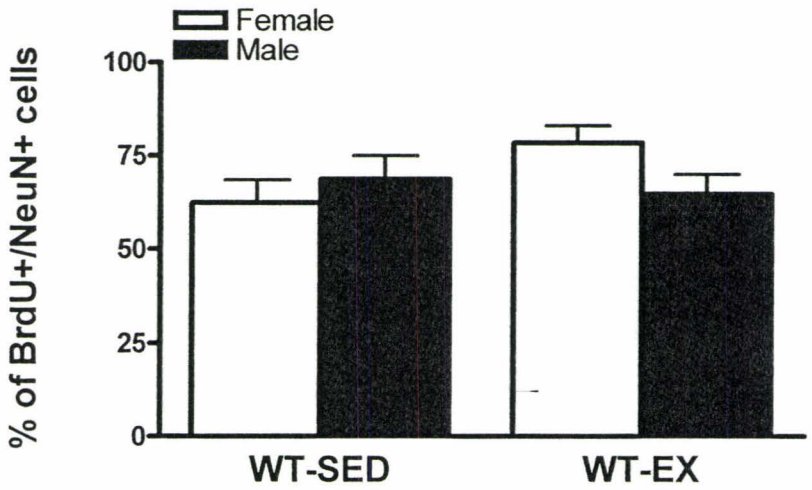

Figure 3.11 The neuronal differentiation of BrdU labeled surviving cells in the DG of WT-SED and WT-EX mice. There was no significant difference in the percentage of BrdU and NeuN double labelled cells between WT-EX and WT-SED mice, n=5-6 per group. Data are means ± SEM.

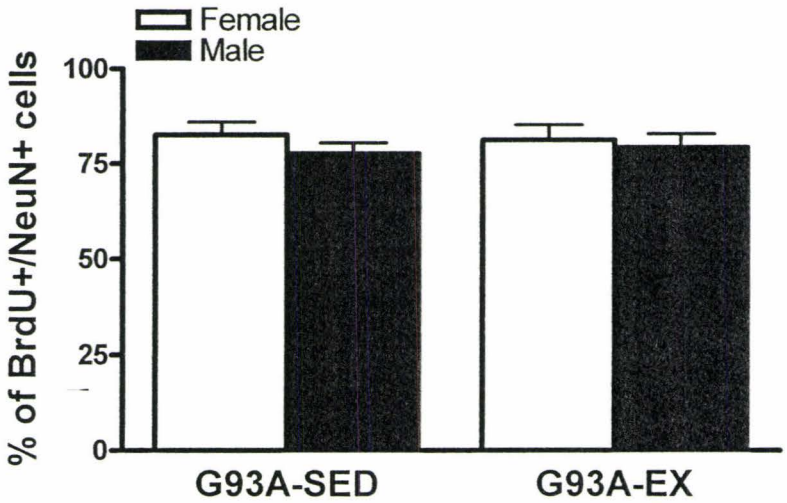

Figure 3.12 The neuronal differentiation of BrdU labeled surviving cells in the DG of G93A-SED and G93A-EX mice. There was no significant difference in the percentage of BrdU and NeuN double labelled cells between G93A-EX and G93A-SED mice, n=5-6 per group. Data are means ± SEM.

Astrocytic differentiation: The percentage of double labeled BrdU and GFAP cells was 47.7% lower in G93A-SED than in WT-SED collapsed across sex (8.0 % ± 6.0 % vs. 15.3 % ± 6.8 %; p=0.022), suggesting that G93A-SED had a lower basal level of astrocytic differentiation as compared to WT-SED mice. Within individual G93A or WT groups in response to treadmill exercise, there was no significant difference in the percentage of BrdU and GFAP double-labeled cells, indicating that there was no effect of exercise or sex on astrocytic differentiation in G93A mice or WT mice.

3.2 mRNA Expression of Growth Factors in the Hippocampus

3.2.1 BDNF mRNA Expression Level

Representative autoradiographs of the hippocampal region for BDNF mRNA in WT and G93A mice are shown in **Figure 3.13.A and B**. The hybridization signal of BDNF mRNA was strong in CA1, CA2, CA3, and DG in the hippocampus, as well as shown in outside of these regions.

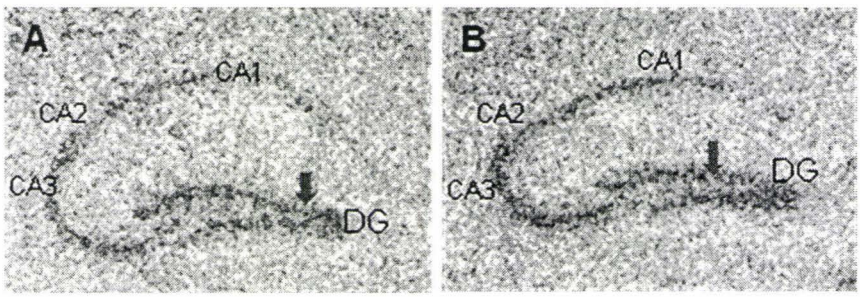

Figure 3.13 Representative autoradiographs of BDNF mRNA hybridization signal in the hippocampus of WT mice and G93A mice. Representative autoradiograph showed BDNF mRNA hybridization signal in the DG in G93A mice (B) is stronger than that in WT mice (A). Red arrows indicated BDNF mRNA hybridization signal in the DG.

BDNF mRNA expression in the DG

There was a significant difference between G93A-SED mice and WT-SED mice collapsed across sex, where the expression level of BDNF mRNA in the DG was significantly higher in G93A-SED mice than WT-SED (187 ± 8 dpm vs 162 ± 6 dpm p=0.016) (**Figure 3.14**). This suggests that the basal level of BDNF mRNA in the DG in G93A mice is higher than that in WT mice.

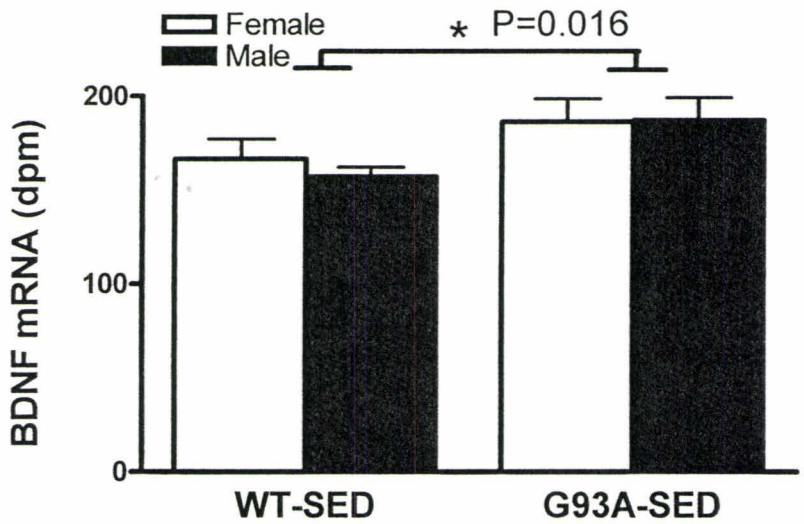

Figure 3.14 The expression of BDNF mRNA in the DG of WT-SED and G93A-SED mice. G93A-SED mice had higher expression of BDNF mRNA than G93A-SED mice. * G93A-SED > WT-SED, p=0.016, n= 10-13 per group. Data are means ± SEM.

Exercised WT mice showed significantly higher BDNF mRNA expression vs WT-SED mice (173.7 ± 3.5 dpm vs 161.8 ± 5.6 dpm; p=0.050) (**Figure 3.15**).

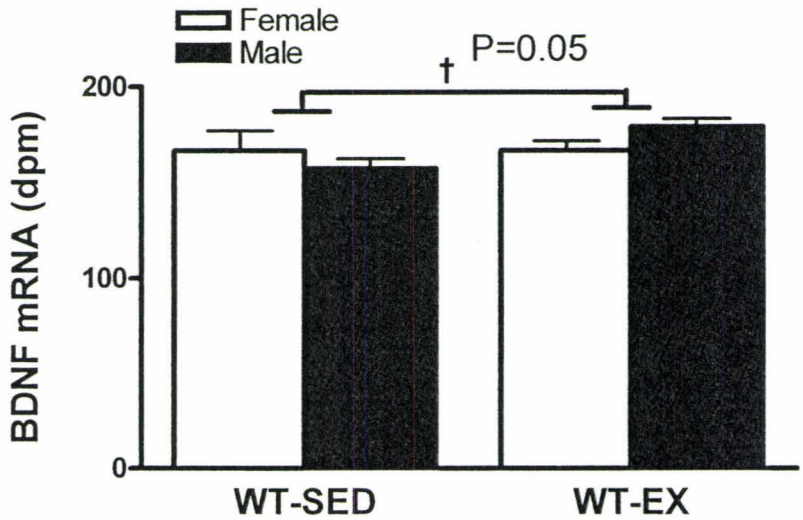

Figure 3.15 The expression of BDNF mRNA in the DG of WT-SED and WT-EX mice. Exercised WT mice had significantly higher BDNF mRNA expression vs WT-SED mice. † WT-EX > WT-SED, p=0.05, n=11-12 per group. Data are means ± SEM.

Exercised G93A mice did not show significant difference vs G93A-SED mice and there were no significant differences between G93A male and G93A female mice with DG BDNF mRNA expression (**Figure 3.16**). It suggested that there is no effect of exercise or sex difference on BDNF mRNA in G93A mice.

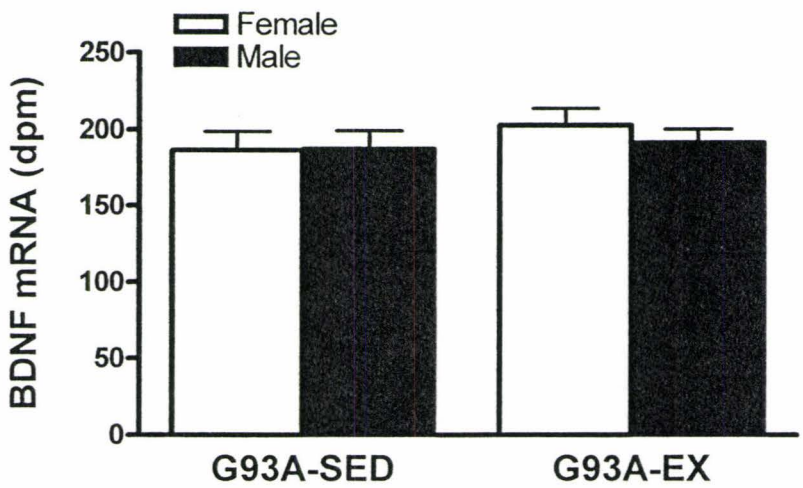

Figure 3.16 The expression of BDNF mRNA in the DG of G93A-SED and G93A-EX mice. There was no significance between G93A-EX and G93A-SED mice, n=10-13 per group. Data are means ± SEM.

BDNF mRNA expression at the CA3 region

In sedentary mice, G93A mice had a significantly higher expression of BDNF mRNA in the CA3 region than that in WT mice collapsed across sex (186.9 ± 8.5 dpm vs 161.8 ± 5.6 dpm; P=0.003). This suggested that G93A mice have a higher basal level of BDNF mRNA expression in the region of CA3 vs WT mice. Within individual G93A or WT groups in response to treadmill exercise, there was no significant difference in BDNF mRNA expression in the region of CA3, indicating that there was no effect of exercise or sex on CA3 BDNF mRNA expression in G93A mice or WT mice.

3.2.2 IGF1 mRNA Expression Level

Representative autoradiographs of the hippocampal region for IGF1 mRNA in WT and G93A mice are shown in **Figure 3.17**.A and B. The hybridization signal for IGF1 mRNA was observed in the regions of CA1, CA2, CA3 and DG of the hippocampus, as well as in outside of these regions.

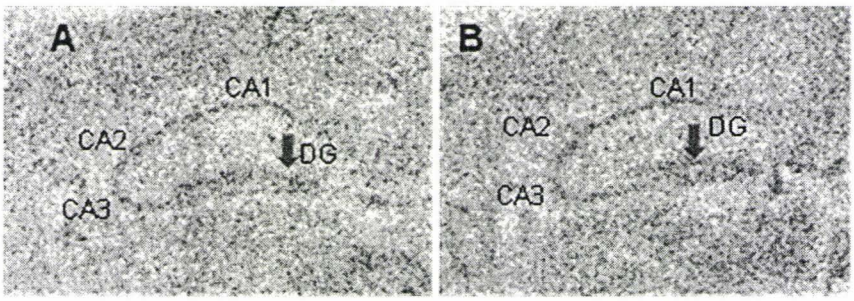

Figure 3.17 Representative autoradiograph of hybridization signal for IGF1 mRNA in the DG of WT and G93A mice. (A) IGF1 mRNA hybridization signal in the DG of WT mice. (B) IGF1 mRNA hybridization signal in the DG of G93A mice. Red arrows indicated IGF1 mRNA hybridization signal in the DG.

IGF1 mRNA expression in the DG

In sedentary mice, there was no significant difference in genotype or sex (**Figure 3.18**), suggesting that G93A mice have the same baseline level of IGF1 mRNA expression in the DG as that in WT mice.

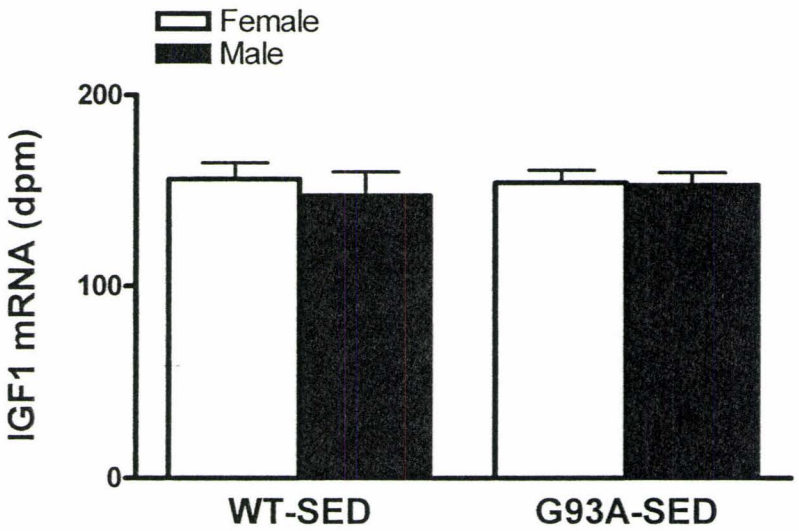

Figure 3.18 The expression of IGF1 mRNA in the DG of WT-SED and G93A-SED mice. There was no significant difference between WT-SED and G93-SED mice, n= 11-13 per group. Data are means ± SEM.

For the WT group, in response to treadmill running, EX mice had a trend to have a higher expression of IGF1 mRNA in the DG vs SED mice (170.0 ± 8.4 dpm vs 151.8 ± 7.6 dpm, p=0.064) (**Figure 3.19**).

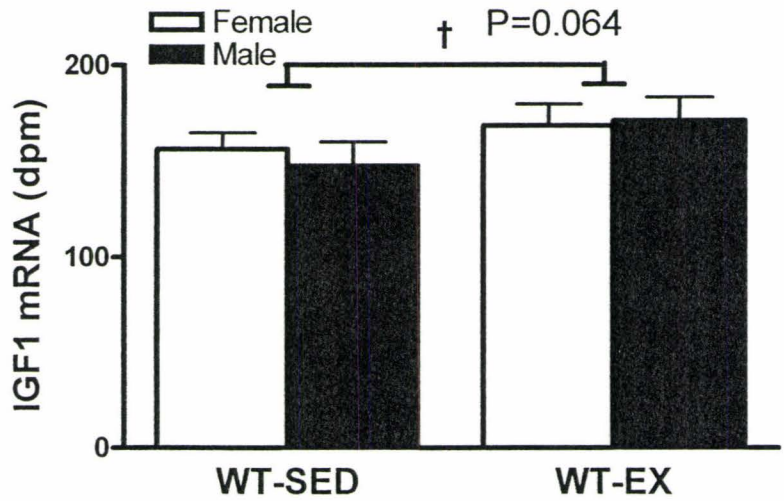

Figure 3.19 The expression of IGF1 mRNA in the DG of WT-SED and WT-EX mice. EX mice had a trend to have a higher expression of IGF1 mRNA in the DG vs SED mice, † p=0.064, n=11-12 per group. Data are means ± SEM.

For G93A mice following treadmill running, there was no any significant difference with IGF1 mRNA expression in the DG (**Figure 3.20**), suggesting that there is no effect of exercise or sex difference on IGF1 mRNA in G93A mice.

