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THE ROLE OF DIETARY ZINC IN THE ADULT RAT LIMBIC SYSTEM:

FROM GENES TO BEHAVIOR

By

NADINE M. TASSABEHJI

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This dissertation is dedicated to my mother Oula and husband Tarek, for their constant support and for being a true inspiration.

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ABSTRACT

Recent work has identified stem cells in the central nervous system that are capable of proliferating into adulthood. While adult neuronal stem cells hold a great deal of therapeutic potential, to be a useful clinical tool, we must first understand the cellular and molecular mechanisms responsible for regulating proliferation and differentiation. Two month old male Sprague-Dawley rats were given one of three dietary treatments. Following 3 wks of zinc-adequate (ZA, 30 ppm), zinc-deficient (ZD, 1 ppm), pair-fed (PF, 30 ppm), or zinc-supplemented (ZS, 180 ppm) diets, immunohistochemistry was used to quantify the number of Ki67 positive cells as a measure of newly proliferated cells in the dentate gyrus. Neurogenesis was determined by the co-localization of the neuronal marker, NeuN, with Ki67. ZD reduced the number of Ki67 positive cells to 50% of ZA controls ($p<0.05$) in both the subgranular zone (SGZ) and the granular cell layer (GCL) of the dentate gyrus. While ZD reduced the total number of cells that co-labeled with Ki67 and NeuN compared to ZS rats, dietary zinc did not alter the percentage of Ki67-positive cells that expressed NeuN. Because impairment in stem cell proliferation has been linked to alterations in mood, we hypothesized that ZD would lead to the development of depression-like behaviors in rodents. Consistent with depression ZD rats displayed anorexia ($p<0.006$), anhedonia (reduced saccharin: water intake, $p<0.001$), and anxiety-like behavior in a light-dark box test ($p<0.05$). Furthermore, the antidepressant drug fluoxetine (10 mg/kg body wt), reduced behavioral despair, as measured by the forced swim test, in ZA and ZS rats ($p<0.002$), but not in ZD rats. Thus, it appears that ZD not only induces depression, but also impairs the efficacy of antidepressant drugs. In an attempt to identify some of the molecular mechanisms that may be contributing to the impairment of drug efficacy in ZD rats, microarray analysis was performed to identify alterations in hippocampal gene expression following chronic fluoxetine administration in ZA and ZD rats. This work showed that ZD disrupts mitochondrial gene expression as well as important mediators of neurogenesis such as the receptor for transforming growth factor beta (TGF-β) and β-carotene 15,15 dioxygenase. Together these studies suggest that ZD-mediated changes in gene expression are responsible for a reduction in

dentate stem cell proliferation, leading to the development of depression-like behaviors that are refractory to antidepressant treatment.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Background

Zinc

Zinc is an important trace metal for many biological functions. Recommended dietary allowances for zinc are shown in Table 1.1. Zinc is found in whole grains, meat, poultry, shellfish, dairy products, nuts and legumes. Functions of zinc are divided into three main categories, catalytic, structural, and regulatory. Main catalytic functions include enzymatic roles. There are at least 80 different mammalian enzymes that require zinc (1). For example, zinc is essential for the activity of DNA and RNA polymerases. Others enzymes include copper-zinc superoxide dismuatse, alkaline phosphatase, and carboxypeptidases. Many of the enzymes in intermediary metabolism including lactate dehydrogenase, pyruvate carboxylase, and alcohol dehydrogenase, all require zinc. In addition to the enzymatic functions of zinc, zinc is also a structural component of many non-enzymatic proteins known as zinc finger proteins. These proteins use cysteine and histidine to bind zinc. The Cys_2-His_2 or Cys_2-Cys_2 motif results in protein folding consistent with the formation of a "finger-like" domain (2). Nuclear hormonal receptors that contain zinc fingers include receptors for estrogen, testosterone, retinoic acid and vitamin D. In addition to these protein-DNA interactions, zinc finger proteins have also been shown to participate in protein-RNA and protein-protein interactions (3). These transcription factors, and many others, are responsible for the main regulatory functions of zinc.

Zinc Deficiency. Unfortunately, dietary zinc deficiency is a worldwide problem. A number of studies have indicated that there is high prevalence of zinc deficiency in countries such as Ethiopia (4), Iran (5), Indonesia (6), and India (7). Furthermore, it is not limited to developing countries. Zinc deficiency has been reported in Europe (8) and in the United States in all age groups including pre-school children (9), adolescents (10), the elderly (11), and in pregnant women (12). There is also evidence that athletes may be at risk for mild or moderate zinc deficiency (13). Main causes of zinc deficiency are consumption of low zinc foods, or foods high in phytates (14, 15) and fiber (16, 17) that inhibit zinc retention. Moreover, certain illnesses

that impair food intake, cause catabolism or malabsorption, or increase zinc excretion can lead to zinc deficiency. Additionally, severe zinc deficiency is seen in a genetic state known as Acrodermatitis Enteropathica (AE). AE is a rare autosomal recessive disorder that is characterized by a defect in ZnT4 transporter, resulting in impaired uptake and transport of zinc. Patients with AE suffer from slow growth and development, delayed sexual maturation, skin rashes, severe diarrhea, immune system deficiencies, impaired wound healing, diminished appetite, night blindness, swelling and clouding of the cornea, and behavioral disturbances. AE has also been shown to be complicated by depression (18). Oral zinc supplementation results in complete remission of symptoms (19, 20).

Zinc deficiency in humans has been linked to a variety of behavioral and physiological abnormalities. Dietary zinc deficiency has been reported to cause decreased food intake that leads to decrease in body weight gain (21, 22) and eventually anorexia, suppressed immune function that increases vulnerability to a wide range of viral, bacterial and fungal infections. Impaired/retarded growth (21, 23), skin lesions in body orifices and extremities, dysphoria, hypogonadism, geophagia, abnormal taste acuity, and cognitive impairment are other common symptoms of zinc deficiency (24, 25). Severe zinc deficiency in adults can result in balance and gait disturbances and neuropsychological disorders including, paranoia, and hallucinations (26). Deficiency has also been linked to learning and memory deficits (27), depression (28, 29), and schizophrenia (30). Low zinc levels have been correlated with reduced cognitive functions. For example, men with low zinc intakes had faster but less accurate performance on a memory digit task (27). Furthermore, there was a positive correlation between hair zinc level and reading ability in elementary school children in Baltimore (31), and low serum zinc was associated with cerebellar dysfunction, and taste and smell dysfunction (26). Moreover, in Egyptian infants a relationship was found between mothers' consumption of foods high in zinc and infants' attention measured by Brazelton Neonatal Development Assessment Scales administered soon after birth (32).

Different animal models have been able to show similar adverse effects of zinc deficiency. Zinc deficiency during development impaired learning and memory (33-37). Effects of zinc deficiency during brain development are not reversible after ad libitum feedings, and offspring exhibit poor performance on shock-induced learning tasks (38, 39). Some have shown that zinc deficiency had effects similar to protein-caloric malnourishment especially during

periods of rapid brain development. For example, severe zinc deficiency during development resulted in altered emotionality, decreased learning, reduced attention span, lethargy and impaired memory (40, 41). Zinc deficiency in early infancy, including prenatal and postnatal rats, led to significantly retarded development of long term memory, however this was not seen in calorically restricted animals and is therefore attributed to zinc deficiency (42). In Rhesus monkeys, zinc deficiency decreased exploration (34), and moderate zinc deficiency reduced short-term memory and task performance (43). In adult rats moderate zinc deficiency reduced cognitive performance (44).

Zinc in the brain. After iron, the highest metal in the brain is zinc. Zinc in the brain increases with growth and development and is needed for myelination, synthesis and release of neurotransmitters, fat metabolism in neurons (ω6 FA), zinc dependent enzymes, and transcription factors. There are two pools of zinc in the brain, free or vesicular zinc, and protein bound zinc. Free zinc is termed "labile" zinc because it can bind to metal chelators. This includes vesicular zinc, ionic zinc in the cytosol, and zinc in the interstitial fluid in brain tissue. Five to ten percent of brain zinc is considered histochemically reactive and is found in synaptic vesicles of a subset of glutamatergic neurons (45). As a result, these neurons have been classified as zinc containing neurons or zinc-ergic, and once zinc is delivered to the axon terminal, it is concentrated into synaptic vesicles with glutamate and becomes histochemically reactive (46). Zinc in synaptic vesicles has been shown to modulate γ-aminobutysic acid (GABA) and N-methyl-D-aspartate (NMDA) receptors. Zinc-containing vesicles are located in the cerebral cortex, amygdalar nuclei, olfactory bulbs and the hippocampal formation (46-48).

Due to high zinc in the hippocampus and related areas it seems that the hippocampal formation may be the most responsive to dietary zinc deprivation in the adult brain (49). Twelve weeks of a zinc depleted diet decreased zinc concentrations in the rat hippocampal mossy fibers by 30% (50). Several studies have studied the cognitive behavioral outcomes related to zinc deficiency (35, 37, 51-54). Zinc deficiency impaired attention tasks (orienting and misdirection), perceptual task (search-count), three memory tasks (letter, shape, cube recognition) and spatial tasks (maze) (55). In rats, zinc deficiency impaired replication of granular cells of the cerebellum (56) retarded extension of neural dendrites (57) and decreased weight and maturity of cerebella (41). In rodents zinc deficiency produces abnormal electrophysiological functions in the hippocampus (58). Maternal zinc consumption can affect fetal brain development and

behavioral outcomes (37). In rats zinc depletion during the latter part of pregnancy led to decreased body and brain size (37). Zinc deficiency from birth untill 21 days of age resulted in impaired growth, decreased brain size, diminished brain DNA, RNA and protein, and reduced cerebellar lipid concentrations compared to control animals (37). Buel et al., has also shown decreased total DNA, and RNA in zinc deficiency animals (41). Indeed, linear growth is sensitive to zinc deprivation. Age groups experiencing rapid growth are most susceptible to zinc deficiency or tissues that require continuous cell replication such as skin, and the immune system.

The roles of zinc in neurological function are reinforced by data showing that zinc supplementation improves brain development and cognitive functions. In Chinese children, zinc supplementation along with an adequate micronutrient state, resulted in significant improvements in recognition memory, reasoning, attention, perception, and psychomotor function when compared to children receiving a micronutrient supplement alone (59-61). Zinc supplementation increased growth velocity (62), improved height and weight velocity (Brown et al., 1998), and enhanced brain development in Indian (63) and Guatemalan children (64). One week of zinc supplementation improved IQ scores from 89-99 (65). Zinc repletion also has beneficial effects on activity levels in children (63). Benefits of zinc repletion can be seen as early as during pregnancy. Zinc supplementation during pregnancy increased body weight, head circumference and length of the babies after birth (66).

Hypotheses

In conclusion, it is well known that zinc is required for a variety of molecular and cellular functions that are essential for the regulation of gene expression of cellular proliferation, and differentiation. Thus, I have hypothesized that dietary zinc will alter the proliferation of stem cells in the SGZ of dentate gyrus in the adult rat (Figure 1.1). This hypothesis has implications for the development and treatment of depression and depression-related disorders. Not only has zinc deficiency been linked to major depression, but we now know that the efficacy of antidepressant drugs is dependent on stem cell proliferation and neurogenesis in the dentate.

The first part of this dissertation will explore the role of different levels of dietary zinc in hippocampal stem cell proliferation and study the efficacy of the selective serotonin reuptake

inhibitor (SSRI) fluoxetine in zinc adequate, deficient and supplemented rats. The third part of this dissertation will use microarray technology to identify genes that are regulated by zinc deficiency and fluoxetine in two areas of the limbic system, the hippocampus and the olfactory bulb.

Table 1.1 Recommended Dietary Allowances (RDA)* for zinc

	Female	Male
Infants (7 month – 3 yrs)	3 mg/day	3 mg/day
4 to 8 years	5 mg/day	5 mg/day
9 to 13 years	8 mg/day	8 mg/day
Adults (>19 years)	8 mg/day	11 mg/day
Pregnancy	11 mg/day	-
Lactation	12 mg/day	-

* mg/kg

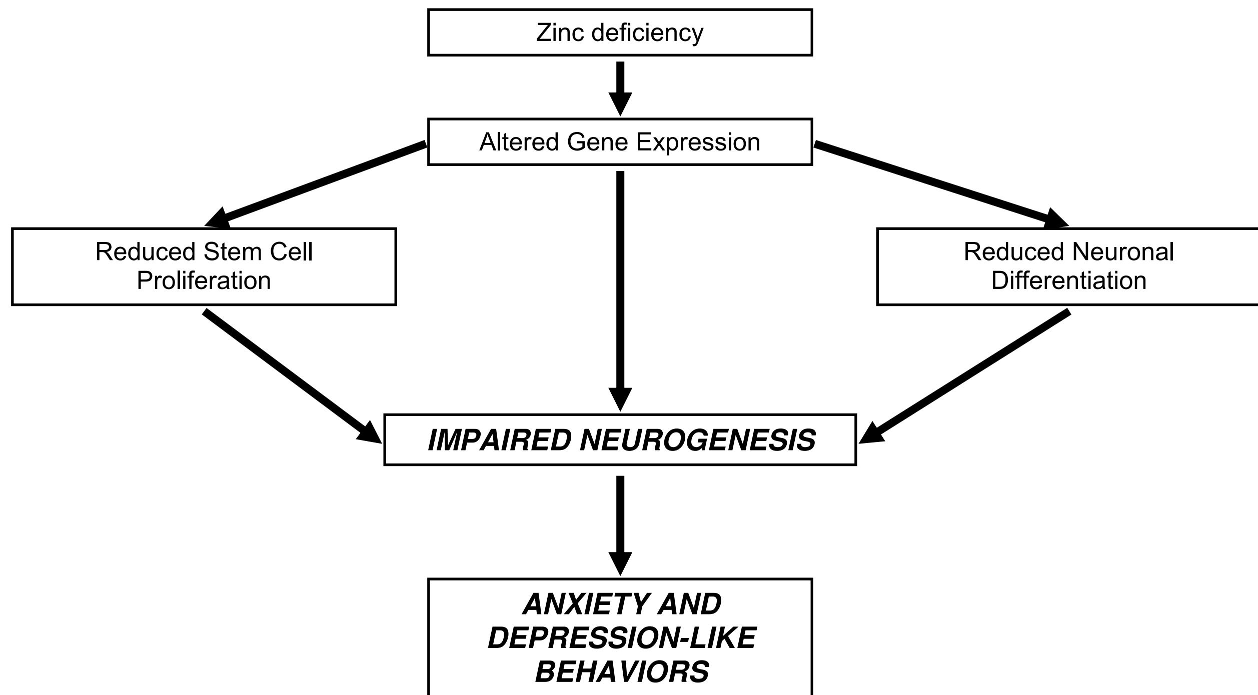

Figure 1.1. Dissertation hypothesis for the role of zinc in neurogenesis & depression.