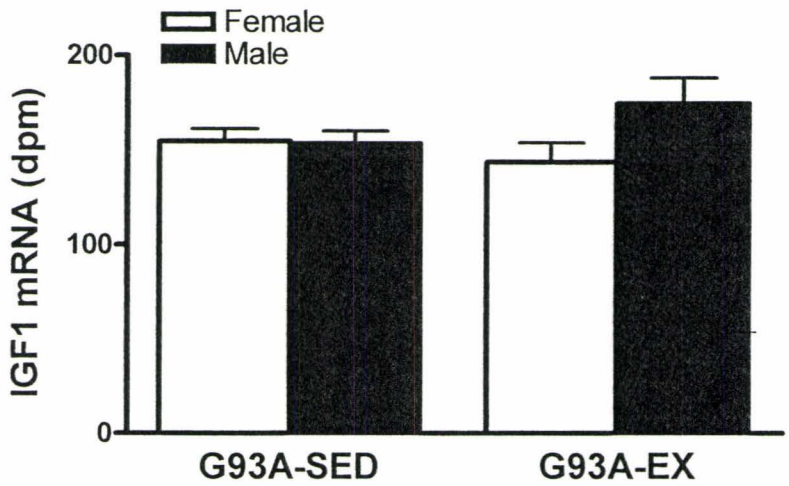

Figure 3.20 The expression of IGF1 mRNA in the DG of G93A-SED and G93A-EX mice. Ther was no significant difference in DG IGF1 mRNA expression between G93A-SED and G93A-EX mice, n=9-13 per group. Data are means ± SEM.

IGF1 mRNA expression in the CA3

In sedentary mice, there was no significant difference in IGF1 mRNA expression in the CA3 region of the hippocampus, suggesting that G93A mice have the same baseline level of IGF1 mRNA expression as compared to WT mice. Following exercise, there was no significant difference in CA3 IGF1 mRNA expression for G93A or WT mice, suggesting that there is no effect of exercise or sex on CA3 IGF1 mRNA expression in G93A or WT mice.

3.3 Correlation between DG BDNF mRNA and the Survival and Neuronal Differentiation of BrdU labeled cells in G93A Mice

Previous studies have shown that BDNF is essential for the cell proliferation, cell survival and cell differentiation in hippocampal neurogenesis (Linnarsson, Willson et al. 2000; Chan, Cordeira et al. 2008), and it plays an important role in the maintenance of the basal level of neurogenesis in the DG of adult mice (Lee, Duan et al. 2002). Therefore, we hypothesized that BDNF would play a role in the maintenance of baseline hippocampal neurogenesis in the G93A mouse model. Thus, BDNF mRNA expression in the DG should be related to cell proliferation, cell survival and neuronal differentiation of brdU labelled cells in G93A sedentary mice. DG BDNF mRNA expression was significantly and positively related to the survival ($r = 0.51$, $p=0.022$) (**Figure 3.21**) and neuronal differentiation of BrdU labelled cells ($r = 0.72$, $p=0.013$) (**Figure 3.22**) in G93A

sedentary mice. This suggests that higher basal levels of survival and neuronal differentiation of BrdU labeled cells are associated with higher baseline level of DG BDNF mRNA expression in G93A mice. However, the correlation of cell proliferation and DG BDNF mRNA expression could not be analysed here, because samples for the cell proliferation study were not available for detection of BDNF mRNA expression in the DG.

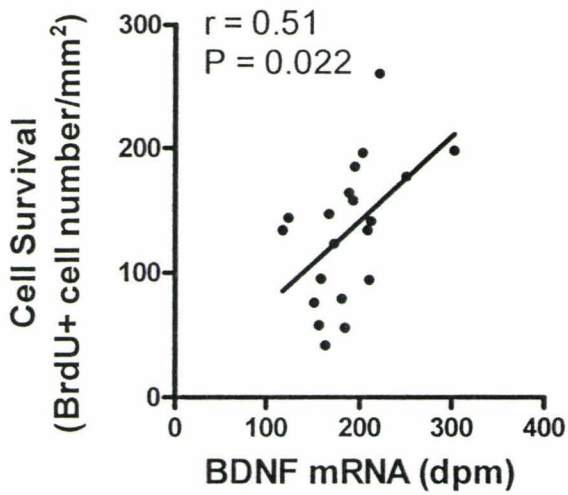

Figure 3.21 Correlation between DG BDNF mRNA and survival of BrdU labeled cells in G93A-SED mice. In G93A-SED mice, DG BDNF mRNA expression was significantly and positively related to the survival (r=0.51, p=0.022, n=20) of BrdU labeled cells. Data are means ± SEM.

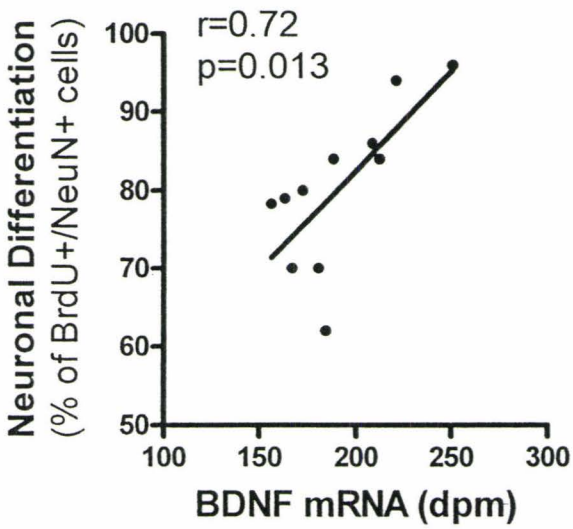

Figure 3.22 Correlation between DG BDNF mRNA and neuronal differentiation of BrdU labeled surviving cells in G93A-SED mice. In G93A-SED mice, DG BDNF mRNA expression was significantly and positively related to neuronal differentiation of BrdU labeled surviving cells (r=0.72, p=0.013, n=11). Data are means ± SEM.

Following exercise, DG BDNF mRNA expression in G93A-EX mice was still significantly and positively related to the survival (r=0.56, p=0.013) (**Figure 3.23**) and neuronal differentiation of BrdU labelled cells (r=0.67, p=0.017) (**Figure 3.24**) in G93A-EX mice.

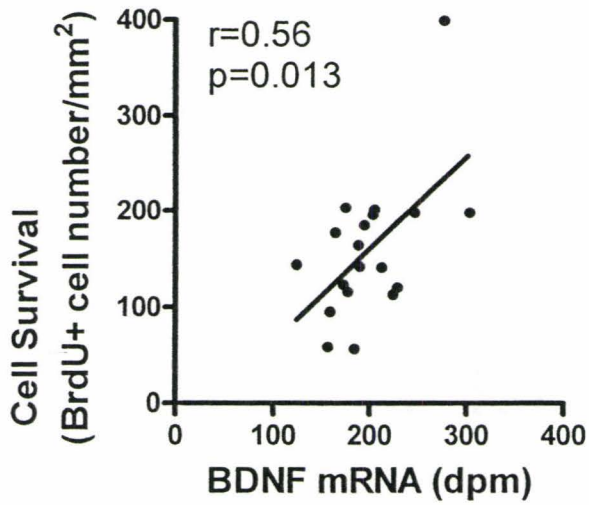

Figure 3.23 Correlation of DG BDNF mRNA and the survival of BrdU labeled cells in G93A-EX mice. In G93A-EX mice, the expression of DG BDNF mRNA was significantly and positively related to the survival of BrdU labeled cells (r=0.56, p=0.013, n=19). Data are means ± SEM.

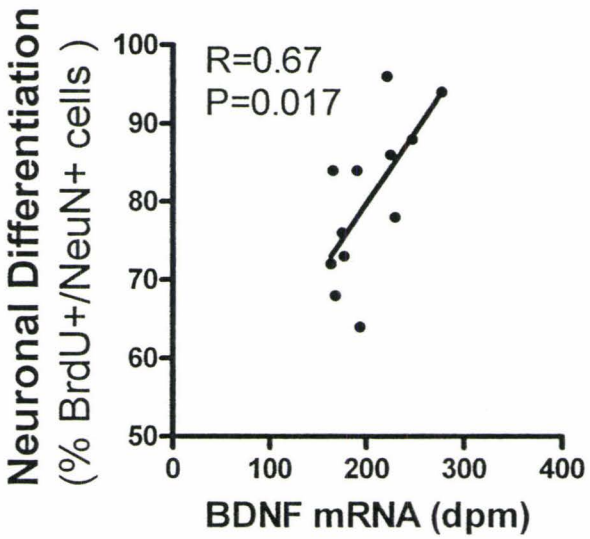

Figure 3.24 Correlation between DG BDNF mRNA and neuronal differentiation of BrdU labeled surviving cells in G93A-EX mice. In G93A-EX mice, DG BDNF mRNA expression was significantly and positively related to neuronal differentiation of BrdU labeled surviving cells ($r=0.67$, $p=0.017$, $n=11$). Data are means ± SEM.

3.4 Oxidative Stress

3.4.1 SOD2 mRNA Expression

Representative film autoradiographs of the hippocampal region for SOD2 mRNA hybridization signal in WT and G93A mice are shown in **Figure 3.25**.A and B. A strong hybridization signal of SOD2 mRNA is shown in the regions of DG, CA1, CA2, and CA3 of the hippocampus for both WT mice and G93A mice.

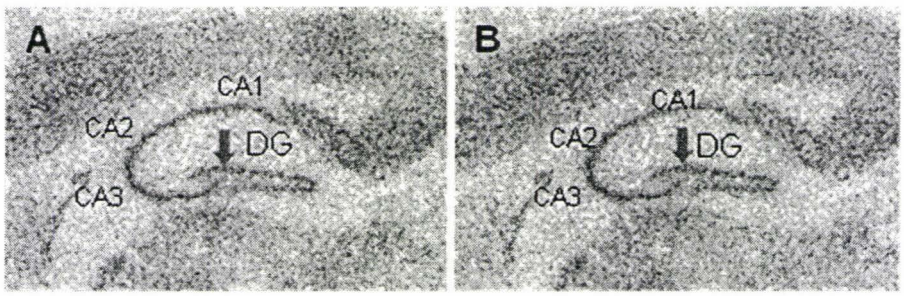

Figure 3.25 Representative autoradiographs of hybridization signal for SOD2 mRNA in the DG of WT (A) and G93A mice (B). Red arrows indicated SOD2 mRNA hybridization signal in the DG.

SOD2 mRNA Expression in the DG and the CA3

In sedentary mice, there was no significant difference between WT and G93A mice in SOD2 mRNA expression in the DG (**Figure 3.26**) or CA3 regions, suggesting that baseline SOD2 mRNA content in the DG or the CA3 of G93A mice is not altered as compared to WT mice. However, females had a trend to have 14.2% higher SOD2 mRNA expression vs. males in the CA3 region (1219.1 ± 61.1 dpm/mg vs. 1067.2 ± 46.7 dpm/mg, $p = 0.074$). Following exercise, there was no significant difference in activity or sex for either WT or G93A mice in SOD2 mRNA expression in the DG (**Figure 3.27, Figure 3.28**) or the CA3 region, suggesting that there is no effect of exercise or sex difference on SOD2 mRNA expression in the DG and no effect of exercise in the CA3 region of the hippocampus in either G93A or WT mice.

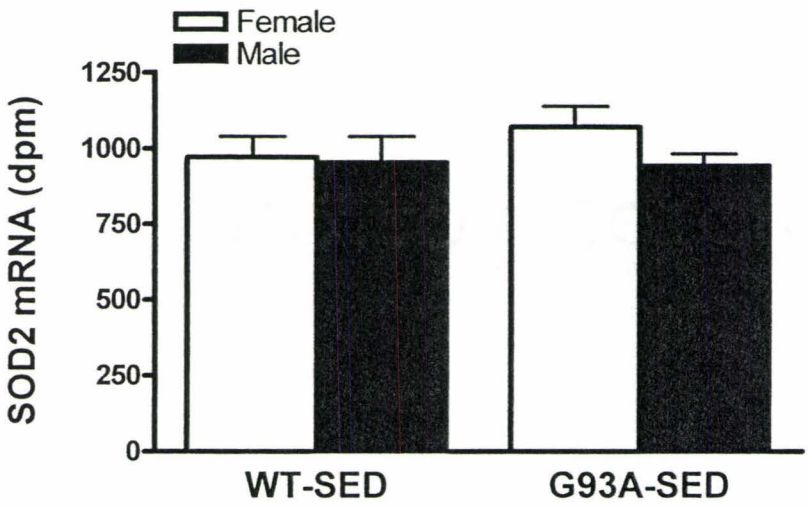

Figure 3.26 The expression of SOD2 mRNA in the DG of WT-SED and G93A-SED mice. There was no significant difference in DG SOD2 mRNA expression between WT-SED and G93A-SED mice, n=11-13 per group. Data are means ± SEM.

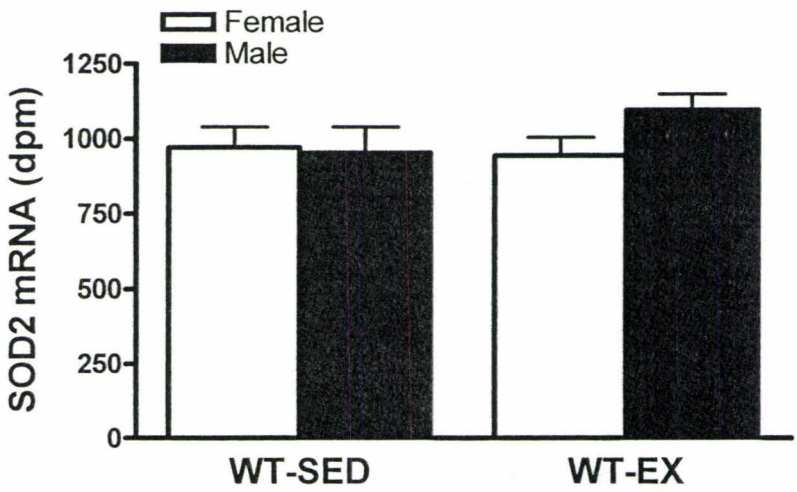

Figure 3.27 The expression of SOD2 mRNA in the DG of WT-SED and WT-EX mice. There was no significant difference in DG SOD2 mRNA expression between WT-SED and WT-SED mice, n=11-12 per group. Data are means ± SEM.

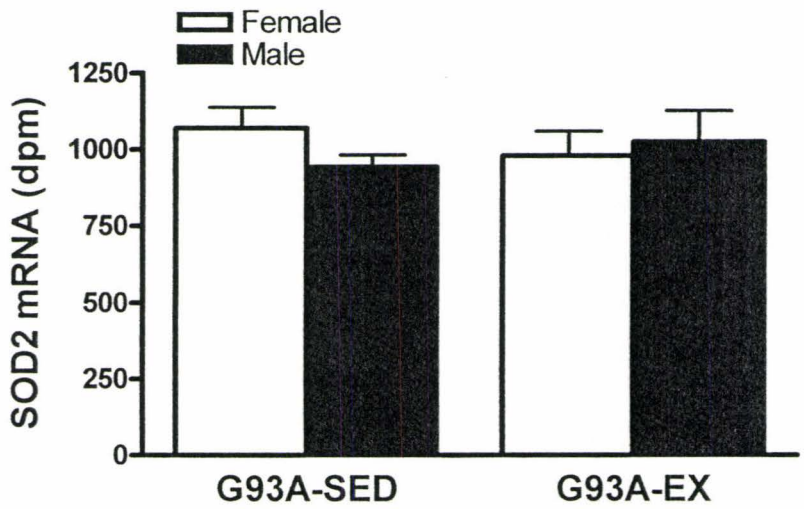

Figure 3.28 The expression of SOD2 mRNA in the DG of G93A-SED and G93A-EX mice. There was no significant difference in DG SOD2 mRNA expression between G93A-SED and G93A-SED mice, n=10-13 per group. Data are means ± SEM.

3.4.2 SOD2 Protein Content

In the hippocampus, SOD2 positively stained cells are distributed in the DG, CA1, CA2, CA3, and hilus. At high magnification, SOD2 positively stained cells presented a granular nature (**Figure 3.29.A**), which is consistent with a previous study (Chung, Joo et al. 2003). Representative images for quantitative analysis of optical density in the DG are shown for WT (**Figure 3.29.B**) and G93A (**Figure 3.29.C**) mice.

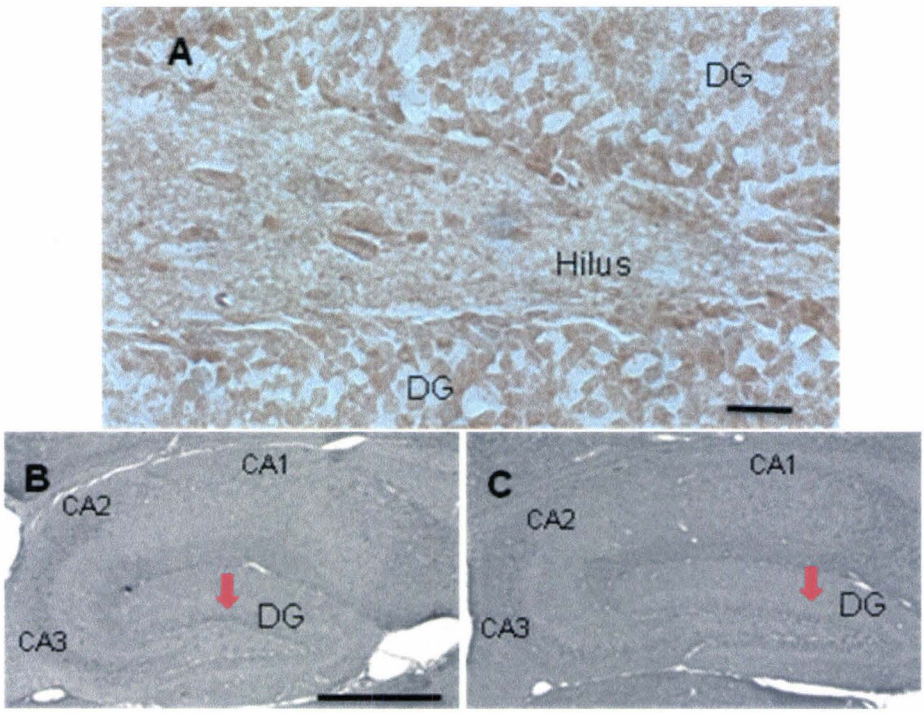

Figure 3.29 Representative images of SOD2 immunereactivity in the DG of WT and G93A mice. (A) A representative image showed that brown cells stained with SOD2 presented a granular nature at higher magnification in the DG and hilus. Representative black-and-white images (used for determination of SOD2 optical density in the DG) showed SOD2 immunoreactivity in the DG of WT (B) and G93A (C) mice. Scale Bar = 25 μM in A, 500 μM in B,C. Red arrows indicate SOD2 immunoreactivity in the DG.