CHAPTER 2: ZINC REGULATION OF STEM CELL PROLIFERATION AND DIFFERENTIATION

Introduction

Neurogenesis

In 1998 Fred Gage and coworkers confirmed the presence of stem cells in the adult human brain that are capable of differentiation into neurons (67). The finding that adult stem cells are capable of participating in neurogenesis is an exciting development with implications for a variety of conditions including brain and spinal cord injury, stroke, Parkinson's and Alzheimer's diseases, and depression and depression-related disorders. Alterations in neurogenesis can occur in two ways, either by regulating the rate of cell proliferation and/or regulating the number of new neurons that survive. For new cells to be functional and become part of the brain circuitry they have to differentiate and form synapses. The process of neurogenesis, which includes proliferation, survival, migration and differentiation, can be regulated by a variety of stimuli (68). Unfortunately, we do not yet understand the mechanisms that control these processes in the adult central nervous system (CNS).

While we have known for some time that neurogenesis takes place in the adult rodent and non-human primate CNS, Gage's report was the first to definitively show, using 4-bromo-2'-deoxyuridine (BrdU) labeling, that neurogenesis takes place in adult humans. These progenitor cells are intermediate daughter cells of stem cells that can only divide for a limited number of times and only give rise to a limited variety of cell types. Neuronal stem cells are cells that can 1) generate neural tissue, 2) are capable of self-renewal and 3) can give rise to cells other than themselves through asymmetric cell division (69, 70). New cells continue to be born in the olfactory bulb (OB), amygdala, dentate gyrus of the hippocampus, and some cortical areas (71, 72). Furthermore, Gage's work showed that adult human stem cell proliferation and neurogenesis occur in both the subventricular zone (SVZ) of the anterior lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, the same regions that were previously

identified in animals. Stem cells in the SGZ of the dentate generate new neurons in the granular cell region of the hippocampus, while stem cells in the SVZ travel through the rostral migratory stream and give rise to granule cells in the olfactory bulb and to neurons in the subcortical forebrain (73). Adult hippocampal neurogenesis has been demonstrated in many different species, rats (67, 74), guinea-pigs (75), monkeys (76), rabbits (77), and humans (67). Neurogenesis can be regulated by several environmental and pharmacological agents. It is increased by estrogen (78-80), caloric restriction (81), exercise, enriched environment (82), and various antidepressants (74, 83-85). Decreased neurogenesis is most commonly seen in different models of stress (86), and with aging (87, 88). The role of zinc in this regulation has not been explored.

The Hippocampus.

The hippocampus is an oval structure located in the medial temporal lobe. It can be divided into five different areas. The dentate gyrus is the dense dark layer of cells in the tip of the hippocampus. From the dentate there are a series of structures, CA1, CA2, and CA3. CA stands for cornu ammonis for the horn shape of these areas. These areas are more diffuse and it is hard to distinguish between them. However, CA1 is the furthest away from the dentate gyrus and CA3 is the closest. The subiculum is located at the base of the hippocampus, and is continuous with the entorhinal cortex. Information flows in one direction. Information enters the hippocampus from the subiculum to the dentate. This tract is called the perforant path. Entorhinal axons synapse on cells in the dentate, dentate neurons then send axons to the CA3, called the mossy fibers. CA3 then sends axons called Schaeffer collaterals to the CA1, which sends fibers to the subiculum. The subiculum is then responsible for output of information from the hippocampus.

The hippocampal formation is most often associated with control of learning and memory. It is involved in episodic, declarative, and spatial learning and memory, but is only critical for declarative memory. Declarative memory is composed of all facts, figures, and names we learn. All of the experiences and conscious memories fall in this category. Damage to the hippocampus will only damage formation of new declarative memories. The formation of memories appears to involve long term potentiation (LTP). The dentate gyrus encodes information to make it usable by CA3 and receives input from many brain regions. Many neurotransmitter systems have terminals in the SGZ. Input from these different brain regions is

‘tagged’ for later storage as part of the ‘consolidation’ process. Information is ‘tagged’ with both temporo-spatial coordinates as well as emotional labels. The latter dictates the priority of memorizing information and temporo-spatial information is the basis of episodic memory. Therefore, the hippocampus permits the storage of memory based on emotional tags, and makes later association possible. The hippocampus provides inputs from other brain regions, including the prefrontal cortex, hypothalamus, cingulate cortex, and amygdala that contribute to altered mood and emotions.

Output of emotional expression is sent to the cerebral cortex, then the cingulate cortex, and eventually to the hippocampus. Once in the hippocampus, information is combined, organized, and sent from the hippocampus to the mammillary bodies that return the information back to the hypothalamus. Also, information from the hippocampus is relayed to the amygdala for portrayal of emotional behavior. Thus, the hippocampus is one region of the brain that has been extensively studied with regards to stress, depression, and antidepressant action. In fact, the hippocampus, and more specifically the CA3, has the highest level of glucocorticoid receptors, and is therefore more sensitive to damage from stress (89). There are two kinds of glucocorticoid receptors; mineralcorticoid (MR), and glucocorticoid (GR) receptors. MR are the most common in the limbic system specifically the hippocampus (90). Furthermore, MR have a higher affinity for corticosterone than GR. Stress hormones produce significant damage to the hippocampus through these receptors. For example, CA3 pyramidal cells revealed significant atrophy when exposed to 3 weeks of high corticosterone doses (91).

Zinc in the hippocampus. I have hypothesized that dietary zinc regulates neurogenesis leading to specific behaviors. The second highest levels of zinc in the brain of neonatal rats are in the hippocampus (92) and in adult animals zinc is highest in the hippocampus (36, 49, 92-94). Heaviest levels of zinc deposits in the hippocampus were in the third week of life (41). Specifically in the hippocampus, levels are highest in the CA3 and dentate gyrus (49, 92, 93). Zinc deficiency leads to impairment of LTP in the CA3 (95, 96). Using a zinc chelator, LTP was significantly impaired. Moreover, zinc containing neurons are associated with episodic memory function and are important for behavior and emotional expression (33). As discussed in chapter 1, there is a strong correlation between zinc deficiency and cognitive and behavioral impairment in animal models and humans. This could be due in part to decreased growth, DNA, RNA, and protein concentration changes seen in zinc deficiency (41). There is also a direct link between the

hippocampus and zinc deficiency. Zinc deficienct animals had smaller hippocampus and less protein in the hippocampus (41).

As introduced in chapter 1, free zinc is predominately localized to the presynaptic vesicles of a subset of glutamatergic neurons (45, 97). This zinc has also been co-localized with glycine and GABA in the mouse (98, 99). Regions rich in "zincergic" neurons include the mossy fibers of the hippocampus, the amygdala, and the olfactory bulb. Free zinc-containing neurons are also abundant in the cortex (100). Zinc has been shown to modulate three different kinds of postsynaptic receptors, γ-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA), and α-amino-3-hydroxyl-5-methyl-14-isoxazolepropionic acid (AMPA) (100). Depolarization and calcium influx result in the release of zinc into the synaptic cleft (101) where its primary function appears to be the modulation of both ionotropic and metabotropic post-synaptic receptors that have specific zinc-binding sites. For example, zinc inhibits $GABA_A$ receptors, reducing their inhibitory action (102, 103). Endogenous zinc inhibited GABA in the CA3 of the hippocampus, and this was reversed by treatment with a zinc chelator (99). Zinc also inhibits the NMDA subtype of the glutamate receptor (102, 104). Furthermore, it appears that zinc modulates the activity of at least a subset of AMPA receptors and that this effect may be cell specific (105-107). There is also evidence that zinc can potentiate glycine-mediated currents (108), regulate voltage gated-calcium channels (109), as well as potassium, sodium, and chloride channels (110), and act as an inhibitory neuromodulator of glutamate release (111, 112).

During pathological conditions such as ischemia, after seizures, or head trauma, zinc is released at glutamatergic synapses in high concentrations (113-116). Released zinc can permeate through NMDA channels, voltage sensitive calcium channels (VSCC) or Ca^{2+}-AMPA/kainate channels (Ca-A/K) into neurons (117-119). However, Ca-A/K channels appear to be most permeable (118). While some zinc manages to be taken back into the bouton and recycled into vesicles (100), the subsequent rise in post-synaptic intracellular zinc is a key factor in neuronal death linked to these syndromes (116, 120, 121) suggesting that the release of pre-synaptic vesicular zinc is responsible for the damage and death. This zinc-mediated neuronal death is the result of a combination of apoptosis and necrosis (122, 123) that can be prevented by zinc chelation (121). There is also evidence that zinc can contribute to neurodegenerative disorders such as Parkinson's disease, some forms of Amyolateral Lateral Sclerosis, and Alzheimer's disease (124). One explanation for the increased neuronal death could be due to the generation of

reactive oxygen species (ROS) by increases in zinc concentration (125). There is also evidence that post-synaptic zinc accumulation leads to the inhibition of NADH dehydrogenase resulting in disruption of energy metabolism and cell death (126).

Zinc and neurogenesis.

Zinc is an essential nutrient that is required for many physiological functions, including growth, immunity, reproduction, and metabolism. The physiological and biochemical implications of zinc deficiency are significant. Zinc is needed for the catalytic activity of over 80 enzymes including DNA and RNA polymerases, enzymes that are clearly critical for cellular replication and gene expression (127). It is also needed for the structural integrity of hundreds of zinc finger transcription factors (128, 129). The importance of these DNA-binding proteins is underscored by the fact that approximately 1% of the entire human genome codes for zinc finger proteins and as many as 220 proteins with over 1300 zinc-finger motifs have been identified. These proteins require the co-ordination of zinc for their secondary structure and function (130). Some of the most ubiquitous and important nuclear proteins are known to utilize zinc-finger motifs for protein-protein and protein-DNA interactions including glucocorticoid and thyroid hormone receptors, Sp1, and cAMP response-element binding proteins (130-133). Thus, it is not surprising that zinc is an essential nutrient for almost all aspects of nutrient metabolism, gene expression, and cellular function.

Neurogenesis is dependent on many growth factors for the growth and maintenance of newly proliferated cells. For example, murine glial cell-derived neurotrophic factor inducible transcription factor (MGIF) is a zinc finger protein (134). Moreover, neuronal differentiation requires the action of retinoic acid (RA), which is mediated by the DNA-binding nuclear receptors, RAR and RXR (135). These receptors, and all of their numerous isoforms, are zinc finger proteins. Zhep, a transcription factor needed for neural development, has multiple zinc fingers (136). Thus, we propose that zinc plays a role in adult stem cell proliferation and neurogenesis because there is strong evidence that it is involved in the mechanisms that direct both cellular proliferation and differentiation.

Materials and Methods

Animal Care. Two month old male Sprague-Dawley rats (n=20) (Charles Rivers Laboratories, Wilmington, MA) were individually housed in temperature controlled, rooms with a 12-hour light-dark cycle. Rats were fed a commercially prepared (Research Diets Inc., New Brunswick, NJ) ZA diet (30 ppm) for 3 days. After this baseline period, rats were fed a ZA (ZA, 30 ppm), zinc deficient (ZD, 1 ppm), or zinc supplemented (ZS, 180 ppm) diet for a period of 3 weeks (Table 2.1). Because zinc deficiency is known to cause anorexia, an additional group of rats was pair-fed to the ZD rats. Pair-fed rats were provided with the weighed amount of ZA food eaten by the ZD rats on the previous day. Body weight, food intake, and water intake were monitored daily.

Table 2.1. Description of the different dietary treatments.