SOD2 protein content in the DG and the CA3.

In sedentary mice, there was no significant difference between WT and G93A mice in SOD2 protein content in the DG (**Figure 3.30**) or CA3 regions, suggesting that baseline SOD2 protein content in the DG or the CA3 of G93A mice is not altered as compared to WT mice. Following exercise, there was no significant difference in activity or sex for either WT or G93A mice with SOD2 protein expression in the DG (**Figure 3.31, Figure 3.32**) or the CA3 region, suggesting that there is no effect of exercise or sex on SOD2 protein expression in the DG or CA3 region of the hippocampus in either G93A or WT mice.

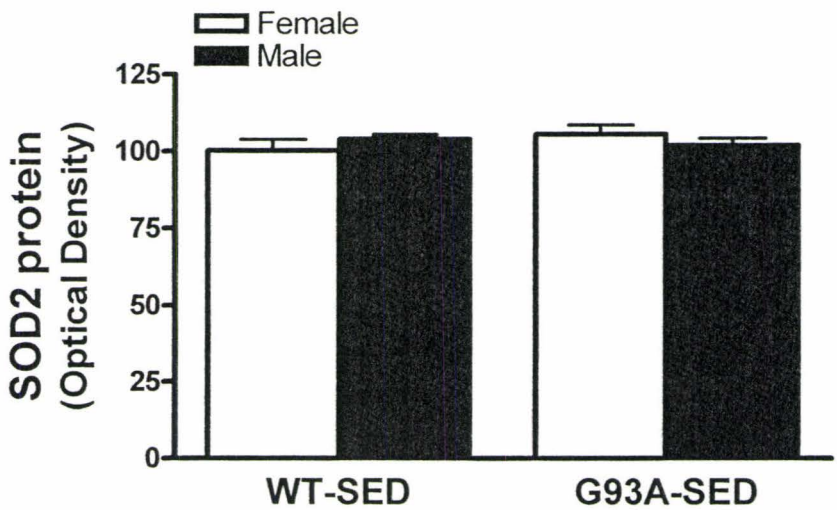

Figure 3.30 The content of SOD2 protein in the DG of WT-SED and G93A-SED mice. There was no significant difference in DG SOD2 protein content between WT-SED and G93A-SED mice, n=9-11 per group. Data are means ± SEM.

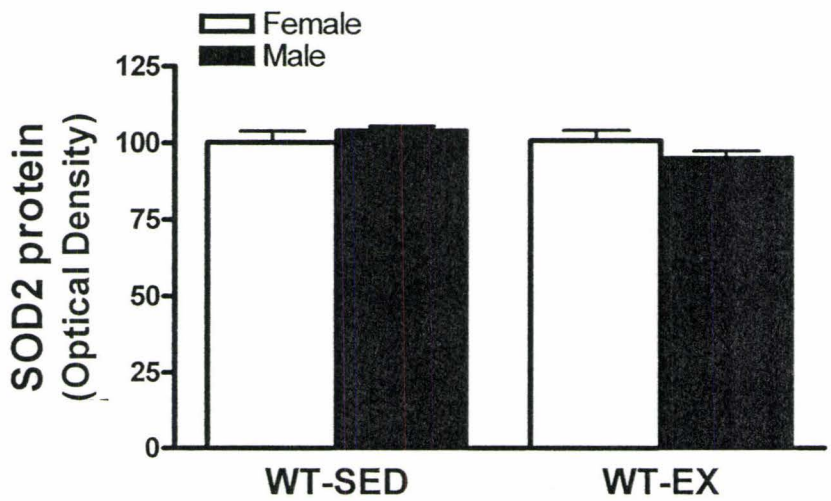

Figure 3.31 The content of SOD2 protein in the DG of WT-SED and WT-EX mice.

There was no significant difference in DG SOD2 protein content between WT-SED and WT-EX mice, n=9-11 per group. Data are means ± SEM.

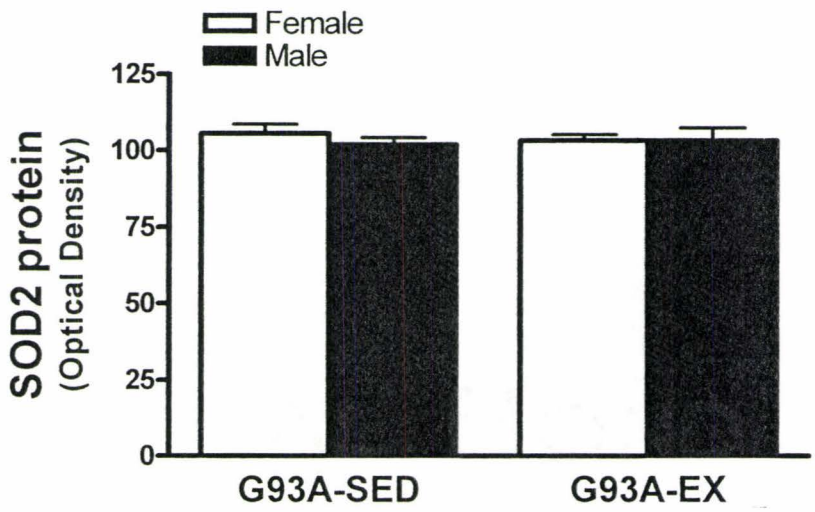

Figure 3.32 The content of SOD2 protein in the DG of G93A-SED and G93A-EX mice. There was no significant difference in DG SOD2 protein content between G93A-SED and G93A-EX mice, n=8-11 per group. Data are means ± SEM.

3.4.3 Catalase mRNA Expression

Representative film autoradiographs of the hippocampal regions for catalase mRNA hybridization signal in WT and G93A mice are shown in **Figure 3.33.**A and B. A weak hybridization signal of catalase mRNA is shown in the region of DG, CA1, CA2, and CA3 of the hippocampus for both WT mice and. G93A mice.

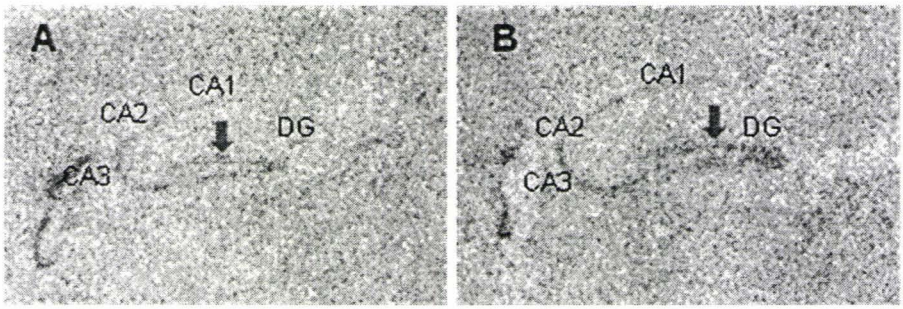

Figure 3.33 Representative autoradiographs of hybridization signal for catalase mRNA in the DG of WT (A) and G93A mice (B). Red arrows indicated catalase mRNA hybridization signal in the DG.

Catalase mRNA expression in the DG and the CA3

In sedentary mice, there was no significant difference between WT and G93A mice in catalase mRNA expression in the DG (**Figure 3.34**) or CA3 regions, suggesting that baseline catalase mRNA expression in the DG or the CA3 of G93A mice is not altered as compared to WT mice. Following exercise, there was no significant difference in activity or sex for either WT or G93A mice in catalase mRNA expression in the DG (**Figure 3.35, Figure 3.36**) or the CA3 region, suggesting that there is no effect of exercise or sex difference on catalase mRNA expression in the DG or CA3 region of the hippocampus in either G93A or WT mice.

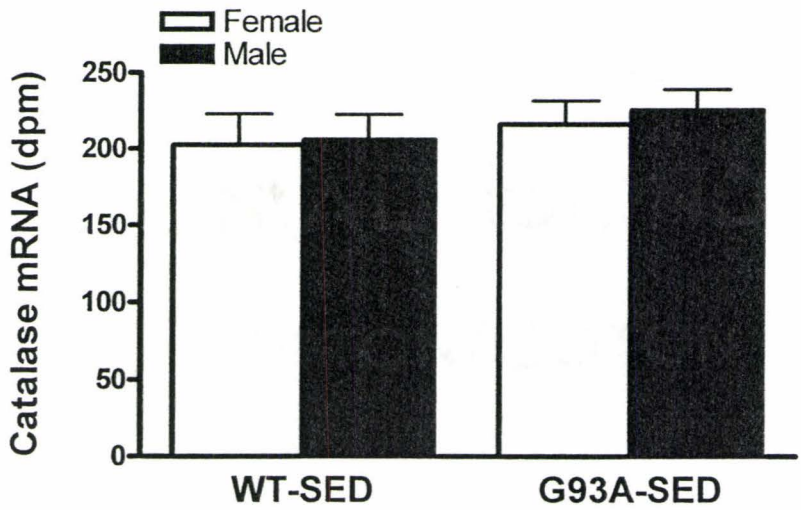

Figure 3.34 The expression of catalase mRNA in the DG of WT-SED and G93A-SED mice. There is no significant difference between WT-SED and G93A-SED mice, n=11-13 per group. Data are means ± SEM.

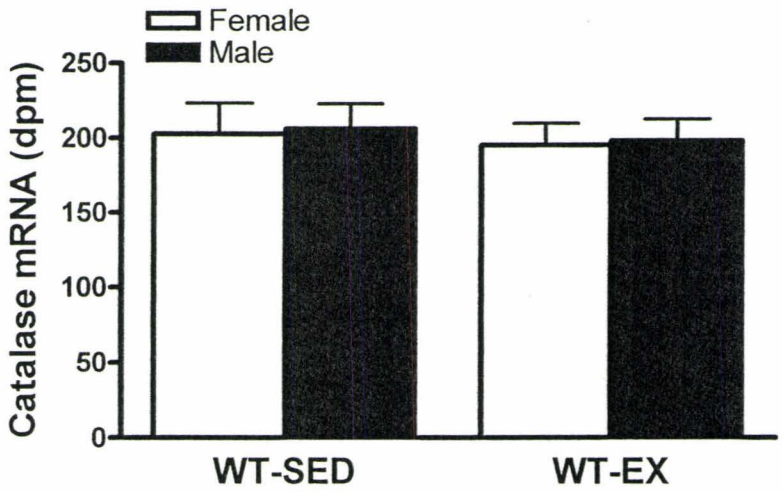

Figure 3.35 The expression of catalase mRNA in the DG of WT-SED and WT-EX mice. There is no significant difference between WT-SED and WT-SED mice, n=11-12 per group. Data are means ± SEM.

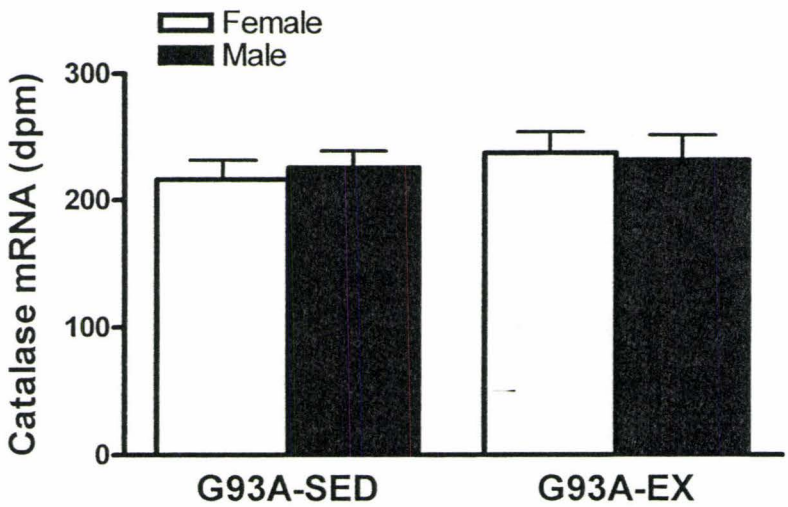

Figure 3.36 The expression of catalase mRNA in the DG of G93A-SED and G93A-EX mice. There is no significant difference between G93A-SED and G93A-SED mice, n=10-13 per group. Data are means ± SEM.

3.4.4 Catalase Protein Content

In the hippocampus, catalase positively stained cells were distributed in the DG, CA1, CA2, CA3, and hilus in WT or G93A mice. At high magnification, catalase positively stained cells presented a granular nature (**Figure 3.37.A**). Representative images for quantitative analysis of optical density in the DG are shown for WT (**Figure 3.37.B**) and G93A (**Figure 3.37.C**) mice.

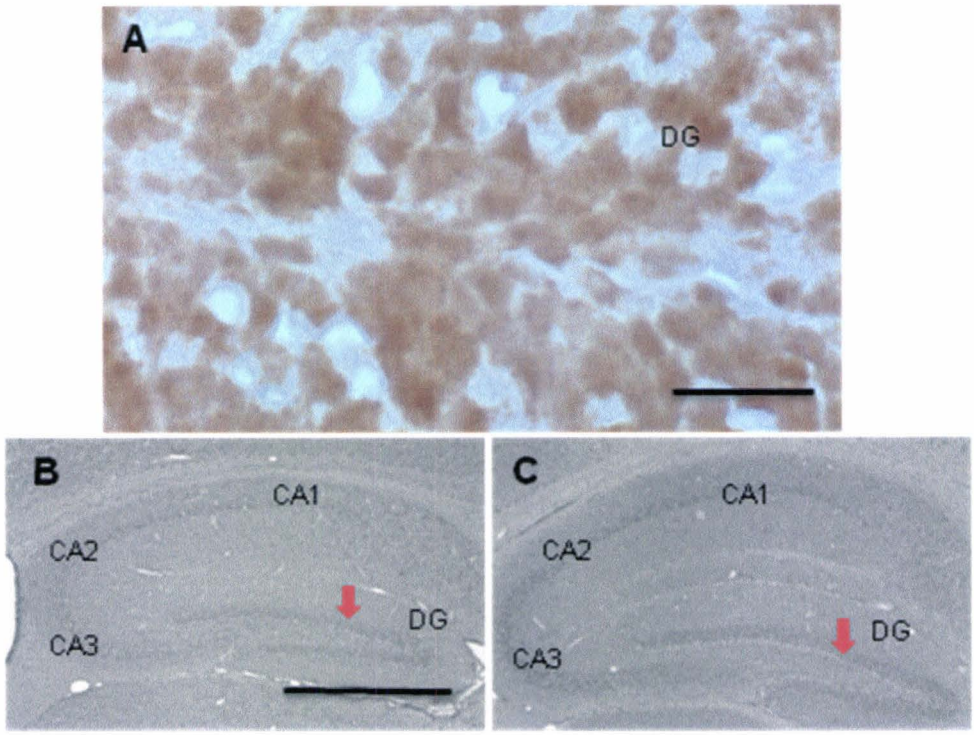

Figure 3.37 Representative images of catalase immunoreactivity in the DG of WT and G93A mice. (A) A representative image showed that brown cells stained with catalase presented a granular nature at higher magnification in the DG and hilus. Representative black-and-white images (determination of catalase optical density in the DG) showed SOD2 immunoreactivity in the DG of WT (B) and G93A (C) mice. Scale Bar = 25 μM in A, 500 μM in B,C. Red arrows indicated catalase immunoreactivity in the DG.

Catalase protein content in the DG and the CA3

In sedentary mice, there was no significant difference between WT and G93A mice with catalase protein content in the DG (**Figure 3.38**) or CA3 regions, suggesting that baseline catalase protein content in the DG or the CA3 is not altered as compared to WT mice. Following exercise, there was no significant difference in activity or sex for either WT or G93A mice in catalase protein content in the DG (**Figure 3.39, Figure 3.40**) or the CA3 region, suggesting that there is no effect of exercise or sex on catalase protein expression in the DG or CA3 region of the hippocampus in either G93A or WT mice.