Diet	Zinc Concentration	Description
Adequate	30 ppm	Control diet
Deficient	1 ppm	Zinc restriction
Pair Fed	30 ppm	Control for food intake at 1 ppm
Supplemented	180 ppm	Dietary supplementation

Brain tissue collection. After the last day of the dietary treatment, rats were anesthetized and perfused via the left cardiac ventricle with 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were post-fixed in 4% paraformaldehyde, followed by 30% sucrose overnight and sectioned at 40 micron thick using a cryostat (HM 440E; Microm, Walldorf, Germany) from approximately Bregma -3.8 to Bregma -5.0 (Figure 2.1) to obtain slices containing the hippocampus and dentate gyrus.

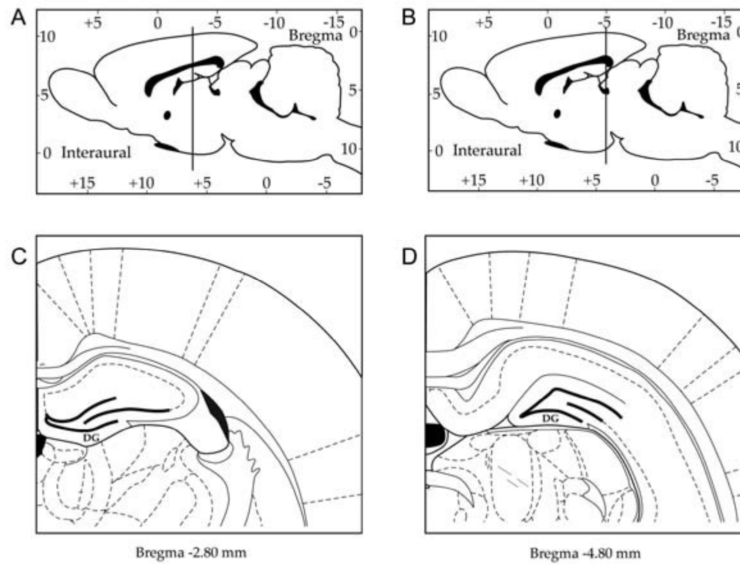

Figure 2.1. Schematic localization for brain sectioning. A: brain sectioning starts at Bregma -3.8, B: sectioning terminates at Bregma -5.0, C: morphology of the dentate gyrus at Bregma -3.8, D: morphology of the dentate gyrus at Bregma -5.0.

Immunohistochemistry. Tissue sections were washed three times with PBS, and then treated with 10 mM citrate buffer, permeabilized with Triton X-100, and blocked with 10 mg/mL bovine serum albumin (BSA) in PBS. They were then incubated with a rabbit anti-rat Ki-67 monoclonal antibody (1:750, Biomeda, Foster City, CA) and a mouse monoclonal anti-neuronal nuclei (NeuN) (Chemicon, Temecula, CA), used to identify neurons, at 4º C overnight with gentle shaking (137, 138) (Table 2.2). Ki67 is a nuclear protein that is produced during late G_1-, S-, M-, and G_2-phases of the cell cycle. Cells in the G_0 (quiescent) phase are negative for this protein. Ki67 antibody was used to identify proliferating cells. After washes with PBS, slices were incubated with anti-rabbit and anti-mouse secondary antibodies labeled with the fluorescent dyes Cy3 or Cy2 (Jackson ImmunoResearch, West Grove, PA) at 4ºC overnight with gentle shaking. Sections were then analyzed by confocal microscopy (Zeiss LSM 510) and newly proliferated cells were quantified by Metamorph software, version 4.5 (Universal Imaging Corp., Westchester, PA) as previously described (139).

Table 2.2. Antibodies for immunological identification of cell phenotype.

Label	Antibodies	Specificity	Source
Neurons	NeuN	Neuronal nuclei	Chemicon
All nuclei	DAPI	Nuclear DNA	Sigma Aldrich
Mitosis nuclear protein	Ki67	Newly proliferated cells	Roche

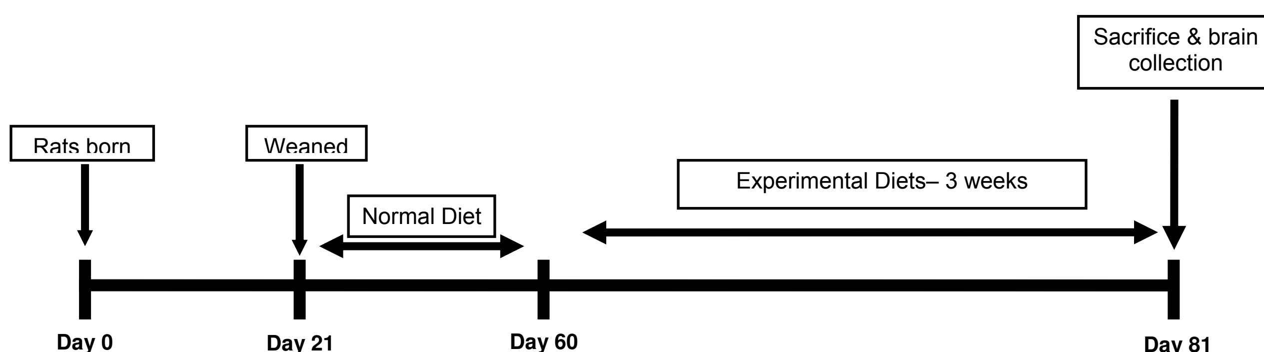

Figure 2.2. Experimental outline for animal sacrifice.

Results

Zinc and cellular proliferation. Figure 2.3 shows the effect of dietary zinc on cellular proliferation (Ki67) and neuronal differentiation (NeuN). Quantification of Ki67 cells revealed 50% ($p<0.05$) fewer labeled cells in ZD (21.7 ± 10.8 cells per section) rats compared to control (43.0 ± 7.2 cells per section) animals in the granular cell layer (Figure 2.5). However, there were no significant differences between ZA, PF, and ZS dietary groups. There were similar patterns, a 50% decrease between ZD and ZA, in Ki67 labeling in the subgranular layer of the hippocampus (Figure 2.4). The percentage of Ki67 positive cells in the GCL (GCL/GCL+SGZ) was approximately 59% in all dietary groups, and did not show differences between groups.

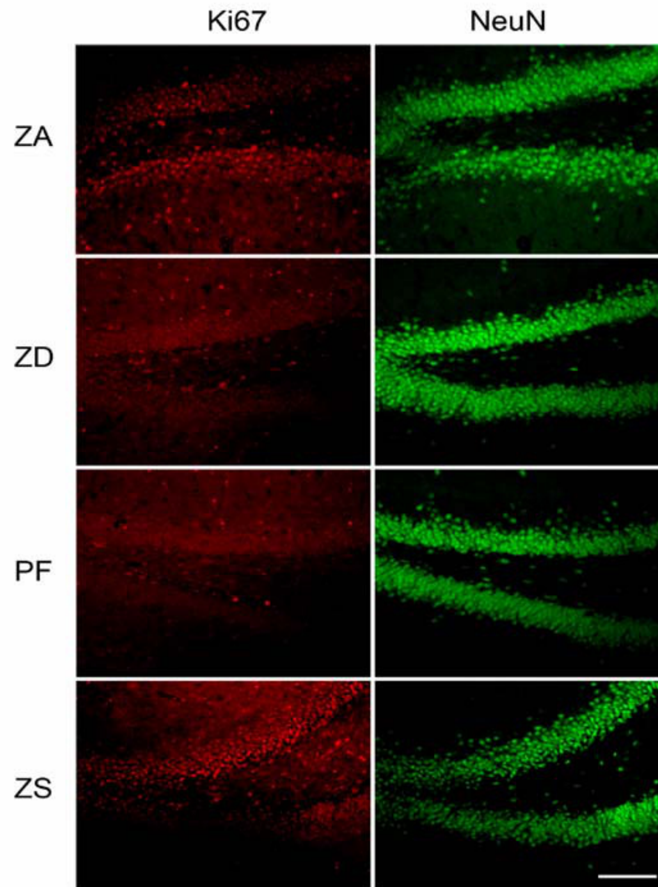

Figure 2.3. ZD decreases the number of Ki67 labeled cells in the dentate gyrus of the hippocampus. The left column of images shows the immunohistochemical localization of Ki67 used to identify newly proliferated cells in hippocampal tissue samples of rats receiving ZA, ZD, PF, or ZS diets for 21 days. The right column of images shows the immunohistochemical localization of NeuN used to label neurons. Photomicrographs are representative of images from adult Sprague-Dawley rats at original magnification 20x, scale bar at 150μM.

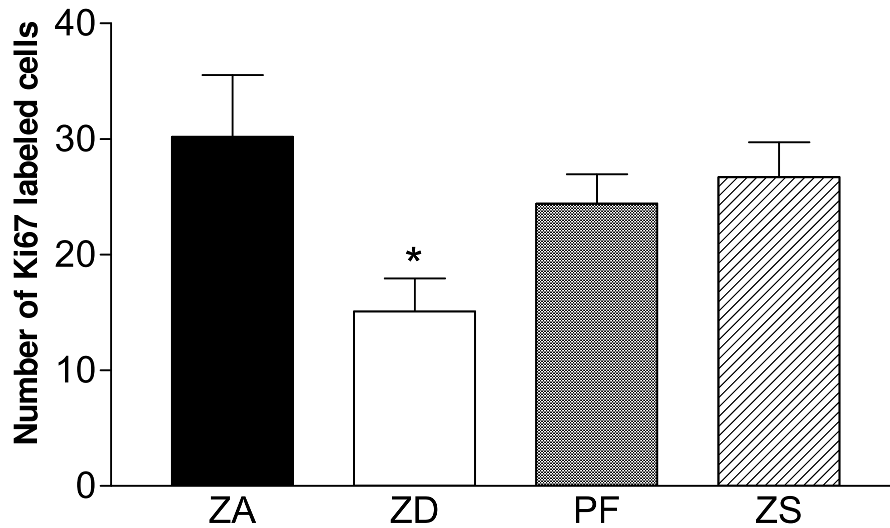

Figure 2.4. ZD decreases the number of Ki67 labeled cells in the subgranular zone of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells in the subgranular zone of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 labeled cells. Bars represent mean±SD number of Ki67 labeled cells per brain section. *Significantly different from control (ZA) $p<0.05$.

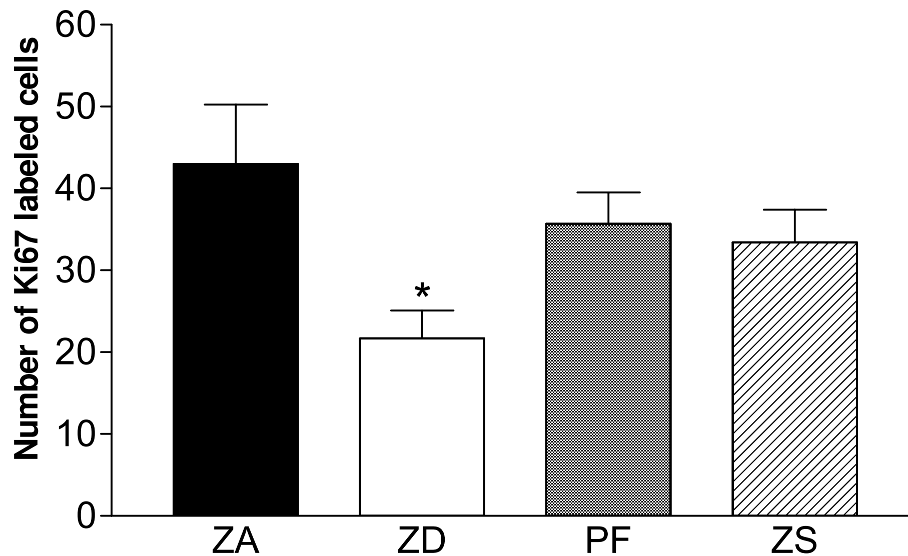

Figure 2.5. ZD decreases the number of Ki67 labeled cells in the granular cell layer of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells in the granular cell layer of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 labeled cells. Bars represent mean±SD number of Ki67 labeled cells per brain section. *Significantly different from control (ZA) $p < 0.05$.

Zinc and Cellular Differentiation. Figures 2.6 and 2.7 show the effect of dietary zinc on neuronal differentiation (NeuN) in newly proliferated cells (Ki67 positive). Quantification revealed that ZD reduced the number of Ki67 and NeuN double labeled cells compared to ZS animals in both the SGZ and GCL (Figure 2.8 and 2.9). However, when the double-labeled cells were expressed as a percent of Ki67-positive cells, no differences were seen among the dietary groups (Figure 2.10). There were similar patterns in the percent of Ki67-labeled cells that co-labeled with NeuN in the subgranular layer of the hippocampus (Figure 2.11).