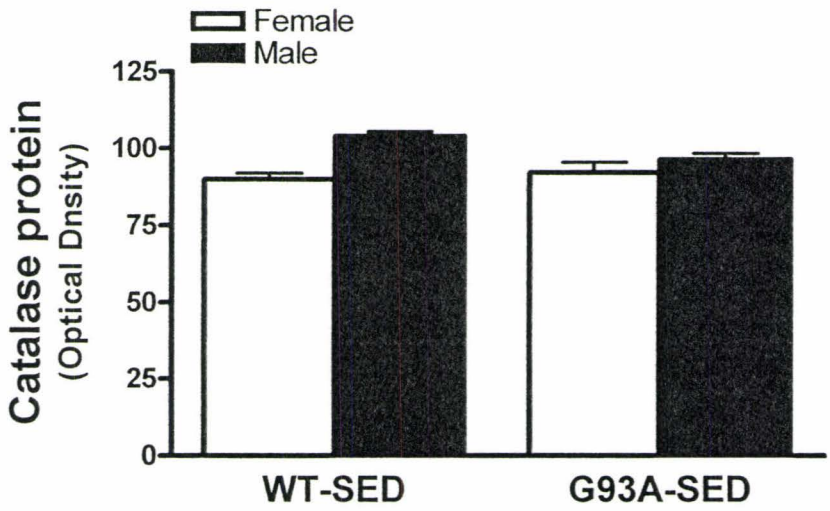

Figure 3.38 The content of catalase in the DG of WT-SED and G93A-SED mice. There is no significant difference between WT-SED and G93A-SED mice, n=9-11 per group. Data are means ± SEM.

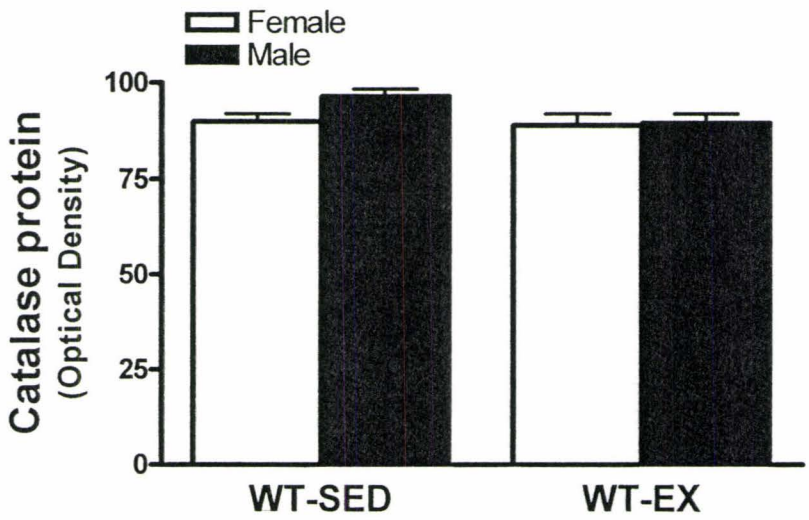

Figure 3.39 The content of catalase protein in the DG of WT-SED and WT-EX mice. There is no significant difference between WT-SED and WT-EX mice, n=9-11 per group. Data are means ± SEM.

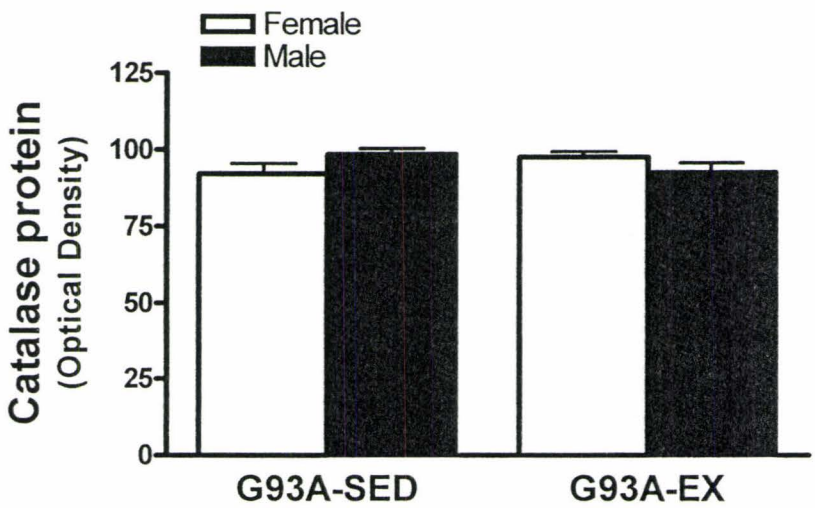

Figure 3.40 The content of catalase protein in the DG of G93A-SED and G93A-EX mice. There is no significant difference between G93A-SED and G93A-EX mice, n=9 per group except for G93A-EX females=12. Data are means ± SEM.

3.4.5 8-OHdG Content

In the hippocampus, 8-OHdG immunoreactivity was detected in granule cells in the DG and pyramidal neurons in the regions of CA1, CA2, and CA3. At high magnification, 8-OHdG immunoreactivity was distributed in nuclear or peri-nuclear areas of granular cells in the DG (**Figure 3.41.A**). Immunoreactivity for 8-OHdG was observed in the DG of WT and G93A mice. Representative images for quantitative analysis of optical density were shown in WT mice (**Figure 3.41.B**) and G93A (**Figure 3.41.C**) mice.

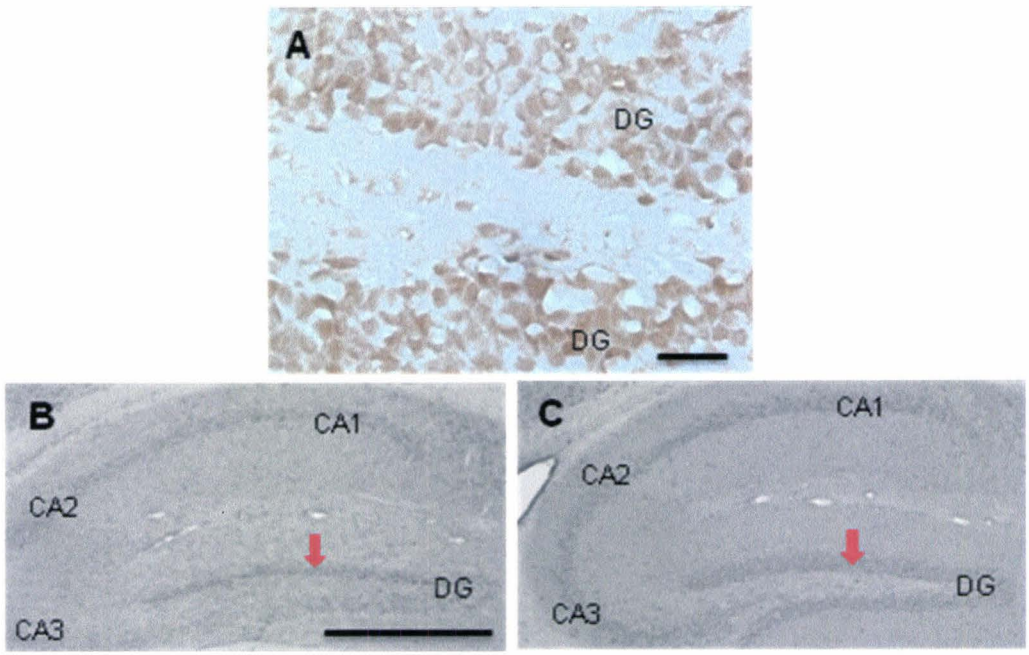

Figure 3.41 Representative images of 8-OHdG immunoreactivity in the DG of WT and G93A mice. (A) A representative image showed granule cells stained with 8-OHdg in the DG. Representative black-and-white images (determination of 8-OHdG optical density in the DG) showed 8-OHdG immunoreactivity in the DG of WT (B) and G93A (C) mice. Scale Bar = 25 μM in A, 500 μM in B,C. Red arrows indicate 8-OHdG immunoreactivity in the DG.

8-OHdG content in the DG

In sedentary mice, optical density of 8-OHdG immunostaining was higher in G93A mice as compared with WT mice (93.4 ± 1.4 vs 90.4 ± 1.8), but it was not significant (p=0.527) (**Figure 3.42**). Following exercise, WT exercising mice showed a lower level of 8-OHdG than WT SED mice (84.9 ± 1.7 vs 90.4 ± 1.8; p=0.022) (**Figure 3.43**), suggesting that exercise lowers DNA oxidative damage in WT mice. No significant difference was found between G93A-EX and G93A-SED mice, suggesting that exercise has no effect on DNA oxidative damage in G93A mice (**Figure 3.44**).

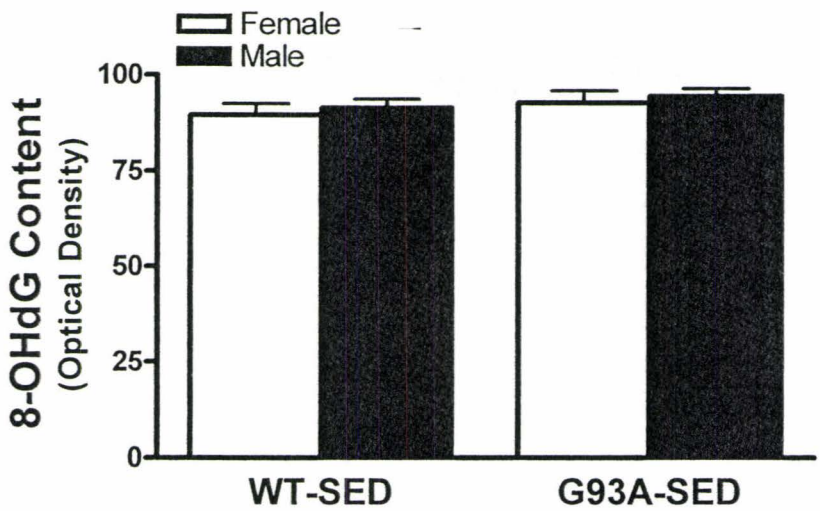

Figure 3.42 The content of 8-OHdG in the DG of WT-SED and G93A-SED mice. There was no significant difference between WT-SED and G93A-SED mice, n=11 per group except for G93A-SED males = 8. Data are means ± SEM.

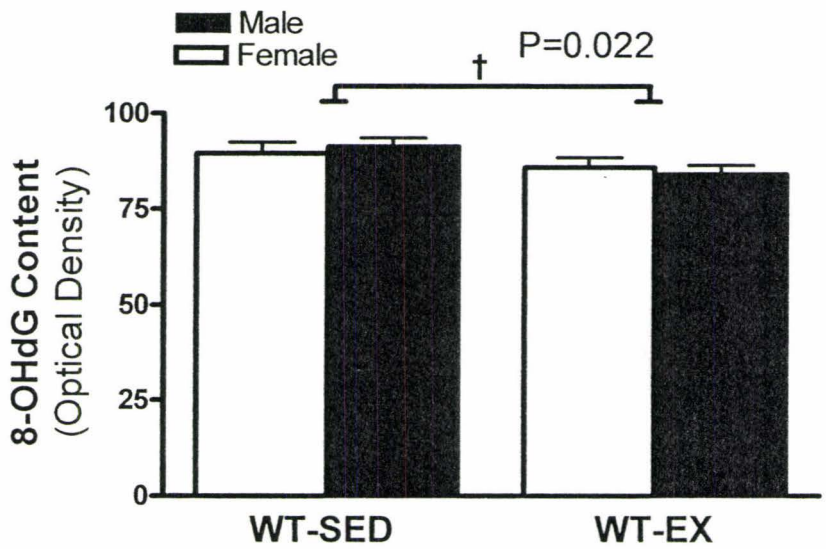

Figure 3.43 The content of 8-OHdG in the DG of WT-SED and WT-EX mice. WT-EX mice shad a lower level of 8-OHdG than WT SED mice, † p=0.022, n=9-11 per group. Data are means ± SEM.

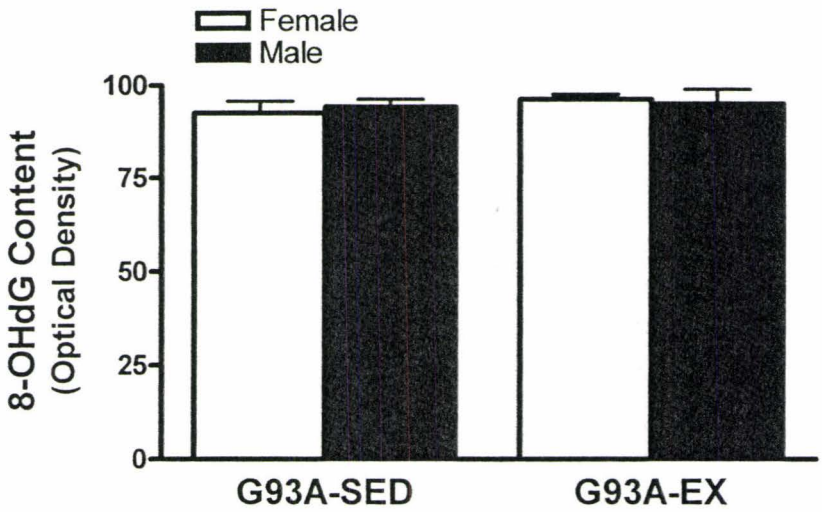

Figure 3.44 The content of 8-OHdG in the DG of G93A-SED and G93A-EX mice. There was no significant difference between G93A-SED and G93A-EX mice, n= 8-12 per group. Data are means ± SEM.

3.4.6 3-NT Content

In the hippocampus, faint 3-NT immunoreactivity was detected in granule cells in the DG and pyramidal neurons in the CA1, CA2, and CA3 regions in WT and G93A mice. At high magnification, cells with 3-NT positively stained presented the granule nature (**Figure 3.45.A**). Representative images for quantitative analysis of optical density are shown in WT (**Figure 3.45.B**) and G93A (**Figure 3.45.C**) mice.

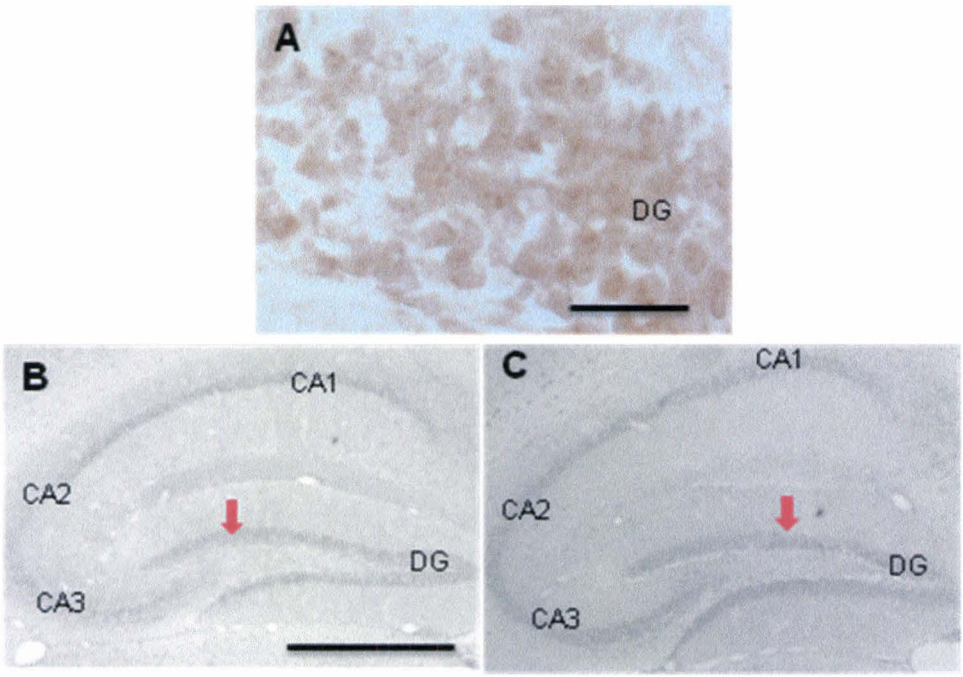

Figure 3.45 Representative images of 3-NT immunoreactivity in the DG of WT and G93A mice. (A) A representative image showed granule cells stained with 3-NT in the DG. Representative black-and-white images (used for determination of 3-NT optical density in the DG) showed 3-NT immunoreactivity in the DG of WT (B) and G93A (C) mice. Scale Bar = 25 μM in A, 500 μM in B,C. Red arrows indicate 3-NT immunoreactivity in the DG.

3-NT content in the DG

In sedentary mice, 3-NT content in the DG of G93A mice was significantly higher than that in WT mice (32.6 ± 2.6 vs 26.1 ± 2.1; p=0.038) (**Figure 3.46**). Furthermore, males had a trend to have high 3-NT content than females (30.9 ± 2.5 vs 26.5 ± 2.4; p=0.076). Following treadmill exercise, the level of 3-NT content in the DG in WT (**Figure 3.47**) or G93A (**Figure 3.48**) mice did not change as compared to sedentary mice, suggesting that treadmill exercise does not have effect on 3-NT content in the DG of WT and G93A mice. However, G93A males had a trend to have high 3-NT content than G93Afemales (35.2 ± 2.5 vs 30.6 ± 2.1; p=0.093).

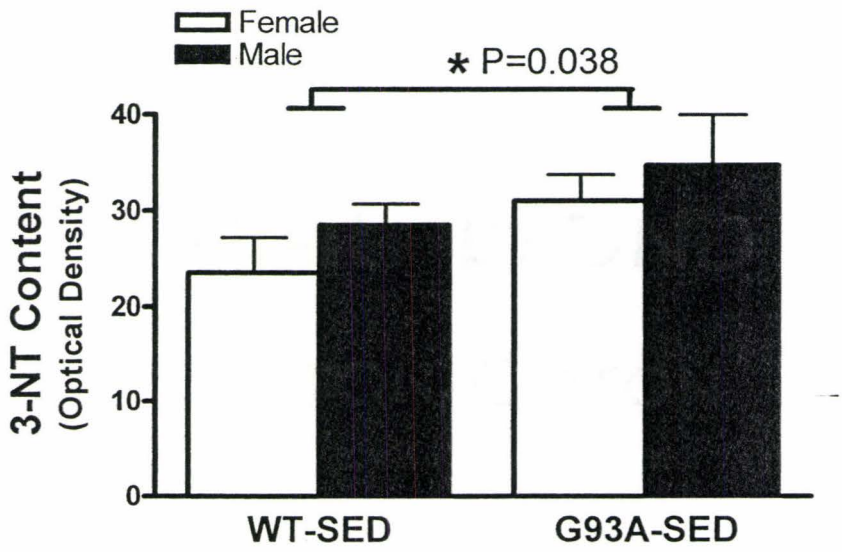

Figure 3.46 The level of nitrotyrosine (3-NT) in the DG of WT-SED and G93A-SED mice. G93A-SED mice had a higher 3-NT content than WT-SED mice, * p=0.038, n= 7-11 per group. Males had a trend to have high 3-NT content than females (p=0.076). Data are means ± SEM.

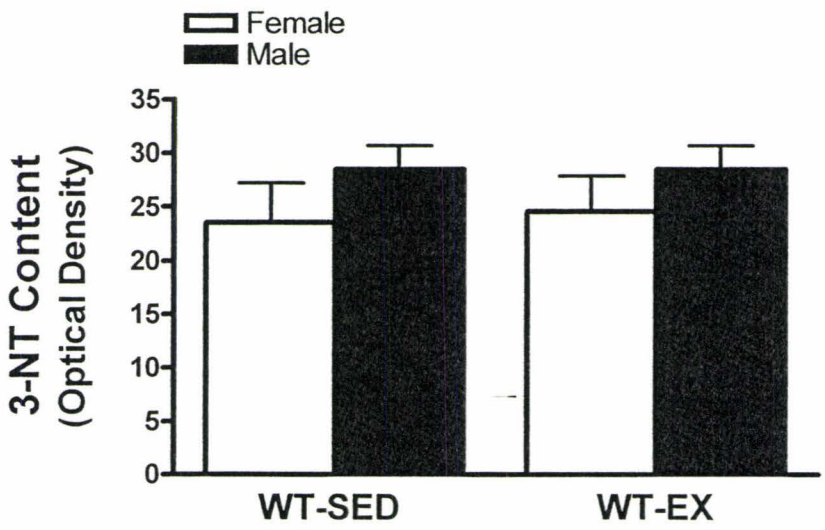

Figure 3.47 The level of 3-NT in the DG of WT-SED and WT-EX mice. There was no significant difference between WT-EX and WT-SED mice, n= 9-11 per group. Data are means ± SEM.

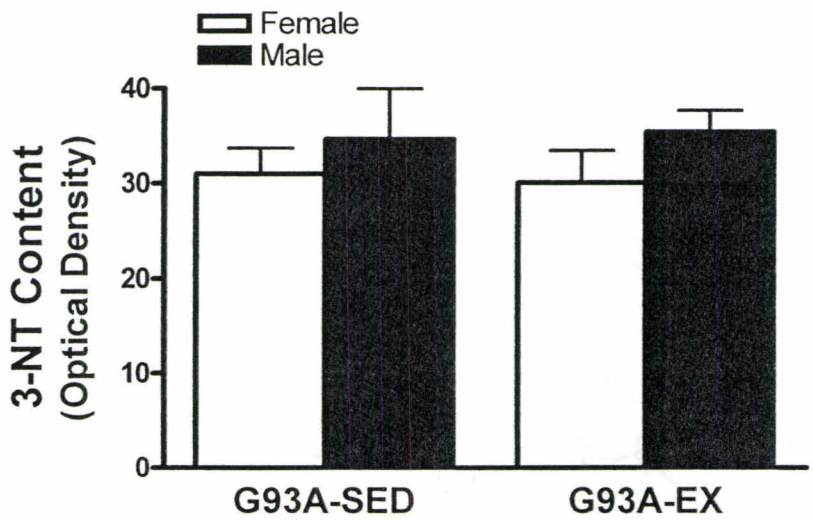

Figure 3.48 The level of 3-NT in the DG of G93A-SED and G93A-EX mice. There was no significant difference between G93A-EX and G93A-SED mice. However, G93A males had a trend to have high 3-NT content than G93A females, p=0.093, n= 7-10 per group. Data are means ± SEM.

4. DISCUSSION

4.1 Overview of Results

The G93A mouse is a model of heightened oxidative stress and is widely used in studies investigating the pathophysiology of ALS. The pathological changes in G93A mice mainly involve the grey regions of areas of the motor neuron system, such as spinal cord ventral horns and brainstem motor nuclei, both of which are usually affected in human ALS. It has been shown that motor neuron loss enhanced the proliferation, migration, and neurogenesis of NPCs in the adult spinal cord in G93A mouse and rat models of ALS (de Hemptinne, Boucherie et al. 2006; Liu and Martin 2006; Guan, Wang et al. 2007), suggesting that enhanced neurogenesis plays a role in the compensatory attempt to replace/repair the degenerated motor neurons and restore the dysfunctional circuitry which result from the pathological effects of mutant SOD1 (Chi, Ke et al. 2006; de Hemptinne, Boucherie et al. 2006). Although ALS is traditionally considered a motor neuron disease, the involvement of non-motor areas of the CNS, including the hippocampus, has been demonstrated in ALS patients and mouse models. In G93A mice, several lines of evidence, including higher oxidative stress and growth factors in the brain as well as enhanced hippocampal dependent function support hippocampal alterations as observed with mutations in the SOD1 gene. A major aim of the present study was to investigate whether the higher level of ROS would affect hippocampal neurogenesis and

whether such changes were associated with changes in hippocampal BDNF and IGF1. Given the evidence that the female sex and/or estradiol may have neuro-protective effects, we also considered selected sex-influenced outcome measures. In addition, exercise has profound benefits for the brain, such as improving learning and memory as well as preventing or delaying loss of cognitive function observed with aging or neurodegenerative diseases, and these benefits might be mediated by enhancement of hippocampal neurogenesis, promotion of growth factors, and mitigation of oxidative stress. Therefore, we also investigated whether treadmill exercise would have beneficial effect on oxidative stress and growth factors, and whether such effects would further affect hippocampal neurogenesis.

The main findings of the present study were that: (1) G93A mice had elevated basal levels of hippocampal neurogenesis of (survival and neuronal differentiation of BrdU labelled cells), growth factor (BDNF mRNA expression), and an oxidative stress (3-NT) as compared to wild type sedentary mice; (2) Treadmill running promoted hippocampal neurogenesis, growth factor expression (BDNF mRNA), and lowered oxidative stress (8-OHdG) in wild type mice, with no changes in G93A mice, as compared to sedentary mice; and (3) There was a sex difference in hippocampal neurogenesis in G93A mice, with male G93A mice exhibiting elevated cell proliferation but reduced cell survival as compared with female G93A mice.

4.2 Heightened Basal Levels of Hippocampal Neurogenesis, Growth Factors, and Oxidative Stress in G93A mice

4.2.1 Heightened Basal Levels of Hippocampal Neurogenesis in G93A Mice

We evaluated basal levels of hippocampal neurogenesis using cell proliferation, cell survival and cell differentiation in sedentary G93A mice. Sedentary G93A mice showed heightened cell survival of nascent cells and neuronal differentiation as compared to sedentary WT mice, suggesting a higher basal level of hippocampal neurogenesis in the G93A mouse model. In addition, G93A sedentary mice showed a trend for higher cell proliferation as compared with WT sedentary mice. This likely is a type 2 error, possibly due to the small sample size per group (5-6 animals/group for 8 groups).

The present study is in agreement with previous studies investigating similar outcome measures in the spinal cord of G93A mice. G93A mice exhibited greater cell proliferation, migration, and neurogenesis in the spinal cord as compared to age-matched controls (Chi, Ke et al. 2006; Guan, Wang et al. 2007). Further, our study is supportive of the enhanced hippocampal-dependent spatial capability observed in the G93A mouse model (Spalloni, Geracitano et al. 2006). Finally, the current results are in line with increased expression of the IGF1 receptor, EPO, and pERK observed in the hippocampus of G93A mice (Chung, Joo et al. 2003; Chung, Joo et al. 2004; Chung, Joo et al. 2005). IGF1, by binding to its IGF1 receptor expressed in the hippocampus, is an important modulator of brain function, ranging from neuroprotection to neural plasticity, and has

been shown to induce hippocampal neurogenesis in normal rodents and is involved in hippocampal neurogenesis in a variety of animal models of brain injury, aging, and disease (Anderson, Aberg et al. 2002; Llorens-Martin, Torres-Aleman et al. 2009). EPO, a well known trophic factor and the major hematopoietic growth factor predominantly produced in the kidney and also highly distributed in the brain, including hippocampus, internal capsule, cortex, and midbrain, has broad neuro-protective properties including preventing hippocampal neuronal and dopaminergic cell death *in vivo* and rescuing neurons from oxidative stress (Lewczuk, Hasselblatt et al. 2000; Kawakami, Sekiguchi et al. 2001; Catania, Marciano et al. 2002), and enhance neurogenesis after brain injury (Lu, Mahmood et al. 2005).

The ERK pathway mediates the neuroprotective activity of growth factors and may act as a compensatory protective mechanism against neuronal damage, as well it enhances brain ischemia-induced neurogenesis (Hetman and Gozdz 2004; Shioda, Han et al. 2009). Together, our and other studies show enhanced neurogenesis to protect against excessive levels of oxidative stress in the brain of G93A mice.

However, the literature is equivocal with respect to neurogenesis in G93A mice. Liu and Martin reported reduced cell proliferation in the DG, unaltered cell proliferation in the spinal cord, and unaltered neurogeneis in both the hippocampus and spinal cord in symptomatic G93A mice (Liu and Martin 2006). The discrepancy between our results and those of Liu and Martin could be due to the sex of animals studied and methodological differences, including the the dose of BrdU administered and the BrdU

administration schedule. In their study, cell proliferation was assessed 2 h or 14 h after a single injection of BrdU at a dose of 50 μg/g body weight, and cell survival and neuronal differentiation were assessed two weeks after the last injection of BrdU at a dose of 25 μg/g, in males only. In contrast, we used daily injections of 50 μg/g for 7 consecutive days, with cell proliferation assessed 24 h after the last BrdU injection and cell survival and differentiation assessed 3 weeks after the last BrdU injection (refer to Methods), in both females and males.

Enhancement of hippocampal neurogenesis has been observed in AD (Jin, Peel et al. 2004), an animal model of AD (Jin, Galvan et al. 2004), and ischemic stroke (Jin, Minami et al. 2001; Kee, Preston et al. 2001; Zhu, Lau et al. 2004). However, as recently reviewed by van Praag, controversy still exists as to whether hippocampal neurogenesis is altered at the basal level in some neurodegenerative diseases and their transgenic mouse models (van Praag 2008). For example, both a reduction (Dong, Goico et al. 2004; Verret, Jankowsky et al. 2007), and an increase (Jin, Galvan et al. 2004; Jin, Peel et al. 2004), in hippocampal neurogenesis have been reported in amyloid precursor protein (APP) mutant transgenic model of AD. Together, these observations suggest that hippocampal neurogenesis in neurodegenerative diseases and their animal models can be affected by many factors, including pathological status, transgenic genes, and animal's background strain.

4.2.2 Heightened Basal Levels of Growth Factors (BDNF mRNA) in G93A Mice

To investigate the mechanism of elevated basal levels of cell survival and neuronal differentiation in G93A mice, we next examined the basal levels of the growth factors, BDNF and IGF1. BDNF, by binding to its receptor TrkB, activates a number of growth and survival-promoting intracellular signalling pathways, including ERK/MAPK and PI3K/Akt (Barbacid 1995; Yuan and Yankner 2000; Zheng, Kar et al. 2000). BDNF plays an important role in the maintenance of hippocampal neurogenesis by regulating cell proliferation, cell survival, and neuronal differentiation (Linnarsson, Willson et al. 2000; Lee, Duan et al. 2002; Sairanen, Lucas et al. 2005). In our study, we showed that the basal levels of BDNF mRNA were higher in the DG of G93A mice as compared to WT mice, which were correlated with higher basal levels of cell survival and neuronal differentiation in this model. In addition, we also showed that BDNF mRNA content was higher in another hippocampal region, the CA3, suggesting that the higher content was consistent throughout the brain in G93A mice. Higher levels of BDNF in the G93A mouse hippocampus are consistent with previous studies showing higher mRNA and protein levels of BDNF in postmortem muscle tissue (Kust, Copray et al. 2002) and higher BDNF mRNA in the spinal cord of ALS patients and G93A mice (Offen, Barhum et al. 2009). Our results are also in line with previous observations showing the activation of ERK in the brain of G93A mice (Chung, Joo et al. 2005). We are the first to report BDNF mRNA content in the hippocampus of G93A mice.

BDNF is a converging molecule pathway. The up-regulation of BDNF in the hippocampus can be triggered by oxidative stress, and hence higher BDNF expression may serve as a compensatory mechanism against the oxidative injury in the hippocampus of G93A mice. Indeed, oxidative stress stimulates the expression of BDNF, and alternatively antioxidants prevent BDNF production in neuron cell line culture (Wang, Yuan et al. 2006). Furthermore, BDNF can protect neurons from oxidative insults (Mattson, Lovell et al. 1995). In glutamate-induced toxicity of hippocampal neuron cultures, BDNF reduced ROS accumulation and increased GPx and SOD activity (Mattson, Lovell et al. 1995). In addition, it has also been found that BDNF can acts as a positive loop with NO to inhibit neural progenitor cell proliferation and up-regulate neuronal differentiation in embryonic and adult neurogenesis (Cheng, Wang et al. 2003). Together, these findings suggest that oxidative stress and BDNF are involved in crosstalk mechanism where mutual feedback assists in regulating hippocampal neurogenesis.

We also investigated whether IGF was involved in the regulation of basal hipppocampal neurogenesis in G93A mice. We showed that IGF1 mRNA expression in both the DG and CA3 regions were not different from WT controls, consistent with a previous study showing that IGF1 immunoreactivity was not altered in the spinal cord in postmortem samples of ALS patients vs controls (Adem, Ekblom et al. 1994). In addition, DG IGF1 mRNA expression was not associated with the survival or neuronal differentiation of BrdU labelled cells (data not shown), suggesting that IGF1 is not related to elevated basal levels of cell survival or neuronal differentiation in G93A mice.

This is in contrast to previous research showing that peripheral IGF1 promotes hippocampal neural progenitor cell proliferation and neuronal differentiation in vivo and in vitro (Aberg, Aberg et al. 2000; Anderson, Aberg et al. 2002), and that it may be involved in brain injury-induced neurogenesis (Anderson, Aberg et al. 2002). However, to date no information is available regarding the possible role of IGF1 in the regulation of hippocampal neurogenesis in G93A mice. Although we showed that locally produced IGF1 in the brain is not related to hippocampal neurogenesis, we cannot however rule out the possibility that peripherally produced IGF1 are involved in hippocampal neurogenesis in G93A mice.

4.2.3 Heightened Basal Levels of Oxidative Stress (3-NT) in G93A Mice

The excessive level of oxidative stress in the spinal cord of G93A mice has been shown in several studies (Andrus, Fleck et al. 1998; Hall, Andrus et al. 1998; Liu, Althaus et al. 1998; Warita, Hayashi et al. 2001; Chung, Joo et al. 2003; Aguirre, Beal et al. 2005; Perluigi, Fai Poon et al. 2005). However, this relationship is not as strongly established in the brain of G93A mice. Chung and colleagues reported that SOD2 immunoreactivity was higher in the brain stem of G93A mice (Chung, Joo et al. 2003), whereas Ferrante and colleagues showed that 3-NT immuoreactivity, was higher in the brain of G93A mice (Ferrante, Shinobu et al. 1997; Cha, Chung et al. 2000), including the CA1 region of the hippocampus (Cha, Chung et al. 2000). However, no research has been conducted regarding oxidative stress in the hippocampus, specifically in the DG. In our study, we

performed in situ hybridization and immunohistochemistry to evaluate the mRNA expression and protein level of antioxidant enzymes (SOD2 and catalase) in the DG of G93A mice. Furthermore, we used immunohistochemical staining to examine the levels of 8-OHdG and 3-NT to evaluate oxidative damage in the DG of G93A mice.

Antioxidant Enzymes

We showed that SOD2 mRNA and protein were abundant in the DG, in both the G93A and WT mice. We also showed that catalase mRNA and protein were not different in the DG of the G93A vs WT mice. The lack of change in antioxidant enzymes in the DG of G93A vs. WT mice suggests that in the DG there is no compensatory increase in antioxidant enzymes in response to heightened levels of oxidative stress caused by SOD1 mutations in G93A mice.