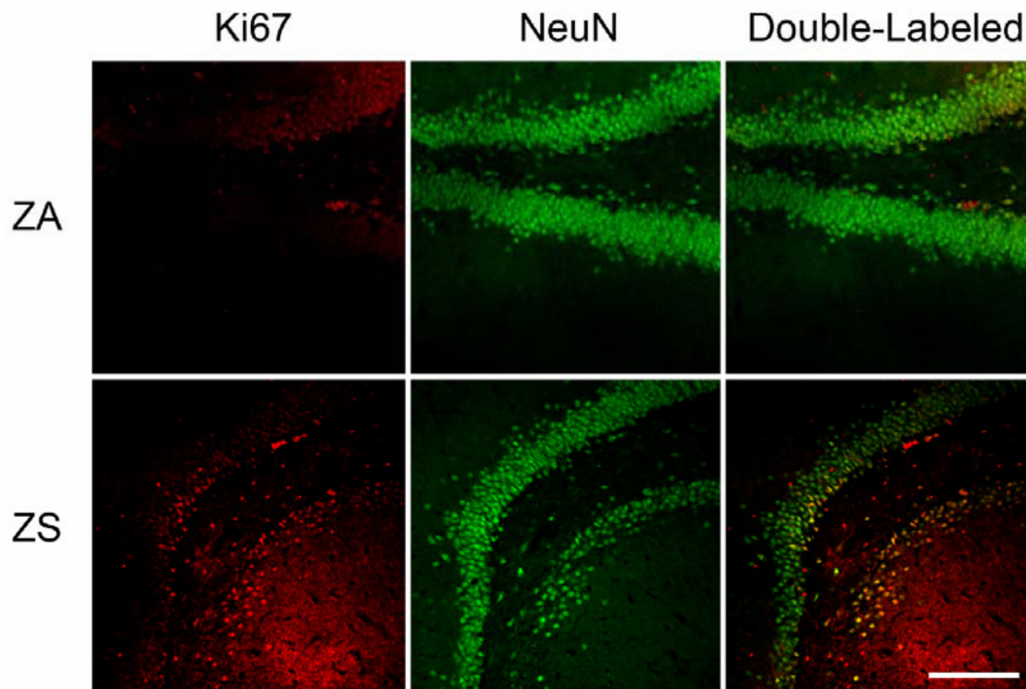

Figure 2.6. Effect of zinc supplementation on the number of Ki67 + NeuN labeled cells in the dentate gyrus of the hippocampus. The left column of images shows the immunohistochemical localization of Ki67 used to identify newly proliferated cells in hippocampal tissue samples of rats receiving ZA, or ZS diets for 21 days. The middle column of images shows the immunohistochemical localization of NeuN used to label for neurons in hippocampal tissue samples. The right column shows double-labeled cells. Photomicrographs are representative of images from adult Sprague-Dawley rats at original magnification 20x, scale bar 150µM.

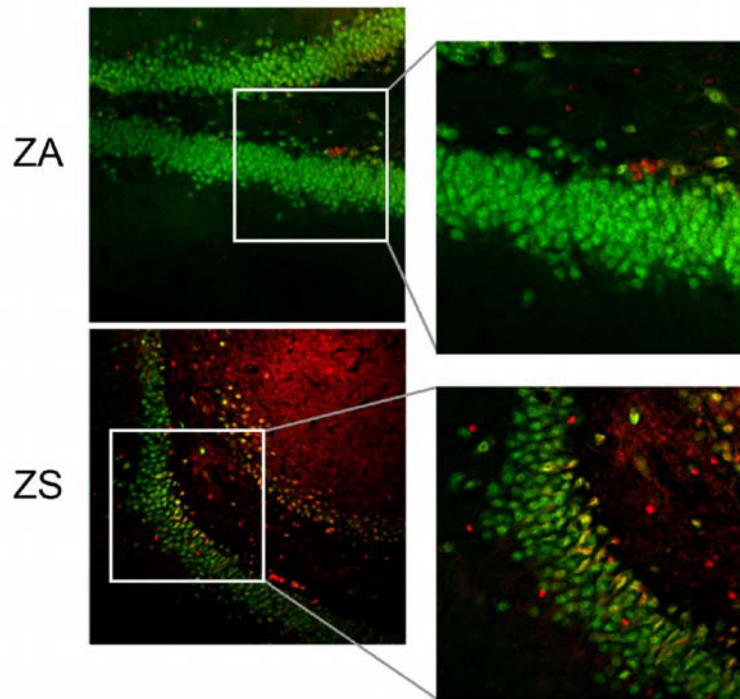

Figure 2.7. Effect of zinc supplementation on the of Ki67 + NeuN labeled cells in the dentate gyrus of the hippocampus. The left column of images shows the immunohistochemical localization of both Ki67 and NeuN (in yellow) in hippocampal tissue samples of rats receiving ZA, or ZS diets for 21 days. Photomicrographs are representative of images from adult Sprague-Dawley rats at original magnification 20x. The right column shows double-labeled cells at 40x magnification.

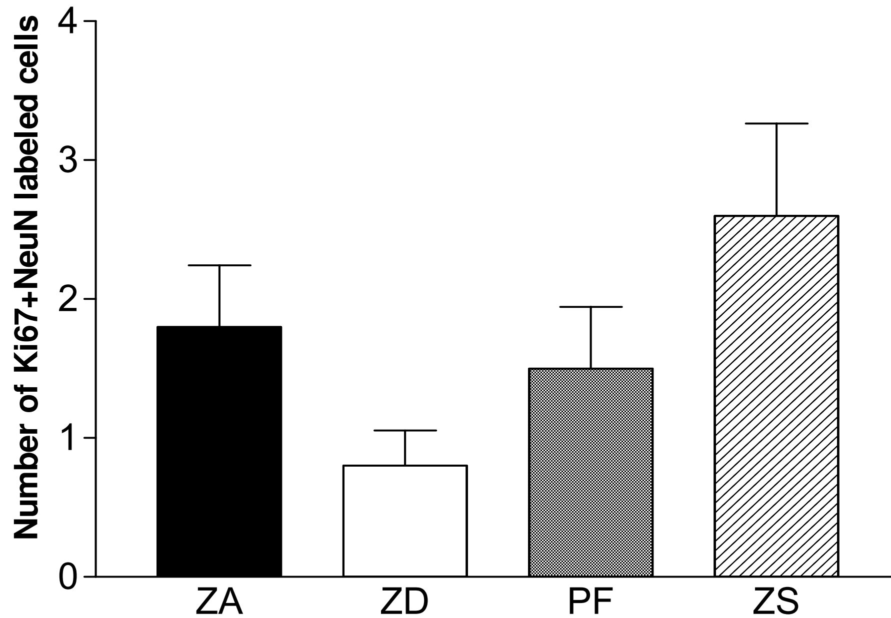

Figure 2.8. Effect of dietary zinc on the number of Ki67-positive cells that are co-labeled with the neuronal marker NeuN in the subgranular zone of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells and NeuN was used to identify neuronal phenotype in the granular cell layer of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 + NeuN labeled cells. Bars represent mean±SEM.

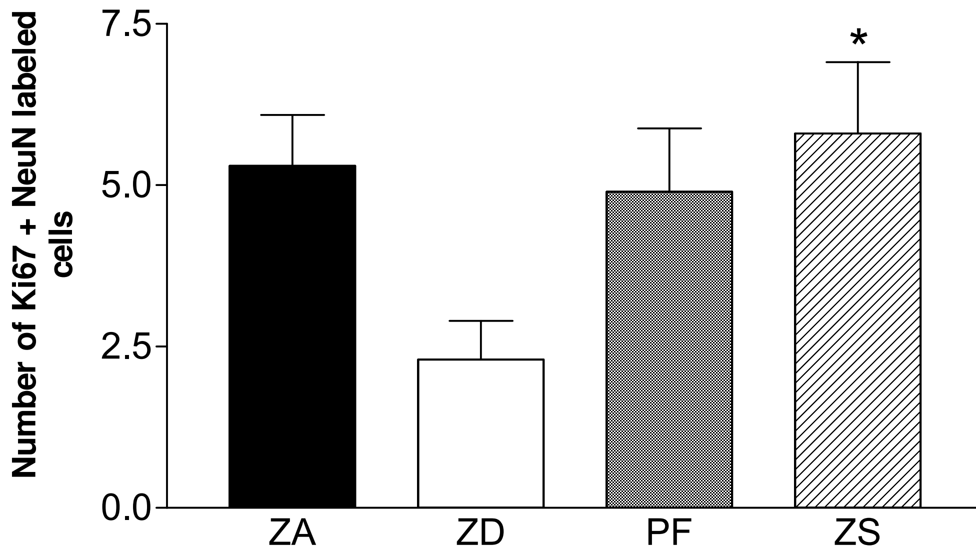

Figure 2.9. Effect of dietary zinc on the number of Ki67-positive cells that are co-labeled with the neuronal marker NeuN in the granular cell layer of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells and NeuN was used to identify neuronal phenotype in the granular cell layer of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 + NeuN labeled cells. Bars represent mean±SEM. *Significantly different from ZD $p<0.05$.

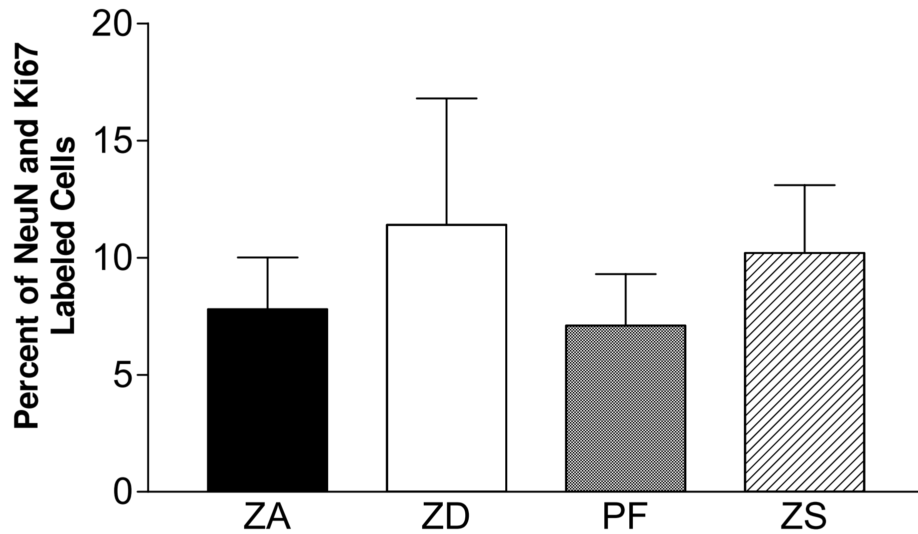

Figure 2.10. Effect of dietary zinc on the percent of Ki67-positive cells that are co-labeled with the neuronal marker NeuN in the subgranular zone of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells and NeuN was used to identify neuronal phenotype in the SGZ of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 + NeuN labeled cells. Bars represent mean±SEM percent of Ki67+NeuN labeled cells per brain section.

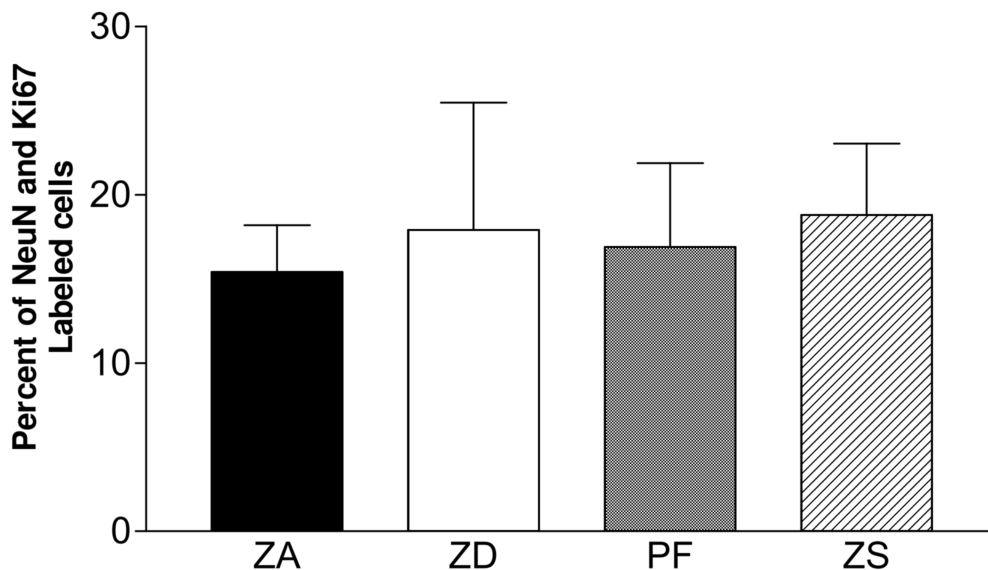

Figure 2.11. Effect of dietary zinc on the percent of Ki67-positive cells that are co-labeled with the neuronal marker NeuN in the granular cell layer of the hippocampus. Ki67 immuno-staining was used to identify newly proliferated cells and NeuN was used to identify neuronal phenotype in the granular cell layer of hippocampal tissue samples of rats (n=5) receiving ZA, ZD, PF, or ZS diets for 21 days. Metamorph was used to quantify the number of Ki67 + NeuN labeled cells. Bars represent mean±SEM percent of Ki67+NeuN labeled cells per brain section.

Discussion

For adult neuronal stem cells to be a useful clinical tool, we must first explore the cellular and molecular mechanisms responsible for regulating their proliferation. Zinc dependent enzymes are critical in cell replication processes and can have consequent effects on brain growth, cognition, and behavior. Based on our observations, severe ZD impairs cellular proliferation specifically in the GCL and SGZ of the dentate gyrus. This is due to ZD and not caloric restriction since our PF group did not show similar decreases in cellular proliferation in either the GCL or the SGZ.

ZS using 180 ppm for 21 days had no effect on stem cell proliferation. It is not known whether or not a longer period of supplementation would have a greater impact on stem cell proliferation. In the experiments reported here we wanted to use a supplementation period that matched the dietary deficiency period. However, previous work from our lab has shown that supplementation of adult rats for this length of time did not alter total zinc concentration (139). Thus, it is possible that a more chronic model of zinc supplementation would be more effective. Clearly any finding suggesting a role for supplementation in stem cell proliferation or neuronal differentiation would need to be followed up by experiments to determine the optimal dose. These studies would need to include measurements of copper because not only is copper essential for CNS function, but its absorption is inhibited by high levels of dietary zinc (140). Stem cell proliferation in the dentate is initiated in the cells of the SGZ. These newly formed cells migrate into the GCL. The finding reported here suggests that ZD impairs proliferation in the SGZ. Furthermore, the number of newly proliferating cells in the GCL is also reduced by ZD. However, it appears unlikely that ZD regulates migration because in both ZD and ZA animals approximately 41% of the Ki67-labeled cells were localized to the SGZ, while approximately 59% were found in the GCL.

The mechanisms responsible for the impairment of stem proliferation in zinc deficiency are not known. However, many transcription factors needed for neuronal proliferation and differentiation are zinc fingers. Thus this may be one mechanism by which ZD may reduce cellular proliferation in the dentate of the hippocampus. For example, as discussed in the introduction MGIF and Zhep are zinc finger proteins involved in cellular proliferation. Zhep has

multiple zinc fingers and is highly expressed in proliferating cells. Furthermore, treatment of cells with Zfhep antisense RNA reduces cellular proliferation. However, Zfhep expression is down-regulated in differentiating cells, further strengthening its role in proliferation and not differentiation. A Zfhep drosophila homologue, ATFB-1, which also has multiple zinc-finger motifs has been identified and seems to be involved in neurogenesis because it is highly expressed in precursor cells in the brain (136, 141). Another zinc finger, the HOF gene, has been localized to post-mitotic neurons (142). During late embryonic and early development HOF expression was confined to CA pyramidal and dentate granular cells. HOF is down-regulated in differentiated neurons because very few NeuN labeled cells expressed HOF. This further strengthens the role of HOF in proliferation and not differentiation.

Several growth factors have been shown to require zinc (143-147). The growth factor needed for epithelial cell proliferation, glial cell-line derived neurotrophic factor (GDNF), has a zinc-finger motif (146). One study investigated the effect of zinc on the brain derived neurotrophic factor (BDNF). It was shown that zinc supplementation, 11.5 mg Zn/kg administrated i.p. once a day for 14 days, increased BDNF mRNA abundance in the cortex (144). Increasing BDNF is one pathway by which chronic antidepressants may elicit their behavioral effects. The BDNF receptor trkB also appears to be regulated by zinc. Zinc activates the trkB signaling pathway needed for formation, maturation, and survival of neurons (148). Furthermore, zinc deficiency impairs neural stem cell proliferation measured by nestin-intermediate filament protein found in stem cells of young neurons in prenatal and postnatal mice brains (143). Zinc deficiency (1 ppm) decreased nestin staining compared to zinc adequate (30 ppm), pair fed (100 ppm), and zinc supplemented (100 ppm) by 40%. Furthermore, nestin protein levels, as measured by western analysis, were lowest in severe zinc deficiency, followed by moderate zinc deficiency (5 ppm) and highest in ZS.

The data reported here show that very few Ki67-positive cells co-labeled with NeuN. Furthermore, dietary zinc did not regulate the differentiation of these adult stem cells into a NeuN-expressing phenotype. These data are consistent with previous findings showing that two days after BrdU injection, less than 10% of the newly proliferated cells in the dentate co-labeled with NeuN (149). In fact, cells that expressed markers were largely differentiated into glia. In the current work, we did not examine other cell specific markers such as GFAP or vimenten, but it

would be interesting in future studies to examine the effect of dietary zinc on the differentiation of stem cells into astrocytes and other glia.

The finding that we did not see zinc regulation of stem cell differentiation in the current work is also consistent with the fact that differentiation from an undifferentiated stem cell into a fully mature neuronal phenotype may take as long as 4 weeks (149). In this time frame others have shown that following antidepressant stimulation of stem cell proliferation, approximately 70% of newly proliferated cells become neurons in the dentate GCL (76). Thus, future studies to explore the role of dietary zinc on stem cell differentiation should use BrdU injections, followed by administration of long term dietary treatment (4-8 weeks), and measurement of multiple markers of cellular differentiation in BrdU-positive cells. This would not only permit the identification of the role of zinc in stem cell differentiation, but would also allow the study of the role of zinc on long-term stem cell survival in the rat.

While zinc deficiency clearly impairs stem cell proliferation in the adult, it should be noted that cellular proliferation is also vitally important in embryos, growing infants and children. Furthermore, developing organisms are more likely to develop nutrient deficiencies, including zinc deficiency. Thus, this work has implications for human health throughout the life span. In particular, it would be interesting to examine the effects of early (prenatal and neonatal) zinc deficiency on the long-term (adult) potential for stem cell proliferation in the dentate gyrus and other regions of the brain.

In conclusion, this chapter has shown that dietary zinc deficiency significantly impairs stem cell proliferation in the dentate gyrus. The next chapter will use the same model of dietary zinc deficiency to examine possible behavioral implications of these changes.

CHAPTER 3: ZINC REGULATION OF DEPRESSION AND ANXIETY-LIKE BEHAVIORS

Introduction

The preceding chapter has shown that ZD leads to an impairment of stem cell proliferation in the subgranular zone of the dentate gyrus. This finding is significant because it has previously been shown that antidepressant drugs function, at least in part, by enhancing stem cell proliferation in this region of the brain (74, 83, 85, 150-152). Thus, based on the data presented in chapter 2, I have hypothesized that ZD will lead to the development of depression-like behaviors. Furthermore, I have hypothesized that deficiency-mediated impairment of stem cell proliferation will limit the efficacy of the selective serotonin reuptake inhibitor (SRRI), fluoxetine.

Etiology of Depression. In 2001 the World Health Organization predicted that in 2020 unipolar major depression will be the second leading cause of disease or injury after ischemic heart disease. Fifteen million people in the U.S. alone have been diagnosed with major depression, while an additional 15 million will experience milder forms of depression during their lifetime. Unfortunately, the development of new treatments for depression and depression-related illness has been hampered by the fact that we do not fully understand the neurobiological mechanisms that underlie the development of these disorders.

Stress has been long thought to precipitate or lead to depression. Depression results from the inability to use adaptive responses to stress and other aversive stimuli. Chronic stress can lead to depression because of extensive damage to the hippocampus and amygdala. Both emotional and cognitive consequences seen in depression can be in part attributed to disturbances in hippocampal function (153-155). These include decreased hippocampal volume (155-160), cell atrophy, and decreased neurogenesis (73, 161-163). Furthermore, impairment of hypothalamic-pituitary-adrenal (HPA) axis results in elevated cortisol levels (164, 165), which could be involved in the pathophysiology of major depression.

Acute stress may be beneficial, but prolonged or intermittenant chronic stress can have detrimental effects. Stress increases cell death and down-regulates neurogenesis in the dentate gyrus (DG) of the hippocampus (166-173). Adrenal hormones, specifically glucocorticoids, mainly mediate the effects of stress. Therefore, clinical depression in humans could be related to disturbances in neural plasticity, inter-neuronal communication, and adaptation that would normally be responsible for the appropriate response to stress and other aversive stimuli (161).

Neurogenesis and Depression. Results from chapter 3 indicate that there is a link between dietary zinc and cellular proliferation. In addition there is now a rather large and growing body of evidence suggesting that there is reduced hippocampal neurogenesis in adult models of depression. Chronic uncontrolled stress often leads to depression in human subjects. In animal models, stress is used to study the predisposing or causative effects that lead to depression. In the last five years there have been at least ten separate studies all showing a decrease in adult neurogenesis with a wide variety of stress models designed to induce depression-like behaviors including intruder stress in marmosets (76), psychosocial stress in tree shrews (174) and in rodents, predator odor (172), chronic restraint stress (173, 175, 176), foot shock stress (167), and prenatal stress (84). For example, 9 days of inescapable shock resulted in an approximately 20% decrease in BrdU labeled cells in the hippocampus compared to unshocked animals ($p<0.05$) (167). Moreover social defeat lead to impaired pyramidal cell synaptogenesis and plasticity in the hippocampus of tree shrews (177). Moreover, tree shrews exposed to psychosocial stress for 28 days had decreased hippocampal volume (174). Additionally the timing of the neurogenesis and onset of behavioral despair led to the conclusion that the reduction in neurogenesis is not simply a reaction to acute stress, but in fact played a role in the development of depression-like symptoms (73, 167). Thus, it has been hypothesized that a reduction in neurogenesis contributes to deficits in the functional capacity of the hippocampus leading to symptoms of depression.

There are several clinical studies that have correlated decreased neurogenesis with the symptoms of depression. Both emotional and cognitive consequences seen in depression can be, in part, attributed to disturbances in hippocampal function. Magnetic resonance imaging (MRI) and postmortem studies have correlated decreased hippocampal volume with major depression (154-157, 159, 160, 178-180). Depressed patients also show a decreased amygdalar volume (160). These effects appear to remain even after patients have recovered. MRI of post-depressed subjects showed mean total, left and right hippocampal volumes were reduced by 9%, 10% and

8% respectively (179). Others have shown that cortical grey matter is reduced in depressed patients compared to control subjects (181). Postmortem studies demonstrate a reduction in neuron size and glial number that could explain the reduction in cortical volume seen in these patients (182-187). Furthermore, depressed patients had smaller caudate nucleus volume compared to non-depressed patients (188). Subjects suffering from familial mood disorders had decreased cortical volume (184, 185). In disorders that have a depression component such as Bipolar disorder, patients had significantly larger ventricles than controls (189).

In addition to a decrease in cell proliferation, increased neuronal cell death, could lead to decreased hippocampal volume. Depressed patients had more pronounced apoptosis seen by increased in situ end labeling (ISEL) in the dentate gyrus, as well as CA1 and CA4 of the hippocampus (158). Postmortem studies of patients with major depression showed that glial density was decreased compared to controls (185, 186), while manic-depressive and schizophrenic patients had 40% reductions in nonpyramidal cells in the CA2 of the hippocampus (190).

Neurogenesis and Antidepressants. New and emerging literature is now showing, very clearly, that antidepressant drugs increase stem cell proliferation and neurogenesis in the hippocampus. Chronic administration (21 days) of fluoxetine resulted in a 3.4-fold increase in BrdU-positive cells in the SGZ of the dentate gyrus (151). Significant increases in BrdU labeling were also seen with a variety of other antidepressant treatments including norepinephrine reuptake inhibitors (83), electroconvulsive shock (191-194), tianeptine (195), and lithium (196), but not morphine or haloperidol that are psychoactive but not antidepressants (83). Interestingly, not only do antidepressant drugs increase neurogenesis, these pharmacological agents are also capable of reversing the deficits in neurogenesis seen in models of chronic stress and depression, an observation made by at least thirteen different published reports since 2001 (74, 83, 84, 150-152, 167, 197-202). When hippocampal neurogenesis was selectively impaired with x-radiation, the behavioral responses of mice to SSRIs and tricyclics were abolished (74), suggesting that the behavioral effects of antidepressant drugs are in fact dependent on stem cell proliferation in the dentate gyrus.

Zinc Deficiency and Depression. Recently, there has been an increased interest in the possible role that nutrient deficiencies may play in the development of depression. There are several links between zinc deficiency and the development of clinical depression. 1) Lower

serum zinc is seen in unipolar depressed patients compared to control subjects (28, 203) and several animal models of depression (204). Patients with major depression had serum zinc levels that were approximately 88-84% of controls ($p<0.0001$) (203). 2) There is a negative correlation between serum zinc and severity of depression (28, 203, 204). 3) Treatment resistant patients appear to have lower serum zinc levels than those who respond to pharmacological intervention (205). 4) Altered serum zinc concentrations are normalized following successful antidepressant treatment (206). 5) Acute zinc administration produces antidepressant-like effects in animals (207-211). 6) Our data show now that ZD decreases cell proliferation in the dentate gyrus of the hippocampus.

There are several reasons why serum zinc may be decreased in depressed patients. One possibility is that zinc deficiency is the result, not the cause, of depression. This is supported by the fact that one of the symptoms of depression is a reduction in food intake (DSM-IV). Thus, it is possible that zinc deficiency is secondary to the development of depression-related reductions in food intake. However, several studies have found no relationship between serum zinc and food intake, anorexia or body weight in depressed patients (28, 203), suggesting that zinc deficiency is not simply a function of reduced food intake. Another possible explanation for reduced serum zinc levels in depressed patients is altered cortisol production. Depressed patients have repeatedly been shown to have elevated cortisol levels that are not suppressed by dexamethasone. It has been hypothesized that increased glucocorticoid production induces the synthesis of the zinc binding protein metallothionein, resulting in cellular sequestration of zinc and reduced serum zinc levels (212). However, serum zinc levels do not appear to have any impact on the response to dexamethasone in depressed patients, suggesting that cortisol dysregulation is not responsible for the serum zinc abnormalities seen in depressed individuals (28). Morning serum corticosterone levels in zinc deficient rats were not significantly different from pair-fed controls at 15 or 22 days (213). Furthermore, animal studies of chronic stress that impair neurogenesis and induce depression-like behaviors show no long term changes in corticosterone levels, suggesting that elevated glucocorticoid hormones are not solely responsible for the reduced neurogenesis associated with depression (167). Moreover, the glucorticoid receptors in the hippocampus are found in the CA1, CA3 and dentate gyrus (89).