Our studies are consistent with previous studies, showing no difference between ALS patients and controls in enzyme activity of SOD2 and catalase in two brain regions, the precentral gyrus of the cerebral cortex, a region affected in ALS patients, and the cerebellar cortex, a region not affected in ALS patients (Przedborski, Donaldson et al. 1996). On the other hand, G93A mice have higher SOD2 protein content in the brain stem (Chung, Joo et al. 2003) and the spinal cord (Shaw, Chinnery et al. 1997; Chung, Joo et al. 2003), both regions affected in ALS patients. In addition, enzyme activity of SOD2 and catalase is higher in skeletal muscle of G93A mice as compared to WT mice (Mahoney, Kaczor et al. 2006). Further, GPx activity is significantly lower in the precentral gyrus, but not the cerebellar cortex (Przedborski, Donaldson et al. 1996), but

higher in the spinal cord of ALS patients as compared to controls (Ince, Shaw et al. 1994). Collectively, these studies suggest that the content of antioxidant enzymes is region-dependent in G93A mice, as observed in the brains of AD patients regarding antioxidant enzymes (Balazs and Leon 1994; Chen, Richardson et al. 1994). Based on this, we could not rule out that G93A mice may have higher levels of SOD2 and catalase in other brain regions as compared to WT mice. And we could not rule out that there may be up-regulation of other antioxidant enzymes, such as GPx and heme oxygenase, in the DG of G93A mice.

Oxidative Stress

We chose to measure 8-OHdG and 3-NT as markers of DNA and protein oxidative modification, since they are consistently reported in both ALS patients and G93A mice (Abe, Pan et al. 1997; Beal, Ferrante et al. 1997; Ferrante, Shinobu et al. 1997) and reflect oxidative damage to two important macro-molecules.

We did not observe an increase in 8-OHdG in G93A vs WT mice, suggesting that the mutant SOD1 induced ROS production did not affect DNA macro-molecules in the hippocampus of G93A mice. In contrast, Aguirre and colleagues found that 8-OHdG was higher in the cortex (at 90, and 120 days) and striatum (at 120 days) of G93A mice as compared to age-matched littermate controls. They also found regional heterogeneity, i.e. no significant changes of 8-OHdG level in the cerebellum at any of the time points studied (at 60, 90, and 120 days) (Aguirre, Beal et al. 2005). In addition, 8-OHdG is most prominent in the ventral horn of spinal cord in ALS patients (Ferrante, Browne et al.

1997) and G93A mice (Warita, Hayashi et al. 2001). Whether the level of 8-OHdG is changed in other brain regions in G93A mice is not clear. On the other hand, the absence of increased DNA damage in the DG of G93A mice may be due to the up-regulation of the DNA repair enzymes, DNA glycosylases, which recognize and remove the damaged base, specifically OGG1 (8-oxoguanine-DNA glycosylase) is a major enzyme responsible for 8-OHdG removal (Klungland, Rosewell et al. 1999; Minowa, Arai et al. 2000). It is possible that OGG1 is up-regulated in the DG of G93A mice, which could explain the lack of change in 8-OHdG in the DG region of the hippocampus.

3-NT is a marker of peroxynitrite-mediated nitration or NO production. Peroxynitrite is formed by the reaction of NO with O_2^-. In the current study, we found significantly higher 3-NT immunoreactivity in the DG of G93A mice as compared to controls, suggesting an excessive peroxynitrite-mediated nitration or NO production in the DG in G93A mice. This is in agreement with previous observations of high 3-NT in other regions of the brain and in the spinal cord of ALS patients and G93A mice. Regional heterogeneity also exist for 3-NT, with higher levels observed in the cerebral cortex but not brain stem, striatum, and cerebellum in G93A mice (Ferrante, Shinobu et al. 1997). Cha and colleagues evaluated 3-NT distribution in the brain of G93A mice, showing intense staining in the brain stem and cerebellum. In addition, they observed intense 3-NT immunreactivity in the pyramidal layer, especially in the CA1 of hippocampus of G93A mice, however they did not measure staining intensity (Cha, Chung et al. 2000). But others have shown greater 3-NT immunoreactivity in the motor

neurons of ALS patients (Beal, Ferrante et al. 1997) and G93A mice (Ferrante, Shinobu et al. 1997). Our novel data suggest greater NO production in the DG of the hippocampus of G93A mice. Excessive NO generation is implicated in neuronal injury after ischemia, trauma, and neurodegenerative disorders, including ALS (Barber, Mead et al. 2006; Valko, Leibfritz et al. 2007).

In summary, our results suggest that G93A mice have high levels of peroxynitrite-mediated nitration or NO production with no compensatory up-regulation of antioxidant enzymes. Since NO acts in tandem with BDNF via a positive feedback loop to regulate neural progenitor cell proliferation and differentiation (Cheng, Wang et al. 2003) or promote hippocampal neurogenesis (Zhang, Zhang et al. 2001), we postulate that oxidative stress could trigger an increase in BDNF mRNA in the DG of G93A mice.

4.3 Effect of Treadmill Exercise on Neurogenesis, Growth Factors, and Oxidative Stress in Hippocampus

Many studies and reviews have addressed the benefits of exercise on brain function (Babyak, Blumenthal et al. 2000; Cotman and Berchtold 2002; Bjornebekk, Mathe et al. 2005; van Praag 2008; van Praag 2009). Exercise may improve learning and memory, postpone age-related cognitive decline, decrease the risk of neurodegenerative diseases, and alleviate depression (Cotman and Berchtold 2002; Cotman, Berchtold et al. 2007; Hillman, Erickson et al. 2008; van Praag 2008; van Praag 2009). The effects of exercise are very complex and could include neurogenesis via growth factors, decreased oxidative

damage, and increased angiogenesis (Johnson and Mitchell 2003; Adlard and Cotman 2004; Adlard, Perreau et al. 2005).

4.3.1 Treadmill Exercise Effect on Hippocampal Neurogenesis

We examined cell proliferation, cell survival, and cell differentiation to evaluate hippocampal neurogenesis in response to treadmill exercise in G93A mice compared to WT mice. Our objective was to investigate if the pulses of oxidative stress induced by exercise that are heavily implicated in the promotion of hippocampal neurogenesis would affect the hippocampal neurogenesis with the oxidative stress seen in the DG of G93A mice. With respect to cell proliferation and cell survival, our results consistently showed that treadmill exercise promoted cell proliferation and cell survival in WT mice (van Praag, Kempermann et al. 1999; Holmes, Galea et al. 2004; Uda, Ishido et al. 2006). However, G93A mice showed a trend for lower cell proliferation and no change in cell survival in response to exercise. In addition, treadmill exercise did not show any effect on neuronal differentiation in both WT and G93A mice. Our data are novel in showing that treadmill exercise did not affect hippocampal neurogenesis in G93A mice, yet it up-regulated hippocampal neurogenesis in WT mice.

Exercise in rodents, including wheel running or treadmill running, promotes hippocampal neurogenesis via cell proliferation and cell survival (van Praag 2008). In pathological states, exercise reverses decreased hippocampal neurogenesis in murine models of normal aging, radiation, and alcohol exposure (Crews, Nixon et al. 2004;

Kronenberg, Bick-Sander et al. 2006). However, in some animal models of neurodegenerative diseases, exercise does not induce hippocampal neurogeneis. Specifically, the amyloid precursor protein (APP)-23 model of AD showed no change in survival of newborn neurons following long-term (8 month duration) exercise (Wolf, Kronenberg et al. 2006). In the R6/2 transgenic mouse model of HD, 4 wk of running failed to stimulate proliferation and survival of newly generated neurons (Kohl, Kandasamy et al. 2007). Under normal conditions, exercise-induced hippocampal neurogenesis is affected by the intensity and duration of exercise (Holmes, Galea et al. 2004; Naylor, Persson et al. 2005), and by the animal strain (Hauser, Klaus et al. 2009). Under pathological conditions, the effect of exercise on hippocampal neurogenesis is complex. Basically, exercise can induce pulses of stress. Under certain conditions, such as social isolation, exercise may increase the susceptibility to glucocorticoid-induced suppression of neurogenesis (Stranahan, Khalil et al. 2006; Leasure and Decker 2009). Consequently, the interaction of exercise and excessive oxidative stress in the G93A mouse may have inhibited hippocampal neurogenesis.

4.3.2 Treadmill Exercise Effect on Growth Factors

Growth factors may be involved in the mechanisms underlying exercise-induced hippocampal neurogenesis. In the present study, we used in situ hybridization to examine whether treadmill exercise would affect BDNF and IGF1 mRNA expression in DG and CA3 regions of the hippocampus. We showed that treadmill exercise increased BDNF

mRNA content in the DG of WT mice, which is in agreement with previous observations (Oliff, Berchtold et al. 1998; Johnson and Mitchell 2003; Farmer, Zhao et al. 2004; Garza, Ha et al. 2004). In contrast, treadmill exercise did not alter BDNF mRNA expression in the DG of G93A mice, possibly due to the 'ceiling effect' of heightened basal levels of BDNF mRNA expression in this disease model. This is consistent with the unaltered hippocampal neurogenesis in response to exercise observed in our study.

Although no change was observed on DG BDNF mRNA expression following exercise in G93A mice, DG BDNF mRNA is still correlated with cell survival and neuronal differentiation in G93A mice following exercise, suggesting the strong association between BDNF and cell survival and neuronal differentiation and also suggesting the further support that treadmill exercise did not have effect on BDNF mRNA and hippocampal neurogenesis in G93A mice seen in our study.

Treadmill exercise tended to promote higher IGF1 mRNA content in the DG of WT mice, but not in the DG or CA3 regions of G93A mice, suggesting that central IGF1 may be involved in hippocampal neurogenesis in exercised WT mice, but not in G93A mice. Previous studies have demonstrated that peripheral IGF1 is required to mediate hippocampal neurogenesis following exercise (Carro, Nunez et al. 2000; Trejo, Carro et al. 2001). Our results suggest that central IGF1 may also be involved in exercise-induced hippocampal neurogenesis in WT mice, however further research is warranted to confirm this observation.

4.3.3 Treadmill Exercise Effect on Oxidative Stress

Antioxidant Enzymes

Exercise generates pulses of ROS due to ATP production via mitochondrial metabolism and/or the xanthine oxidase reaction. Pulses of oxidative stress following exercise could lead to compensatory up-regulation of antioxidant enzymes in muscle (Powers, Criswell et al. 1994; Parise, Phillips et al. 2005). Whether exercise induces a similar response in antioxidant enzymes in the brain is not clear.

In the present study, we observed no change in mRNA expression or protein level of antioxidant enzymes (SOD2 and catalase) in the hippocampus of WT or G93A mice, suggesting that treadmill exercise does not lead to compensatory antioxidant adaptation in the hippocampus of WT or G93A mice. This is in agreement with previous studies showing no alteration in antioxidant enzymes in the hippocampus following exercise (Radak, Toldy et al. 2006; Cechetti, Fochesatto et al. 2008). Cechetti and colleagues reported that daily moderate intensity exercise (2 weeks of 20 min/day of treadmill training) did not modify the level of DNA repair enzyme OGG1 in the brain (Cechetti, Fochesatto et al. 2008), suggesting that moderate exercise did not induce adaptations of the cellular antioxidant system in the hippocampus. Moreover, swimming training (8 week, 5 times/week, 2 h/day) did not modify antioxidant enzyme activities of OGG1 in the rat brain (Radak, Toldy et al. 2006). In contrast, Romani found that a treadmill exercise for 7.5 weeks led to higher enzyme activities for SOD and GPx in the brainstem and corpus striatum of rats (Somani, Ravi et al. 1995). However, these higher SOD and

GPx activities did not happen in the hippocampus and cerebral cortex (Somani, Ravi et al. 1995). These studies suggest that the effect of exercise on antioxidant enzymes is also dependent on brain regions (Somani, Ravi et al. 1995). In addition, a recent study reported that treadmill exercise did not affect antioxidant enzyme activities of SOD1, catalase, and GPx in the brain of normal rats; whereas, it increased the activity of GPx in diabetic rats (Ozkaya, Agar et al. 2002), suggesting the effect of exercise on antioxidant enzymes was also dependent on animal conditions. Therefore, the response in the activities of antioxidant enzymes may be determined by many factors, including brain regions, animal status, and exercise type. In our study, we could not exclude whether there is the adaptive up-regulation of antioxidant enzyme in other brain regions in G93A mice.

Oxidative Stress

We have shown that treadmill exercise lowered 8-OHdg immunoreactivity in the DG of the hippocampus in WT mice, but not G93A mice. These results suggest that treadmill exercise attenuate DNA oxidative damage in WT mice. These results are in agreement with previous observations showing that exercise reduced oxidative damage in the brain (Radak, Kaneko et al. 2001; Radak, Taylor et al. 2001; Toldy, Stadler et al. 2005) and skeletal muscles (Radak, Kaneko et al. 1999; Parise, Phillips et al. 2005). On the other hand, we did not find any effect of exercise on 3-NT immunoreactivity in WT or G93A mice, suggesting that treadmill exercise did not reduce damage for peroxynitrited protein. Our study showing that treadmill exercise attenuated DNA oxidative damage only and

this attenuation was not mediated by compensatory adaption to up-regulate antioxidant enzymes in WT mice seems paradoxical. However, we could not rule out the possibility that exercise would up-regulate the repair enzyme for oxidatively damaged DNA, i.e. OGG1, to counteract the DNA damage in the DG of the hippocampus in WT mice.

The effect of exercise on oxidative stress in the DG of the hippocampus, a susceptible brain region to neurodegenerative disease and a specific region for adult neurogenesis, is less established and findings reported on oxidative stress variables in the hippocampus are conflicting as compared with other brain regions. A recent study demonstrated that moderate exercise (2 weeks of 20 min/day of treadmill) did not affect free radical content in the hippocampus, suggesting that exercise does not acutely induce a significant oxidative stress (Cechetti, Fochesatto et al. 2008). In addition, exhaustive treadmill exercise did not change levels of DNA damage or protein oxidation in the hippocampus (Radak, Toldy et al. 2006). In other brain regions, however, chronic swimming exercise 2 hours for 6 weeks lowered the level of free radical in the cerebellum (Toldy, Stadler et al. 2005). In contrast, swimming at different intensities and durations has shown no significant change on lipids and DNA oxidative damage in the whole brain of normal rats (Ogonovszky, Berkes et al. 2005). Moreover, it has been reported that voluntary wheel-running exercise resulted in increased lipid peroxidation in rat brain (Suzuki, Katamine et al. 1983), while a swimming regime of 1 h a day, 5 days a week for 9 weeks attenuated the protein oxidative damage in rat brains (Radak, Kaneko et al. 2001). Together, these suggest that exercise, especially swimming exercise, can

attenuate oxidative stress in the brain. However, the effect of exercise induced attenuation of oxidative stress is subject to many factors including regional heterogeneity, animal status, and the type of exercise.

4.4 The Effect of Sex Difference on Hippocampal Neurogenesis

There are several observations that led us to speculate that sex difference would affect hippocampal neurogenesis in G93A mice. First, the concept that there is the sex difference in hippocampal neurogenesis is beginning to be established (Galea, Spritzer et al. 2006; Galea 2008). Second, it has also been reported that there is sex difference in the progression of diseases in neurodegenerative disease including ALS (Czlonkowska, Ciesielska et al. 2006). Furthermore, our previous study showed that there was a sex difference in the progression of the disease in response to exercise (Mahoney, Rodriguez et al. 2004).

Our results showed that neurogenesis in cell proliferation was significantly higher in male G93A mice than in female G93A mice ($p=0.009$) but cell survival tended to be higher in female vs. male G93A mice ($p=0.057$). In addition, exercised female G93A mice displayed more surviving cells than exercised male G93A mice ($p=0.033$). There was no difference in neuronal differentiation between male and female G93A mice. In contrast, we did not find sex difference in hippocampal proliferation, cell survival, or cell differentiation in WT mice. Our current study suggest that a sex difference was appeared only in G93A mice associated with excessive oxidative stress whereby cell proliferation

and cell survival in an opposite way, with male mice showing a higher cell proliferation but a lower cell survival as compared to female mice. The sex differences in cell proliferation and survival for the G93A mice are supportive of our previous observation that female G93A mice have better survival and resistance to excessive running stress as compared with males (Mahoney, Rodriguez et al. 2004). Our current study showing no sex difference in hippocampal neurogenesis in WT mice is consistent with a recent study, which failed to find evidence for a significant sex difference in basal rates of hippocampal cell proliferation in mice (Lagace, Fischer et al. 2007). Although our and Legace's studies are consistent in showing no sex difference in wild type animals, it is possible that estrous cycle could influence the ability to detect a sex difference (Tanapat, Hastings et al. 1999).

Evidence for a sex difference in hippocampal neurogenesis has been shown in several studies. Two studies showed that female had higher levels of cell proliferation, but not cell survival, than male depending on the endocrine of the female (Galea and McEwen 1999; Tanapat, Hastings et al. 1999). For example, only proestrus female rats (with high estradiol levels) showed higher levels of cell proliferation than male rats (Tanapat, Hastings et al. 1999). However, female meadow voles had higher levels of cell proliferation than male meadow voles only during the non-breeding season (when estradiol levels were low) (Galea and McEwen 1999). Further studies showed that reproductively active female meadow voles with high endogenous levels of estradiol had suppressed rates of cell proliferation in the DG compared with reproductively inactive

female with low estradiol, yet more new cells survived in females with high endogenous levels of estradiol (Ormerod and Galea 2001). A recent study failed to show that there was a sex difference in basal rates of hippocampal cell proliferation between non-proestrus female mice and male mice (Lagace, Fischer et al. 2007). Together, these suggest that sex difference in hippocampal neurogenesis could be related to species and endogenous levels of estradiol in animals.