Zinc and Antidepressants. There are a number of studies showing that acute zinc administration has antidepressant effects in laboratory rodents (207, 208, 211, 214). When zinc

is injected at concentrations of 30 mg/kg body weight, zinc significantly reduces immobility time in the Porsolt swim test (207, 211, 214). The Porsolt swim test measures behavioral despair which correlates with depression-like behavior in the rodent model. In this test behavioral despair is quantified by the amount of time the rat spends floating (immobile) rather than actively swimming. In most studies the antidepressant effects of zinc were comparable to those seen with similar doses of the imipramine (207, 211, 214). When low, ineffective doses of zinc (1 mg/kg) and imipramine (5 mg/kg) were combined, there was again a significant reduction in immobility (207), suggesting that zinc is either acting via similar mechanisms as tricyclic antidepressants, or augmenting the mechanisms of imipramine action. Human trials designed to test the efficacy of zinc as an adjunct to antidepressant drug therapy have been consistent with the findings in rodents (209). Measures of depression status showed that 6 weeks of zinc supplementation augmented antidepressant drug therapy by over 50%. This difference was not only statistically different ($p<0.05$), but was sustained through the full 12 weeks of the study (209).

Therefore, the aim of these experiments was to test the direct link between different zinc diets and the development of depression. A series of standardized behavioral tests for depression and anxiety were used in adult male rats.

Materials and Methods

Animal Care. Two month old male Sprague-Dawley rats were individually housed in temperature controlled rooms with a 12-hour light-dark cycle. Rats were fed previously described ZA, ZD, PF and ZS diets for a period of 3 weeks. Body weight, food intake, and water intake were monitored daily. Rats were handled daily to reduce anxiety prior to behavioral testing.

Drug administration. Chronic fluoxetine administration was administered as follows. Animals (n=40) were divided as previously described into four dietary zinc groups (n=10/group). For each dietary group, half of the animals (n=5) were given ad libitum access to deionized water. The remaining animals (n=5) were provided with the antidepressant fluoxetine administered daily in drinking water for the duration of the 3 week dietary treatment. Water

intake was monitored daily to permit the administration of 10 mg fluoxetine/kg/day. After three weeks of dietary and drug treatment, rats were subjected to the light dark box or the forced swim test. For each behavioral test a new group of animals were used. For the elevated plus arm maze animals were only subjected to different dietary zinc treatments and no drug treatment.

In a separate set of experiments, animals fed the zinc diets for three weeks were given single, acute injections of fluoxetine (10 mg/kg body weight, i.p., n=5) after the pre-swim portion of the Porsolt swim test according to the method of Santerelli et al., 2003 (74). For each dietary group control animals (n=5) were given saline vehicle injections (i.p.).

Elevated plus-arm maze. Rats were subjected to this paradigm to test for novelty-seeking behavior, which has been shown to correlate with anxiety-like behaviors in animals (215). The apparatus is elevated 70 cm above the floor and exposed to dim illumination (Figure 3.1). The maze consists of two opposite open arms (45 cm x 12 cm) and two opposite closed arms of the same size with walls 45 cm high. The arms are connected by a central square (12 cm x 12 cm). Rats are introduced into the maze facing the closed arm, and spend a total of 5 minutes in the maze. The number of entries in to the open arms and the total time spent on the open arms were recorded.

Figure 3.1. Elevated plus-arm maze used to measure exploratory behavior for anxiety-like behaviors in rats.

Light-dark box. Novelty-seeking behavior was also measured using standard protocols utilizing the light-dark box (215). A 20 x 16 x 8 cm Plexiglass apparatus was equally divided into two compartments. One compartment was covered and dark while the other remained open and illuminated. A divider, with a 4x4 opening in the middle center allowing for free movement between compartments, separated the dark and light sides of the apparatus. Each rat was placed in the light side of the apparatus. The number of times the rat entered into the light side (head and both front limbs) and time spent in the light side were measured for a period of 10 min.

Porsolt Swim Test. The Porsolt swim test is based on the observation that depression-like behaviors can be measured in rats when they exhibit behavioral despair and resort to floating in a tank of water (immobility) rather than actively swimming. This method has been well documented in the literature as a test for antidepressant drug efficacy (74, 216, 217). On day 81, (Figure 3.2) rats from the 4 different dietary groups were placed individually into a temperature controlled (25°C) water tank measuring 25 cm in diameter and 75 cm high (Figure 3.3) for a period of 15 minutes.

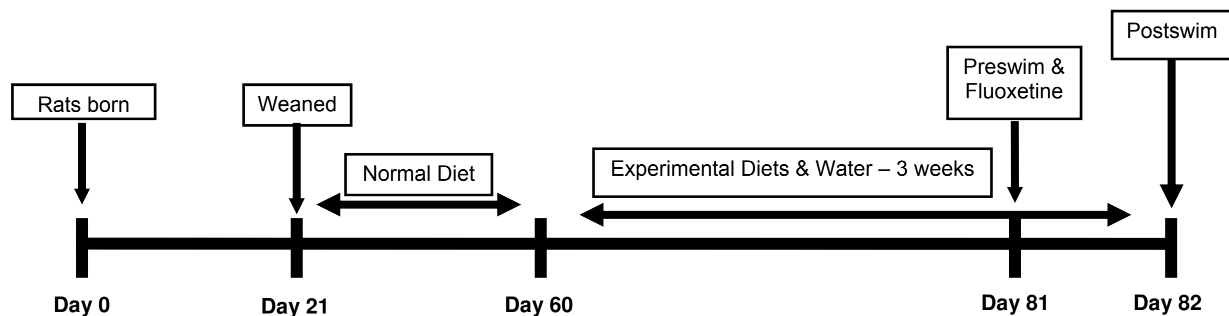

Figure 3.2. Experimental outline for acute fluoxetine administration and Porsolt swim test.

The water was deep enough so that rats were not able to rest on the bottom of the tank. At the end of the preswim, rats (n=5) were injected i.p. with either 10 mg/kg fluoxetine or an equal volume of vehicle (saline). The following day, rats were again placed in the swim tank for 5 min. Immobility, latency to immobility, time spent actively swimming and diving were recorded for each animal as a measure of behavioral despair.

For all behavioral tests, rats were recorded using a video camera to allow careful analysis. Differences between dietary groups were analyzed by ANOVA and a Tukey's post-hoc test.

Figure 3.3. Porsolt swim tank used to measure immobility for depression-like behaviors.

Results

Food Intake and Body Weight. The effect of dietary zinc treatment on food intake over the 3-week experimental period is shown in Figure 3.3A. At the beginning of the experiment food intake was similar for all groups. As expected, food intake maintained in the ZA and ZS group with time. A decline in food intake in ZD and PF animals was observed by day 8 of dietary treatment when compared to ZA and ZS. Differences remained throughout the dietary treatment. For rats consuming the ZD diet, food intake and body weight were significantly decreased compared to control by day 13 ($p<0.05$). This reduction was maintained throughout the study such that by day 21, body weight of ZD food was approximately 30% of ZA ($p<0.0004$). ZD (and thus PF) rats developed the previously characterized (22) 4-day feeding cycle. The reduction in food intake by ZD and PF rats led to a significant reduction in body

weight in these groups compared to ZA and ZS (Figure 3.3B). By day 8, body weights of ZA and ZS rats were greater than ZD or PF rats. And from day 13 to 21 PF animals body weight were greater than ZD animals by 12% (day 21). Water consumption patterns in all groups followed that of food consumption (data not shown).

A

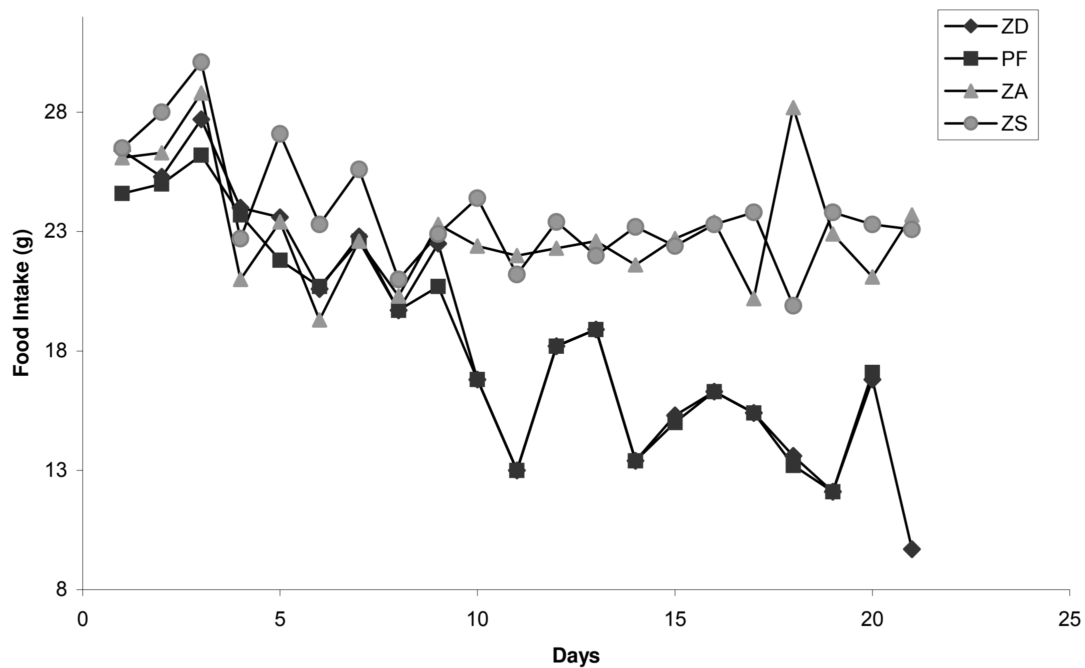

Figure 3.4. Effect of dietary zinc on (A) food intake and (B) body weight. Adult male rats were fed zinc adequate (ZA, grey triangle, n=5), zinc deficient (ZD, black diamond, n=5), pair-fed (PF, black square, n=5), or zinc supplemented (ZS, grey circle, n=5) diet for 21 days. Food intake and body weight for each day were reported as means.

B

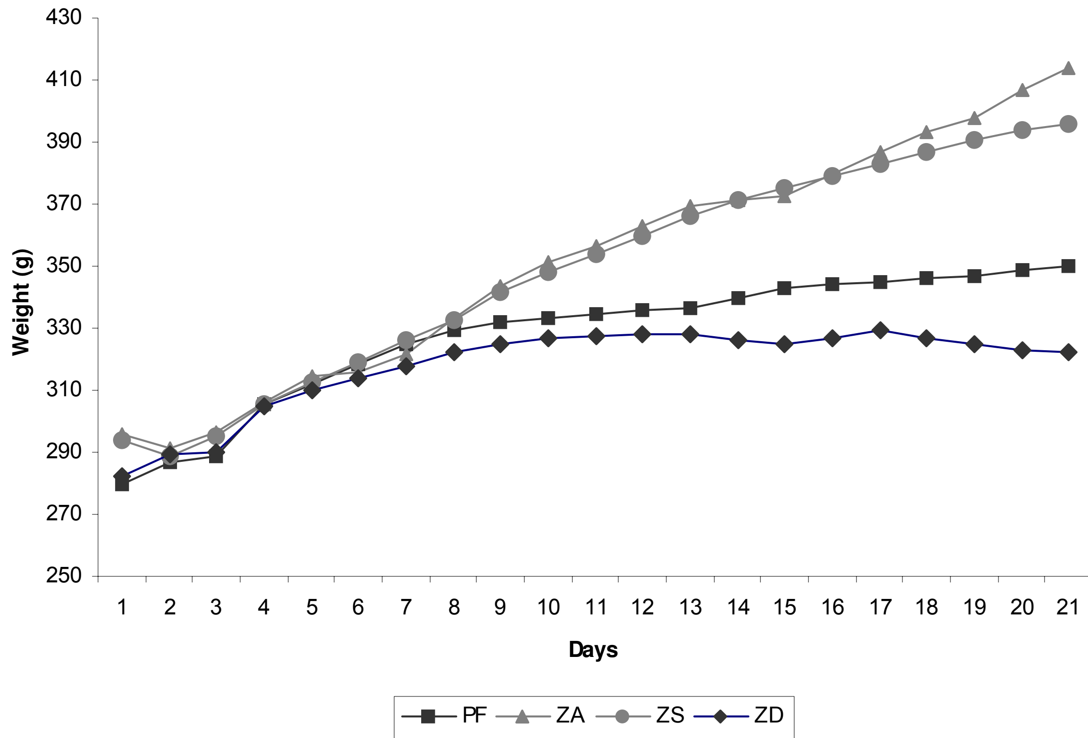

Figure 3.4. Continued

Elevated Plus-Arm Maze. Previous work has correlated time spent in the open arms of the elevated plus-arm maze with decreased anxiety-like behaviors in rodents (215). Control animals (ZA) spent approximately half the time in the open arms (Figure 3.4). ZD and ZS animals spent the same amount of time in the open arms. Mean difference between ZA, ZD, ZS animals did not reach significance. However, PF animals spent significantly less time in the open arm, indicating increased anxiety-like behaviors in this group ($p<0.03$).

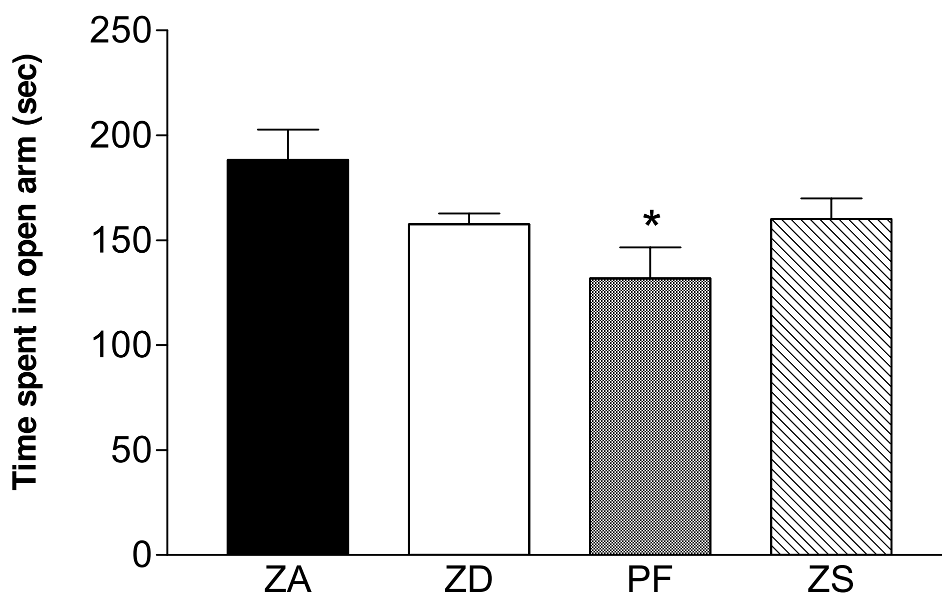

Figure 3.5. Effect of zinc diets on time spent in the open arm of the EPM. Adult male rats were fed zinc adequate diet (ZA, black, n=5), zinc deficient diet (ZD, white, n=5), pair-fed diet (PF, hashed, n=5), or zinc supplemented diet (ZS, lined, n=5) for 21 days. Rats were then tested in the elevated plus-arm maze for 5 min. Time spent in the open arms was reported as mean ± SEM. *Significantly different from ZA $p < 0.05$.

Light-Dark Box. The light-dark box is well a established test for anxiety-like behaviors (215, 218). The number of explorations and time spent in the light side are negatively correlated with anxiety-like behaviors (218, 219). PF rats spent less time in the light side than ZS ($p<0.001$) and more time than the ZD rats, but were not significantly different from control animals (Figure 3.6). ZD rats spent the least time in the light side of the box (29 ± 28 seconds) compared to control ($p<0.01$), PF ($p<0.001$) or ZS ($p<0.001$) (Figure 3.6). Furthermore, ZS animals spent significantly more time in the light box than control animals ($p<0.05$) (Figure 3.6). ZD animals spent significantly more time (105 seconds more) on the light side (134 ± 49 seconds) when fluoxetine was added to drinking water (10 mg/kg) for 21 days ($p<0.01$) (Figure 3.7). Moreover, ZD + FLX was not significantly different from ZA controls.

Number of explorations followed the similar trend as time spent in the light side. However PF animals explored the same number of times as the ZS, and more than ZA and ZD ($p<0.001$) (Figure 3.8). ZD animals explored less than ZA ($p<0.01$) and significantly less than ZS ($p<0.001$). ZD rats that were chronically treated with fluoxetine spent significantly ($p<0.03$)

more time in the open arm with increased number of exploration compared to control ZD rats ($p<0.001$) receiving regular deionized water (Figure 3.9). Fluoxetine treated ZD animals explored more than control animals as well ($p<0.05$).

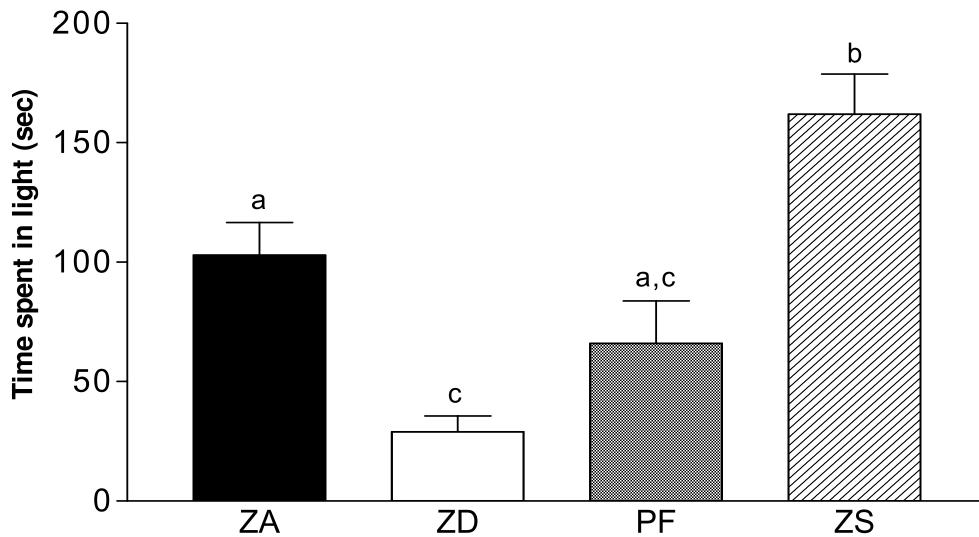

Figure 3.6. Effect of different zinc diets on time spent in light side in the light dark box. Adult male rats were fed zinc adequate diet (ZA, black, n=14), zinc deficient diet (ZD, white, n=10), pair-fed diet (PF, grey, n=10), or zinc supplemented diet (ZS, lined, n=10), for 21 days. Rats were then tested for novelty-seeking behavior in the light-dark box for 10 min. Time spent in the light box was reported as mean±SD. Bars with different letters are significantly different by $p<0.05$.

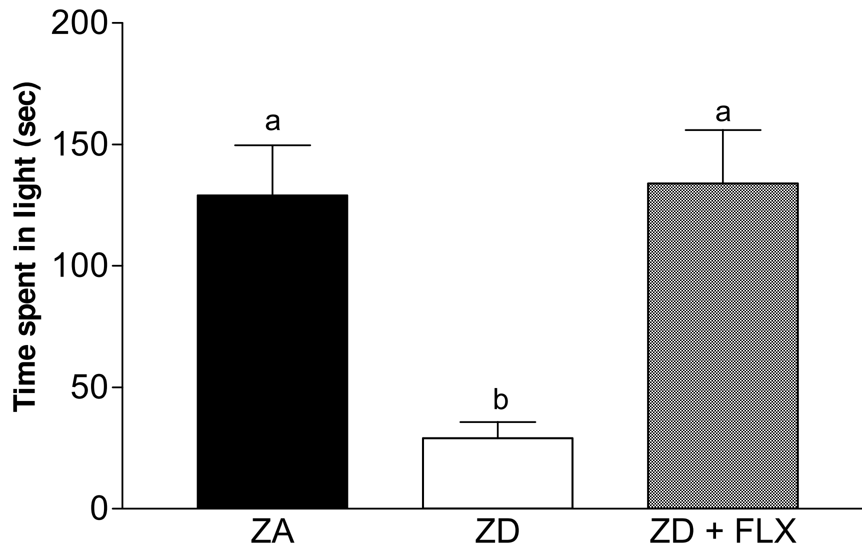

Figure 3.7. Effect of fluoxetine on time spent in light side in the light dark box. Adult male rats were fed zinc adequate diet (ZA, black, n=14), zinc deficient diet (ZD, white, n=10), or zinc deficient with fluoxetine (ZD+FLX, crossed, n=5) for 21 days. Rats were then tested for novelty-seeking behavior in the light-dark box for 10 min. Time spent in the light box was reported as mean±SD. Bars with different letters are significantly different from each other at $p<0.01$.

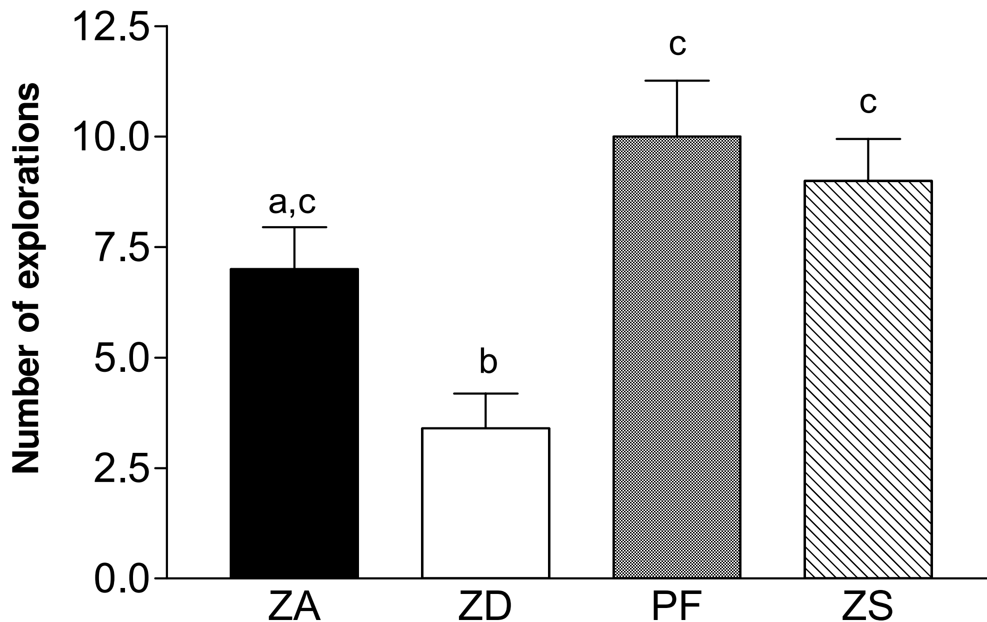

Figure 3.8. Effect of different zinc diets on number of explorations in the light dark box. Adult male rats were fed zinc adequate diet (ZA, black, n=14), zinc deficient diet (ZD, white, n=10), pair-fed diet (PF, grey, n=10), or zinc supplemented diet (ZS, lined, n=10) for 21 days. Rats were then tested for novelty-seeking behavior in the light-dark box for 10 min. Number of explorations was reported as mean±SD. Bars with different letters are significantly different by $p<0.01$.

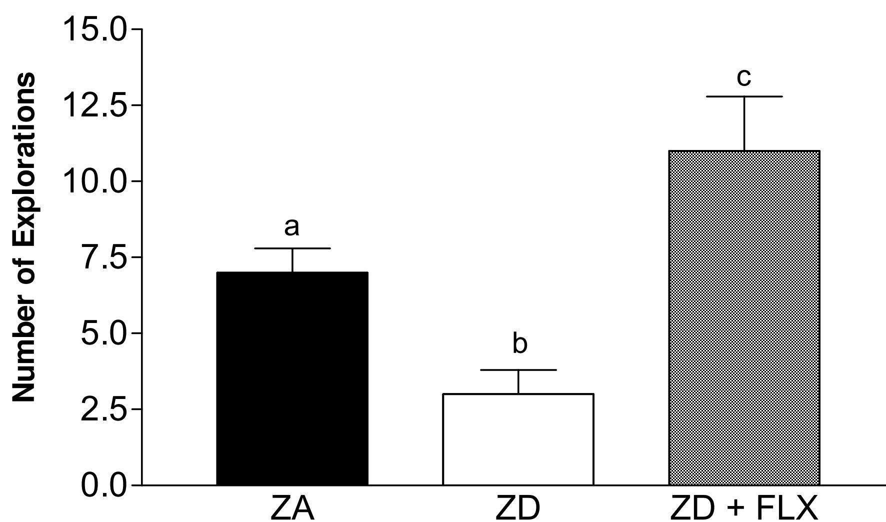

Figure 3.9. Effect of different fluoxetine on number of explorations in the light dark box. Adult male rats were fed zinc adequate diet (ZA, black, n=14), zinc deficient diet (ZD, white, n=10), pair zinc deficient with fluoxetine (ZD+FLX, crossed, n=5) for 21 days. Rats were then tested for novelty-seeking behavior in the light-dark box for 10 min. Number of explorations was reported as mean ± SD. Bars with different letters are significantly different by $p<0.01$.

Porsolt Swim Test. The Porsolt swim test has established a positive correlation between behavioral despair measured by increased immobility time with increased depression-like behaviors in rats (214, 220, 221). Immobility times for different dietary groups were not significantly different when these animals were injected with saline. However, acute fluoxetine treatment significantly decreased immobility time ($p<0.01$) in ZA and ZS rats but proved to be ineffective in reducing immobility time in ZD rats (Figure 3.10).