The mechanisms underlying possible sex difference neurogenesis are not completely understood, but estradiol may be involved. Estradiol affects both cell proliferation and cell survival in the dentate gyrus of adult rodents; however, these effects differ depending on the time of exposure to estradiol, amount of estradiol, the presence of proestrus, species, and sex of subject (Galea 2008). Estradiol exposure for 4 hours enhances; whereas, estradiol exposure for 48 hours, suppresses cell proliferation in the dentate gyrus of adult female meadow voles (Ormerod and Galea 2001). The suppression of cell proliferation was demonstrated to be mediated by adrenal steroid corticosterone (Ormerod, Lee et al. 2003). In male meadow voles, estradiol enhances cell survival only if administered during the 'axon extension phase' of cell maturation (Ormerod, Lee et al. 2004).

Taken together, G93A mice have a heightened basal level of hippocampal neurogenesis, which is associated with heightened basal level of BDNF mRNA. Oxidative stress might indirectly affect hippocampal neurogenesis by regulating BDNF level. Thus, at the presence of oxidative stress in the hippocampus (Cha, Chung et al.

2000; Chung, Hong et al. 2003; Chung, Joo et al. 2004), BDNF may serve as a compensatory mechanism to against the oxidative injury via promoting hippocamal neurogenesis. Treadmill running has different effects in the hippocampal neurogenesis, growth factors, and oxidative stress between G93A mice and WT mice. Treadmill exercise did not have effect on hippocampal neurogenesis, growth factors, and oxidative stress in G93A mice, although it is beneficial in WT mice. Therefore, it should be cautious when the beneficial effect of exercise on hippocampal neurogeneis is interpreted in the motor neurodegenerative disease.

4.5 Overall Conclusions

In the present study, we investigated the basal level of hipppocampal neurogenesis, hippocampal mRNA of growth factors, hippocampal mRNA and protein of antioxidant enzymes, and oxidative stress markers in WT and G93A mice in response to treadmill exercise. We also evaluated whether there was sex difference in the above mentioned variables.

We showed that G93A mice had an elevated basal level of hippocampal cell survival and neuronal differentiation as compared to WT mice, which is consistent with previous studies that G93A mice had increased neurogenesis in the spinal cord. The observation was also in line with other hippocampal changes in G93A mice, including elevated hippocampal function, elevated activation of ERK, and EPO. With respect to growth factors, we found that G93A mice have elevated basal level of hippocampal

BDNF mRNA as compared to WT mice, while no significant difference in IGF1 mRNA. In addition, we found that the higher basal level of hippocampal cell survival and neuronal differentiation in G93A mice was associated with higher basal level of DG BDNF mRNA content, suggesting that BDNF, but not IGF1, may be influencing basal level of hippocampal neurogenesis in G93A mice. We confirmed that G93A mice had elevated basal levels of 3-NT with no change in antioxidant enzymes (SOD2 and catalase, mRNA and protein) and no change in 8-OHdG as compared to WT mice. The latter findings suggest that peroxynitrite damage or excessive NO production is pathogenic in G93A mice. The unchanged level of antioxidant enzymes and DNA oxidative damage markers in the DG in G93A mice is in contrast with observations of elevated antioxidant enzymes and DNA oxidative damage marker in the spinal cord and skeletal muscles of G93A mice. This observation is generally consistent with clinical presentations and reflects the different extent of pathological involvement between DG and spinal cord. We could not rule out whether there is oxidative damage for protein, lipid, or DNA in other brain regions in G93A mice.

Our study showed that treadmill running did not alter hippocampal proliferation, cell survival, or neuronal differentiation in G93A mice, yet treadmill training promoted hippocampal cell proliferation and cell survival in WT mice. The latter finding may be associated with higher BDNF mRNA content following treadmill exercise in WT mice. In contrast, G93A mice showed no change in BDNF mRNA content following treadmill running, possible due to 'ceiling effect' of heightened basal levels of BDNF mRNA

expression in this model. Our study showed that treadmill running tended to increase DG IGF1 mRNA content in WT mice, but not in G93A mice. This suggests that central IGF1 might be involved in the regulation of hippocampal neurogenesis in exercised WT mice but not in G93A mice. We showed that treadmill exercise does not lead to compensatory antioxidant adaptation in the hippocampus in WT or G93A mice, which is consistent with the study showing that treadmill exercise did not modify antioxidant enzyme level in the hippocampus in rats. We could not rule out whether there is antioxidant adaptation in other brain regions. We showed that treadmill exercise lowered 8-OHdG (not 3-NT) content in the DG of the hippocampus in WT mice, but not G93A mice, suggesting that treadmill exercise attenuate DNA oxidative damage in WT mice. These results are agreement with previous observations showing that exercise reduce oxidative damage in the brain of normal rats. Further study is needed to investigate whether exercise attenuated DNA oxidative damage is associated with other antioxidant enzymes such as SOD1, GPx or OGG1.

We showed that there was a sex difference in basal level of hippocampal neurogenesis and also a sex difference in response to exercise in G93A mice, but not in WT mice, in which male G93A mice had a higher cell proliferation but a lower cell survival as compared to female G93A mice and male exercised male G93A mice had lower cell survival as compared to exercised female G93A mice. These suggest that a sex difference was evident only in G93A mice associated with excessive oxidative stress. The sex differences in cell proliferation and survival for the G93A mice are supportive of

our previous observation that female G93A mice have better survival and resistance to excessive running stress as compared with males.

4.6 Future Directions

The main aim of our study was to evaluate whether excessive oxidative stress caused by mutant human SOD1 would trigger the modification of neurogenesis in G93A mice. We showed a higher basal level of hippocampal cell survival and neuronal differentiation in G93A mice. Further studies investigating neurogenesis in another neurogenic region SVZ/olfactory bulb would help us to have a comprehensive notion regarding the neurogensis in G93A mice. It has been suggested that the effect of exercise on hippocampal neurogenesis is modulated by the intensity and duration of exercise in normal conditions and the effect of exercise on hippocampal is more complicated under pathological conditions. Therefore, exercise at different intensities (low or moderate) and durations should be performed before we clearly conclude that exercise does or does not promote hippocampal neurogenesis in G93A mice.

The G93A mouse model is specially targeted for excessive oxidative stress, thus, we speculated that antioxidant enzymes (SOD2 and catalase) would be up-regulated to compensate oxidative stress in the DG as compared to WT mice. However, we did not show higher levels of antioxidant enzymes in the DG of the hippocampus in G93A mice. Based on the regional heterogeneity for the level of antioxidant enzymes in the brain, we should examine antioxidant enzymes in other brain regions. In addition, other

antioxidant enzymes, such as GPx, also need to be examined. We also need to investigate whether DNA damage repair enzymes (i.e., OGG1) are involved in the repair of DNA damage in the DG of G93A mice and exercise induced attenuation of DNA damage in WT mice. Given that pulses of oxidative stress following exercise could lead to compensatory adaption to up-regulate antioxidant enzymes and this consequence is affected by many factors including brain regions, animal status, and exercise type, we should carry out treadmill training at different intensities or try swimming training to clarify whether the adaption is present in the DG of hippocampus in WT mice or G93A mice. Finally, only immunohistochemistry staining *in situ* was used to evaluate oxidative stress marker and antioxidant enzyme contents in the DG of the hippocampus. This technique might not be sensitive enough to detect the difference between G93A mice and WT mice. Other techniques, such as western blot, should be considered when it is possible to isolate the tissute of dentate gyrus from brain.

We found that there was sex difference in hippocampal proliferation and cell survival in G93A mice, in which female G93A mice had a lower cell proliferation but higher cell survival as compared to male G93A mice and exercised female G93A mice had higher cell survival as compared to exercised male G93A mice. It suggested that estrogen might be the candidate to mediate the sex difference in hipppocampal neurogenesis in G93A mice. We should investigate whether administration of estradiol in male G93A mice would lead to lower hippocampal proliferation but higher cell survival as compared to controls. Given that our studies are in contrast with some of

previous studies showing that there is a sex difference in hippocampal neurogenesis between WT pro-estrous female mice and WT male mice, we should evaluate whether pro-estrous female WT mice would show a sex difference in hippocampal neurogenesis as compared to male WT mice.

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5. APPENDICES

METHOD DEVELOPMENT

5.1 Brain Tissue Preparation

5.1.1 Brain Perfusion Procedure

1. Mice were anesthetized with isoflurane inhalation and wait until unconscious.
2. Expose thoracic cavity by inserting blunt end of scissors
3. Clamp on xyphoid process and lift up
4. Cut the diaphragm and cardiac ligaments
5. Cut ribs on either side to create a flap and lift up (be careful not to pierce the lung)
6. Squeeze the heart gently and place the 20 gage needle into the left ventricle (the right of apex of the hear)
7. A small cut of the right atrium to let the blood bleed out
8. Perfuse with PBS till the mouse liver turns right
9. Switch the perfusant to 4% PFA until mice were stiff

5.1.3 Brain Removal and Cryoprotection

1. Remove brains and store for 24 hours in 4% PFA at 4°C.
2. Wash three times in PBS
3. Store for 24 hours them in 30% sucrose at 4°C
4. Wash three times in PBS

5. Store in PBS at 4°C

5.1.3 Preparation of Frozen Sections for Sectioning

1. Label the base mold and partially fill the mold with OCT.
2. Place a stainless steel beaker of 2-methylbutane in liquid nitrogen and allow to cool adequately.
3. Place brains into the beaker of cold 2-methylbutane and quickly immerse brains.
4. Allow brains to solidify completely and remove block from 2-methylbutane and place on dry ice. NOTE: If block is left in 2-methylbutane too long (usually less than 6 seconds), the block may crack.
5. Place solidified brains in pre-labeled base molds partially filled with OCT.
6. Mount brains vertically (on top of dry ice) (olfactory bulb part on top and cerebellum part at the bottom of the mold) in OCT and covered the brains with cold OCT and dry ice powder.
7. Store blocks in the -80°C freezer until ready for sectioning.

5.1.4 Sectioning of Frozen Tissues

1. Before cutting sections, allow the temperature of the brain block to equilibrate to the temperature of the cryostat (-20°C).
2. Place the brain block on the cryostat specimen disk. Adjust the positioning of the block to align the block with the knife blade. Cut brain block until the desired tissue is exposed.

3. Cut and collect sections.

Note: In the cell proliferation group, brains were cut to coronal sections (40 μm /section) throughout the entire rostral-caudal extent of the hippocampus (Bregma -0.94 ~ -3.88) (Paxinos and Franklin 2001)for BrdU IHC. In the cell survival study group, half of brains were cut into coronal sections (40 μm/section) throughout the entire rostral-caudal extent of the hippocampus (Bregma -0.94 ~ -3.88) for BrdU IHC and for analysis of cell phenotype by immunofluorescent staining. Coronal sections were collected into 12-well-plates and stored at –20°C in cryoprotectant. Another half of brains were cut to sagittal sections (16 μm/section), collected in gelatin-coated slides, dry 2 hours at room temperature and kept at -80°C for mRNA expression of BDNF, IGF1, SOD2,and catalase by in situ hybridization and for protein level of SOD2, catalase, 8ohdg, and NT by IHC. Frozen section slides can be stored for a short period of time at -80 °C in a sealed slide box. When ready to stain, remove slides from freezer and warm to -20 °C in the cryostat or -20 °C freezer, fix for 2 minutes in cold fixative (acetone or other suitable fixative) and allow coming to room temperature to continue with the staining.

5.2 Construction of cRNA Probes

The complete procedure of construction of cRNA probes includes many experiments. The outline of these experiments is as the following:

1. Mini-Prep of plasmid DNA (purchased cDNA clones)
2. Running PCR to amplify cDNA fragment desired

3. Purify cDNAs
4. Ligation
5. Transformation
6. Screening colonies
7. Mini-prep of plasmid DNA for plasmid DNA sequencing
8. Maxi-prep of plasmid DNA
9. Linearization of Plasmid
10. Purify the linearized DNA
11. Make radioactive cRNA riboprobe using 35S UTP and ΔNTP's

5.2.1 Mini-Prep of plasmid DNA (purchased cDNA clones)

Materials

- Mouse SOD2 cDNA clone: ATCC, MGC-6144
- Mouse catalase cDNA clone: ATCC, MGC-18464
- Mouse IGF-1 cDNA clone: Open Biosystems, clone ID: 4194295
- Disposable sterile loop
- Luria-Bertani (LB) medium
- Ampicillian stock solution (100mg/ml
- LB agar plate (with ampicillian)
- QIAGEN Plasmid Mini Kit: QIAGEN, CAT#12125, Mississauga, Ontario

Procedure (following the instruction of QIAGEN Plasmid Mini Kit described below)

1. Pick up some cells (using sterile loop) from each colony on the agar tube of the cDNA clone.

2. Streak onto a fresh LB agar plate.

3. Incubate at 37°C overnight.

4. Inoculate a single colony to a culture of 5 ml of LB medium containing ampicillian (50 or 100 ug/ml) overnight at 37°C with vigorous shaking (250 rpm).

5. Harvested bacterial cell pellets in 15ml tube by centrifuging at 4000 rpm for 15 min at 4°C.

6. Resuspend bacterial pellet in 0.3 ml Buffer P1

7. Add 0.3 ml Buffer P2 and mix thoroughly by inverting the tube 4–6 times and incubate at room temperature for 5 min

8. Add 0.3 ml chilled Buffer P3 and mix immediately and thoroughly by inverting the tube 4–6 times and incubate on ice for 5 min

9. Centrifuge for 10 min at 13,000 rpm and remove supernatant

10. Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow.

11. Apply the supernatant to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow.

12. Wash the QIAGEN-tip 20 with 2 X 2 ml Buffer QC

13. Elute DNA with 0.8 ml Buffer QF.

14. Precipitate DNA by adding 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of

room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately 13,000 rpm for 30 min. Carefully decant the supernatant.Wash DNA pellet with 1 ml of 70% ethanol and centrifuge at 10,000 rpm for 10 min.

15. Carefully decant the supernatant without disturbing the pellet
16. Air-dry the pellet for 10 min, and redissolve the DNA in 20-30μl water
17. Measure the concentration of plasmid DNA by using Spectrophotomer (ND-1000, Thermo Scientific, Wilmington, DE).
18. Dilute plasmid DNA into 50 ng/μl and store at -20°C.

5.2.2 PCR

Materials

- mouse SOD2 primers: Sigma-Genosys

  forward 5'-CGC CAC CGA GGA AAG TA-3',

  reverse 5'-CAG TCA TAG TGC TGC AAT GC-3'

- Mouse catalase primers: Sigma-Genosys

  forward 5'-GCT ATG GAT CAC ACA CCT T-3'

  reverse 5' -GTT CAC AGG TAT CTG CAG-3'

- mouse IGF1 primers: Sigma-Genosys

  forward 5'–TGG ATG CTC TTC AGT TCG TG-3'

  reverse 5'-TCC TGC ACT TCC TCT ACT TGT-3'

Note: All primers were prepared to be at a concentration of 100μM by adding certain amount of water and stored at -20°C. Then these stock primers were further diluted into 20 μM for PCR reaction.

- 2X PCR Master Mix: Fermentas, #K0171, Glen Burnie, MD
- Ethidium bromide
- Agarose: Bioshop AGA405.1, Burlington, Ontario
- Loading buffer
- 100bp DNA ladder: Fermentas, Cat# SM1143 , Glen Burnie, MD
- TBE buffer
- QIAquick PCR Purification Kit (QIAGEN)

Procedure

DNAs extracted from purchased cNDA clones were used as templates to amplify cDNA fragments desired by PCR. For mouse SOD2, forward 5'-CGC CAC CGA GGA AAG TA-3' and reverse 5'-CAG TCA TAG TGC TGC AAT GC-3' primers generated a 559bp cDNA fragment. For mouse catalase, forward 5'-GCT ATG GAT CAC ACA CCT T-3' and reverse: primer 5' -GTT CAC AGG TAT CTG CAG-3' primers generated a 488bp cDNA fragment. For mouse IGF1, forward 5'–TGG ATG CTC TTC AGT TCG TG-3' and reverse 5'-TCC TGC ACT TCC TCT ACT TGT-3' primers were used to amplify a 318bp cDNA fragment. After optimizing the concentration of primers and templates and the temperature of annealing, 30 cycles of PCR reactions were performed by using 2 X PCR Master Mix. The size of PCR fragments were identified on a 1.5% agarose gel

stained with ethidium bromide under UV light. Then PCR products were purified with the kit.

All PCR reactions were optimized.

SOD2

2XPCR master Mix	25 µl
Forward Primer (0.25µM)	0.625 µl
Reverse Primer (0.25µM)	0.625µl
H2O	19.75 µl
cDNA template (1ng/µl)	4 µl
total	50 µl

95° C – 5 min.
95° C – 30sec.
58° C – 30 sec.
72° C – 30 sec.
72° C – 7 min.
} 30 cycles

Catalase

2XPCR master Mix	25 µl
Forward Primer (1µM)	2.5 µl
Reverse Primer (1µM)	2.5µl
H2O	16 µl
cDNA template (1ng/µl)	4 µl
total	50 µl

95° C – 5 min.
95° C – 30sec.
50° C – 30 sec.
72° C – 30 sec.
72° C – 7 min.
} 30 cycles

IGF1

2XPCR master Mix	25 µl
Forward Primer (0.5µM)	1.25 µl
Reverse Primer (0.5µM)	1.25 µl
H2O	18.5 µl
cDNA template (1ng/µl)	4 µl
total	50 µl

95° C – 5 min.
95° C – 30sec.
60° C – 30 sec.
72° C – 30 sec.
72° C – 7 min.
} 30 cycles

5.2.3 Purify PCR Products

Materials

- PCR products of SOD2, Catalase, and IGF1
- QIAquick PCR Purification Kit, QIAGEN

Procedure (following the instruction of the kit described below)

1.Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix in 1.5 ml tube.

2. Place a QIAquick spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 s.

4. Discard flow-through. Place the QIAquick column back into the same tube.

5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60 s.

6. Discard flow-through and place the QIAquick column back in the same tube.

7. Centrifuge the column for an additional 1 min.

8. Place QIAquick column in a clean 1.5 ml tube.

9. To elute DNA, add 30 µl water to the center of the QIAquick membrane and let the column stand for 1 min, and then centrifuge.

10. Measure the concentration of purified PCR products by using Spectrophotomer (ND-1000).

5.2.4 Ligation

Materials

- pGEM®-T Vector System: Promega, Cat# A1360, Madison, WI
- PCR products

Procedure

A 3:1 molar ratio of the Insert DNA to the vectors was used.

1. Vortex the 2X Rapid Ligation Buffer vigorously before each use.
2. Set up ligation reactions as described below;

Component	SOD2	Catalase	IGF1
2X Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	2 µl	3 µl	2 µl
Water	1 µl	0	1 µl
T4 DNA ligase	1 µl	1 µl	1 µl
Total	10 µl	10 µl	10 µl

Note: Molar ratio of PCR product:vector of 3:1 was used.

3. Mix the reactions by pipetting. Incubate the reactions 2 hour at room temperature.

5.2.5 Transformation

Materials

- Competent cell: Invitrogen, Carlsbad, CA
- pGEM®-T Vector System: Promega, Cat# A1360
- LB plate with ampicillian
- SOC medium

Procedure

1. Measure DNA concentration of ligation reaction and diluted to 10 ng/μl.

2. Add 10 μl (100 ng) of ligation reaction to 50 μl of pre-thawed competent cells on ice and mix gently

3. Place on ice 30 min on ice.

4. Heat shock at 42 °C for 20 sec

5. Return to ice for 2 min

6. Add 950 μl of prewarmed SOD medium.

7. Incubate for 1 hr at 37 ° C with shaking (300rpm).

8. Place 200 μl of transformation culture onto LB plates.

9. Incubate plates overnight at 37 °C.

5.2.6 Screening colonies

DNAs from growing plasmid colonies were used as templates to run PCR reactions. Primers for PCR reactions of SOD2, catalase and IGF1 described above were used to make sure that plasmids of SOD2, catalase and IGF1 were placed in the bacteria colony. PCR profiles were the same as PCR previously described. The size of PCR products was examined by running 2% agarose gel. Six colonies were picked up for the screening of colony for each plasmid. Within 6 colonies, half of one colony was processed with the preparation of colony DNA for PCR. Meanwhile, another half was placed onto the new LB plate and incubated overnight at 37 °C.

Preparation of colony DNA

1. Press sterile loop onto half of the colony and disperse into 30 µl of water.
2. Heat at 95 °C for 5 min.
3. Place on ice for 5 min.
4. Centrifuge at 13000rpm for 3 min.
5. Take 5 µl as the template to run PCR (total volume of PCR reaction is 25 µl).

5.2.7 Mini-Prep of Plasmid DNA for Plasmid Sequencing

After screening colonies, two of colonies from each plasmid were picked to amplify plasmid DNA by doing Mini-prep of plasmid DNA (QIAGEN Plasmid Mini Kit). The procedure was followed as previously described. Then plasmid DNAs were purified by using the kit (QIAquick PCR Purification Kit, QIAGEN) and measure the DNA concentration. To be sure that the plasmid DNAs are in accordance with the mRNAs of interest, Plasmid DNAs were sent to MOBIX lab at McMaster for sequencing. The sequence of each plasmid was confirmed.

5.2.8 Maxi-prep of Plasmid DNA

Materials

- QIA Hispeed Plasmid Maxi Kit
- LB agar plates (containing plasmid of SDO2, catalase, IGF1, and BDNF)

Procedure (follow the instruction of kit described below)

1. Pick a single colony from a plate and inoculate a starter culture 5 ml LB medium containing ampicillian. Incubate for approx. 8 h at 37°C with vigorous shaking (260 rpm).
2. Inoculate 160 ml of LB medium with 600 µl of started culture and grow overnight at 37C with shaking at 260 rpm.
3. Transfer bacterial culture to the 250 ml centrifuge tube and harvest the bacterial cells by centrifugation at 4700 *g* for 15 min at 4°C.
4. Resuspend the bacterial pellet in 10 ml Buffer P1.
5. Add 10 ml Buffer P2 and mix thoroughly by inverting the tube 4–6 times and incubate at room temperature for 5 min
6. Add 10 ml chilled Buffer P3 and mix immediately and thoroughly by inverting the tube 4–6 times.
7. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10 min.
8. Equilibrate a HiSpeed Maxi Tip by applying 10 ml Buffer QBT and allow the column to empty by gravity flow.
9. Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the QIAfilter Maxi Cartridge and filter the cell lysate into the previously equilibrated HiSpeed Tipffer QBT and allow the column to empty by gravity flow.
10. Allow the cleared lysate to enter the resin by gravity flow.
11. Wash the HiSpeed Maxi Tip with 60 ml Buffer QC.

12. Elute DNA with 15 ml Buffer QF.
13. Precipitate DNA by adding 10.5 ml (0.7 volume) of room-temperature isopropanol to the eluted DNA. Mix and incubate at room temperature for 5 min.
14. During the incubation remove the plunger froma 30 ml syringe and attach the QIAprecipitator Maxi Module onto the outlet nozzle.
15. Place the QIAprecipitator over a waste bottle, transfer the eluate/isopropanol mixture into the 30 ml syringe, and insert the plunger. Filter the luate/isopropanol mixture through the QIAprecipitator using constant pressure.
16. Remove the QIAprecipitator from the 30 ml syringe and pull out the plunger. Re-attach the QIAprecipitator and add 2 ml 70% ethanol to the syringe.
17. Wash the DNA by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.
18. Remove the QIAprecipitator from the 30 ml syringe and pull out the plunger. Attach the QIAprecipitator to the 30 ml syringe again, insert the plunger, and dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step.
19. Dry the outlet nozzle of the QIAprecipitator with absorbent paper to prevent ethanol carryover.
20. Remove the plunger from a new 5 ml syringe and attach the QIAprecipitator onto the outlet nozzle.

21. Hold the outlet of the QIAprecipitator over a 1.5 ml collection tube. Add 0.5 ml of water to the 5 ml syringe. Insert the plunger and elute the DNA into the collection tube. Remove the QIAprecipitator from the 5 ml syringe, pull out the plunger and reattach the QIAprecipitator to the 5 ml syringe.
22. Transfer the elute from step above to the 5 ml syringe and elute for a second time into the same 1.5 ml tube using constant pressure.
23. After Maxi-prep plasmid DNA and running 1.5% agar gel to confirm plasmid DNAs, the concentration of plasmid DNA was measured by using Spectrophotomer (ND-1000).

5.2.9 Linearization of Plasmid DNA

Materials

- Plasmid DNA of SOD2, catalase, IGF1, BDNF
- Restriction enzymes (RE) (NEW ENGLAND BioLabs): APAI, PSTI, SacI, SacII

Procedure

Dr. Jane A Foster lab's (McMaster University) protocol was followed as described. APAI and PSTI were use to digest SOD2 and IGF1 plasmids. SacI and SacII were used to digest catalase plasmid. Before digesting big amount of plasmid DNAs (25μg), small amount of plasmid DNAs (1 μg) was digested to make sure that enzymes could cut plasmid DNAs properly.

A. Mini Digest

1. DNA (1 μg)	1 μL
2. RE(10U) (+ 1 μL 1:10 BSA if nec.)	1 μL
3. 10X RE Buffer	1 μL
4. Water	7 μL

5. Mix well, incubate 1-2 hrs at appropriate enzyme temperature.

B. Overnight restriction enzyme digest

Amount of enzyme (glycerol concentration) must be limited to no more than 10% of total volume of digest volume.

1. DNA (room temperature)	25 μg
2. Restriciotn enzymes (5x amount DNA, keep on ice)	125 unit/μl
3. 10X RE Buffer (+ 2 μL 100X BSA if nec.)	20 μl
4. Sterile water	make up to 200μL
5. Flick well, spin and digest at specified temperature	overnight

C. Prepare uncut DNA to run on 1% gel

1. DNA	1 μg
2. 6X Tracking Dye	1 μL
3. H_20	make up to 6 μl
4. Load all	6 μL

D. Prepare cut DNA to run on 1% gel

1. reaction mixture	5 μL
2. 6X Tracking Dye	1 μL
3. Load all	6 μL

E. Load 1 kB ladder 5 μL

F. Run gel and checked for linearization of DNA, if DNA is linearized, its band run slower than the supercoiled circular DNA.

G.Purification of Linearized DNA
(Phenol/chloroform Method)

1. Start with 25 μg DNA digest	200 μl
2. Extract with 1 vol ume P:C:I	200 μl

PCI: phenol:chloroform:isoamyl alcohol (25:24:1)

Flick well and centrifuge 12,000g, 5 min

Transer upper aqueous phase to new tube

Repeat

3. Extract once with 1 volume chloroform 200 µl

   Flick well and centrifuge 12,000g, 2 min

   Transfer upper aqueous phase to new tube

4. Precipitate DNA

   Add 1/10th volume 3M NaOAc (pH 5.2) 20 µl

   Add 2 volumes of 100% ethanol 400 µl

   Flick well, at -20°C >2 h

   Centrifuge 12,000g, 20 min, 4°C

   Carefully pipette off liquid

   Dry in oven (52°C) 10-15 min

5. Resuspend DNA. 10 ul

6. Measure concentration and adjust to 1 µg/µl if possible.

7. Store digested plasmid at 4°C.

Materials

- Linearized cDNA plasmid of SOD2, catalase, IGF1,BDNF
- Transcription Optimized 5X Buffer (Promega, P1181)

- DTT (Promega, P1171)
- rATP, rCTP, rGTP (Promega, P1221)
- T7 RNA Polymerase (Promega,P2075)
- SP6 RNA Polymerase (Promega,P1085)
- RQ1 RNase-Free DNase (Promega,M6101)
- RNasin Ribonuclease Inhibitor (Promega,N2111)
- α-^{35}S-UTP (specific activity >1,000 Ci/mmol; Perkin Elmer)
- ProbeQuant G-50 Micro Columns: GE Healthcare Bio-Sciences Corp, 27-5335-01

Procedure

1. If needed, prepare ΔNTP, final concentration of 2.5 mM each
   a. 10 mM ATP, CTP, GTP 50 µl of each
   b. sterile water 50 µl

2. Mix at 25° C

a. 5X transcription buffer	6 µl
b. 100 mM DTT	3 µl
c. RNAsin (40 U/µl)	0.6 µl
d. ΔNTP	5.5 µl
e. DNA (1 µg/µl)	1 µl
f. ^{35}S UTP (187.5 µCi)	**15** µl
g. T7 or SP6 RNA Polymerase (15-20 U/µl)	1 µl
Total	**32.1** µl

3. Incubate at 37° C 30 min

4. Add 1 µl T7 or SP6 RNA Polymerase 1µl
   Vortex and spin down

5. Incubate at 37° C 30 min

6. Add sterile DEPC H_2O to make up 50µl 14.9 µl

7.. Remove DNA template:

RNAsin	1 µl
RQ1 DNAse	1 µl
Total volume	50.0 µl

8. Incubate at 37° C 10 min

9. Remove unincorporated NTPs:

a. Resuspend resin in column: Vortex (5 Sec)
b. Loosen cap 1/4 turn and snap off bottom
c. Place columns in the empty 1.5 ml eppendorf tube
d. Pre-spin (3000 RPM or 735 g) 1 min
e. Place column in new, (empty) 1.5 ml tube and apply 50µl sample to center of resin bed carefully
f. Spin (3000 RPM or 735 g) 2 min

10. Determine Labeling:
    a. add 5M DTT 1 µl
    b. count 1 µl in 10 ml Cytoscint

11. Calculate Hybridization Mixture:
    Input cpm and # of sections + 20% in Excel Worksheet "Ribo Calcs"

5.3 In situ hybridization Procedure

The procedure included preparation of tissue for hybridization, hybridization, and post-hybridization washing.

Preparation of tissue

1. Fix tissue
    a. 4% formaldehyde in 1x PBS (25° C) 5 min
    b. rinse in 1x PBS

2. Acetylate tissue

a. rinse in TEA-HCl
b. prepare TEA-HCl/acetic anhydride
   acetic anhydride (final conc 0.25%, Sigma A6404) 2.5 ml/1000 ml
   add immediately before use, mix well
c. incubate 10 min
d. rinse in 2X SSC

3. Dehydrate/delipidate tissue
   70% Ethanol 1 min
   80% Ethanol 1 min
   95% Ethanol 1 min
   100% Ethanol 1 min
   chloroform 5 min
   100% Ethanol 1 min
   95% Ethanol 1 min

4. Air dry slides

Hybridization

1. Prepare ribo hybridization buffer (HB) mixture (as determined by ribocalcs)

a. 2X ribo HB	0.5 x total volume
b. add probe to ribo HB	
c. heat denature probe and HB	5 min at 90° C
d. cool in ice	5 min
e. add formamide	0.5 x total volume
f. 10% SDS	0.01 x total volume
g. 10% Na thiosulfate	0.01 x total volume
h. 5 M DTT	0.02 x total volume

2. Apply probe to tissue
   a. line trays with Whatman paper saturated with 4X SSC/50% formamide
   b. put x µl HB mixture/mouse brain sections
   c. put cap of 4X SSC/50% formamide in each tray
   d. incubate tissue overnight 55° C

Post-hybridization Washing

1. Remove coverslips 2X SSC

2. RNase treatment
   a. prepare RNase A (final conc: 20 μg/ml)
      RNase A (20 mg/ml) 1 ml
      RNase buffer 1000 ml
   b. incubate RT 30 min
   c. rinse in RNase buffer 30 min

3. High stringency washes
   a. 2X SSC 1 h at 50° C
   b. 0.2X SSC 1 h at 55°C
   c. 0.2X SSC 1 h at 60°C

4. Dehydrate tissue
   a. 50, 70, 80, 90% EtOH with 0.3 M NH_4OAc 1 min each
   b. 100% EtOH 1 min
   c. air dry slides

5.4 Immunohistochemistry for detection of SOD2, catalase, 8-OHdg, and 3-NT

Sagittal sections (16 um/section, 320 um apart throughout the hippocampus) collected were used.

Materials

- 1X PBS, pH 7.4

a.	10X PBS, pH 7.4	200 ml
b.	dH_2O	1800 ml
	Total	2000 ml

- 1X PBS, 0.1% Triton-X

a.	10X PBS, pH 7.4	200 ml
b.	10% Triton-X	20 ml
c.	dH_2O	1780 ml
	Total	2000 ml

- Blocking solution

a.	Normal goat serum (Vector SP-5020)	10 ml
b.	1X PBS, 0.1% Triton-X	90 ml
	Total	100 ml

- Primary antibody solution (diluted in blocking solution)
  rabbit anti-mouse SOD2 (abcam, 1:600 dilution),
  rabbit anti-mouse catalase (abcam, 1:1000 dilution),
  rabbit anti-mouse 8ohdg (Secrotec,1:400 dilution)
  rabbit anti-mouse NT (Upstate, 1:200)

- H_2O_2/methanol (make freshly)

30% H_2O_2 (stock liquid)	333 µl
methanol	100 ml

- Secondary antibody (diluted in blocking solution)
  biotinylated goat anti-rabbit IgG (Jackson lab, 1:500 dilution)

- Vectastain Elite ABC Kit (Vector Lab, Cat# PK-6100)

1X PBS	5 ml
Solution A	2 drops
Mix by inverting.	
Solution B	2 drops
Mix by inverting.	
Let stand for a minimum of 30 minutes.	

DAB Kit (make immediately before use): Vector lab, Cat# SK-4100

dH_2O	5 ml
Buffer solution	1 drop
Mix by inverting.	
DAB reagent	2 drops
Mix by inverting.	
H_2O_2	1 drop
Mix by inverting.	

Procedure

1. Transfer slides to staining jars.

2. Rinse in 1XPBS 5 min

3. Rinse in 1XPBS/0.1% TritonX-100 5 min

4. Rinse in 1XPBS 5 min

5. Incubate in blocking solution 1 h, RT

6. Incubate in primary antibody, overnight at 4°C

7. Rinse slides in PBS.

8. Quench in 0.1% H_2O_2 in 100% methanol 30 min

9. Wash in PBS. 3 x 5 min

10. Incubate in secondary antibody, 200 µl /slide, in humid chamber. 1hr

Note: make Vectastain (must sit at room temperature for 30 min before use)

11. Wash in PBS. 3 x 5 min

12. Incubate in Vectastain, 200 µl per slide. 1 hr

13. Wash in PBS. 3 x 5 min

14. Incubate in DAB colour reagent. Time Varies

15. Rinse in tap water for 5 minutes.

16. Dehydrate tissue.

    a. 50, 70,70% ethanol 30 s each

    b. 90, 90, 100, 100% ethanol 2 min each

17. Coverslipping

    a. Xylene 2 x 5 min

    b. Mount with permount.