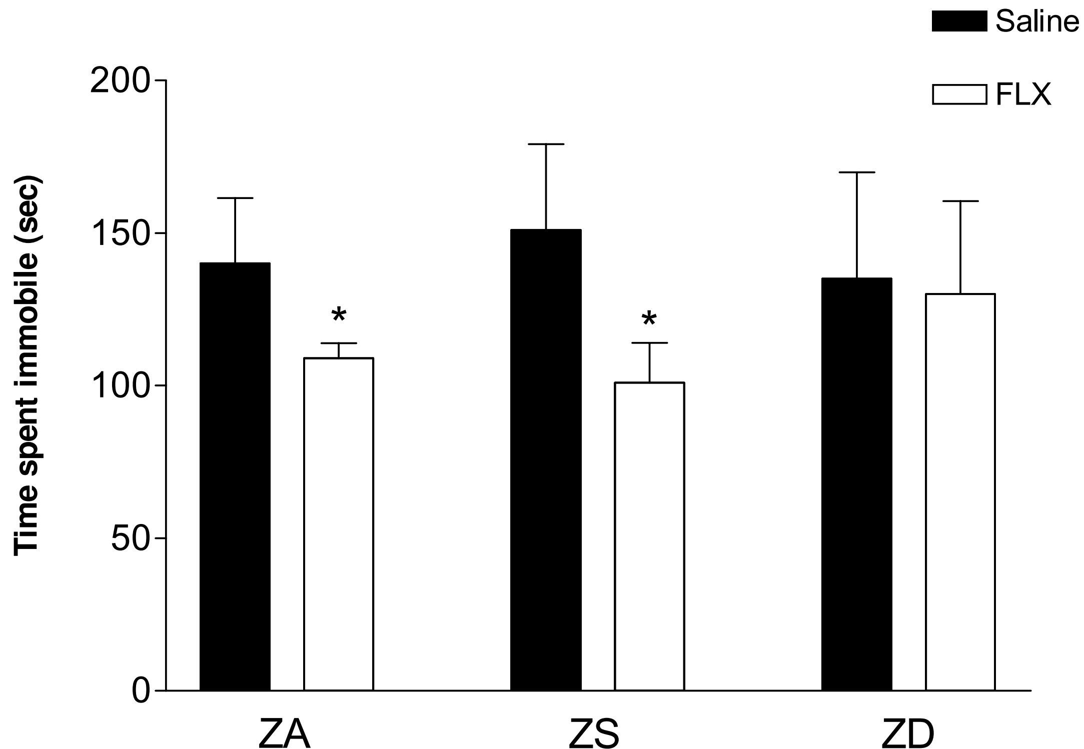

Figure 3.10. Effect of zinc on efficacy of the antidepressant fluoxetine in the forced swim test. Bars indicate mean duration (seconds) of immobility during the 5 min test phase. Adult male rats were fed zinc adequate (ZA, n=5), zinc supplemented (ZS, n=5), or zinc deficient (ZD, n=5) diet for 21 days. Each dietary group had additional rats that were treated with fluoxetine (n=5). Immobility time was reported as mean±SD. * Significantly different from ZA saline treated or ZD saline treated $p < 0.01$.

Discussion

Depression in the United States has a high rate of occurrence. Ten to fifteen million people are depressed in the US in any year. In addition, patients suffering from the symptoms of depression also suffer from suicide attempts, accidents, resultant illness, lost jobs, failure to advance in a career or school, and in many cases, substance abuse. Despite the severe consequences of this disorder, we do not have full understanding of the etiology of depression making the development of effective treatments difficult. Mechanisms underlying current pharmacological treatments such as SSRIs, and monoamine inhibitors (MAOI) are by no means completely understood. Furthermore, they elicit many side effects such as gastric discomfort

(222), nausea (222, 223), vomiting (222), dry mouth (222), headache (222, 223), tremor (222), insomnia (223), and dizziness (223).

Symptoms of depression listed in the DSM-IV-TR include feeling sad or empty, insomnia, fatigue, feeling of worthlessness, suicidal thoughts, diminished interest in pleasurable activities, and significant weight loss or gain. In rodent models of depression it is hard to measure some of these outcomes; however there are established behavioral tests that we have used to measure depression-like behaviors in zinc deficient rats. While there have been previously reported links between zinc deficiency and depression, the results shown here, provide the first evidence of a causative relationship between zinc deficiency and depression-like behaviors. Each will be discussed in detail below.

First, as shown in figure 4.6 A and B, dietary ZD leads to decreased food intake and eventually anorexia. This is a hallmark of ZD that we and others have repeatedly shown (22, 213) and is consistent with weight loss seen in depressed patients.

Second, using the standardized test for anhedonia, the two-choice model of sucrose vs. deionized water (224-226), rats decrease their consumption of, and preference for, highly palatable sweet solutions (225). Zinc deficient rats reduced their preference for water sweetened with 0.05% of saccharin, suggesting anhedonia consistent with depression (227). Similar results have also been seen in anxiety (228). Using standardized behavioral tests, ZD appears to produce anxiety-like behaviors in the rat, which may partially account for the anorexia. Indeed, ZD animals displayed significantly less time and made fewer entries into the novel light environment than either ZA or PF rats in the light-dark box. These behaviors are consistent with anxiety in rodent models (229). Chu et al., also reported anxiety-like behaviors in zinc deficient rats subjected to the water maze test. While they were unable to detect deficits in memory and learning, they did note thygmotaxia, a sign of anxiety (213). The finding that ZD rats display not only depression-like behaviors but also anxiety-like behaviors suggests that zinc deficiency may be a useful model for future studies of depression. Indeed a significant number of patients with major depression have co-morbid anxiety (230).

The mechanisms responsible for anhedonia seen in depression and co-morbid anxiety in zinc deficiency are not yet understood. It has been speculated that anhedonia in depression is related to noradrenergic insufficiency (231). Zinc deficiency in rats has been reported to affect total brain catecholamine levels (232). Furthermore when 20 μg of norephinephine (NE) was

centrally injected into Zn-deficient rats they displayed partial resistance to the NE by eating significantly less food than controls ($p<0.001$) (233). Thus it is plausible that Zn-deficiency induces anhedonia by noradrenergic mechanisms.

To explore the mechanism by which ZD may induce anxiety and depression-like behaviors, ZD rats were treated chronically (in drinking water) with 10 mg/kg of fluoxetine. ZD rats treated with fluoxetine showed significantly ($p<0.01$) reduced anxiety-like behavior compared to ZD animals receiving regular drinking water, suggesting that this ZD-mediated anxiety-like behaviors could be treated with fluoxetine. This is consistent with the finding that anxiety in humans is commonly treated using antidepressant agents. The antidepressant tianeptine has been shown in several studies to be an effective treatment for anxiety (222, 223, 234). Six weeks of tianeptine treatment in patients suffering from anxiety led to significant improvement on Montgomery and Asberg Depression Rating Scale (MADRS) (235). Furthermore, tianeptine and amitriptyline, when administered separately to patients suffering from depressive disorders, showed significant decreases in anxiety associated with depression after only 7 days of treatment (234). Similar effects were seen with the antidepressant we used for our studies, fluoxetine. Adolescents and children with anxiety disorder, generalized anxiety disorder, or separation anxiety had reduced anxiety after 1 year of fluoxetine treatment (236). Other investigators have shown similar anxiolytic effects of fluoxetine when administered chronically (237).

Because many patients with major depression do not respond well to antidepressant drug therapy, we tested the efficacy of the SSRI fluoxetine in ZD rats using the Porsolt swim test. This test is commonly used by pharmaceutical companies to test the efficacy of antidepressant drugs. In this well established model, drugs that acutely reduce immobility time are frequently effective in depressed humans when administered chronically. This test has proven very effective in drug development, despite the fact that the mechanism of acute action in rodents is unknown. Therefore, we performed the swim test in conjunction with acute fluoxetine treatment. When each dietary group was treated with 10 mg/kg fluoxetine, ZA and ZS rats showed a significant decrease in immobility time, suggesting that both ZA and ZS animals respond to this SSRI. However, fluoxetine proved to be ineffective in ZD animals, suggesting that zinc is necessary for the activity of SSRIs. Fluoxetine also proved to be ineffective in reversing anorexia in ZD

animals. This is consistent with the fact that antidepressant drugs are not an effective treatment for anorexia nervosa (238).

Based on the finding presented here that ZD impairs stem cell proliferation, it is reasonable to speculate that ZD impairs the ability of fluoxetine to induce neurogenesis, preventing the SSRI from functioning. This finding has potentially significant clinical implications. While current drug therapies can be very effective for the treatment of depression, the data presented here suggest that inadequate zinc intake may explain some cases that are refractive to traditional treatment. Future studies will be needed to determine whether zinc deficient diets prevent the increase in stem cell proliferation normally associated with antidepressants.

In rodents, antidepressant treatment increased serum zinc (206). Specifically antidepressants increased hippocampal zinc levels by 20% and decreased zinc in other regions of the brain (206). Therefore it appears that chronic antidepressant treatment redistributes zinc in the brain, and links zinc, antidepressant treatment, and depression. Acute zinc injections (30 mg/kg) significantly decreased immobility time in the forced swim test (207). Imiprimine, citalopram, or SSRI antidepressant, combined with zinc decrease immobility time in a dose-dependent manner in mice, and prove to be more effective in reduction of behavioral despair in the swim test than previous ineffective doses of zinc or citalopram alone (207). Even different amounts of zinc injection, acute (one injection), subchronic (3 doses) or chronic (2 weeks of treatment) decreased immobility time in the forced swim test (209). Zinc supplementation showed improvements in the HDRS in depressed patients (28). Similar to the combined effects of antidepressant and zinc in the forced swim test, zinc and antidepressants proved to be more effective in the HDRS and Beck Depression Inventory, than antidepressants alone (214). Given that zinc supplementation up to levels of 40 mg/day are not toxic, the data presented here suggest that supplementation should be prescribed in conjunction with antidepressant therapy, particularly when patients are slow to respond to traditional pharmacological therapy.

<u>Possible Mechanisms of AD actions</u> - Because depression may result from an impairment of neurons to make appropriate adaptations and/or synaptic connections, recent approaches to understanding depression have focused on pathways involved in cellular plasticity and survival (153). For example, it has been shown that expression of brain derived neurotropic factor (BDNF) and its receptor, trk B, is increased in the hippocampus after treatment with

antidepressant drugs such as imipramine, trancylcypromine, and desipramine (239, 240), suggesting that this neurotrophic factor may play a role in the regulation of adult neurogenesis. Since zinc treatment (65 mg/kg) significantly increased BDNF mRNA in the cortex of rats compared to control rats (144), it would be reasonable to hypothesize that ZD produces behaviors consistent with depression, in part, through down-regulation of BDNF.

A key modulator in differentiation, maturation and synaptogenesis is the NMDA receptor (241), which consists of two subunits, a core subunit (NR1) and an additional subunit (NR2B). Decreased NMDA transmission produces antidepressant effects. NMDA is one mechanism by which zinc may produce antidepressant effects. When zinc chloride was injected into mice, immobility time decreased in the forced swim test compared to control. However when an NMDA antagonist was used (ascorbic acid or guanosine 5'-monophosphate), the decreased immobility seen with zinc was inhibited (241). Furthermore, zinc appears to be involved in receptor regulation and binding. For example, on postnatal day 2 (PND), zinc deficiency decreased mRNA of NR1, NR2A and NR2B in the entire brain, similar effects were seen on PND 11 (242). Protein levels of NR1, NR2B, and NR2A were also decreased in moderate zinc deficiency in the whole brain on PND 2, 11 and 65. Protein levels of these receptor subunits were also decreased significantly in the hippocampus of zinc deficienct animals compared to controls (242). Zinc has also been found to be a potent non-competitive antagonist of NMDA responses in cultured hippocampal and cortical neurons (102, 243). Zinc decreased NMDA receptor-activated channel currents opening frequency and by interfering with ion passage (243). Furthermore, zinc deficiency decreased glutamate receptor antagonist, MK801 binding sites by 20% but had no effect on binding affinity (244). Antidepressants such as imipramine have been shown to increase the potency of zinc to inhibit MK801 binding to the NMDA receptor (245, 246).

Possible causes for efficacy of fluoxetine to reverse zinc deficiency-induced anxiety but not reverse depression seen in ZD could be because fluoxetine functions in two different areas for each behavior. Fluoxetine works on the amygdala (247) to induce anxiolytic effects (236). The amygdala plays an important role in fear conditioning as well as a variety of anxiety disorders. In the amygdala serotonin receptors are present and may be responsible for the effects of fluoxetine treatment. Fluoxetine acts as an antidepressant by increasing stem cell proliferation

in the dentate gyrus. The next chapter will describe preliminary work designed to identify mechanisms responsible for the differential effects of Fluoxetine in ZA and ZD rats.

CHAPTER 4: ZINC REGULATION OF HIPPOCAMPAL GENE EXPRESSION

Introduction

Dietary zinc manipulation is reflected in the brain. Zinc deficiency decreases zinc levels in whole brain (66) as well as specific areas of the brain such as the hippocampus which is part of the limbic system. The olfactory bulb, also part of the limbic system, has been shown to respond to dietary zinc. We have shown that zinc levels in the olfactory bulb decrease with dietary ZD (227). Zinc deficiency also reduces DNA, mRNA and protein levels in the brain (41). Moreover, brain size of zinc deficient animals was smaller than pair-fed and control animals (41).

As discussed earlier, zinc is needed for many biological enzymes. There are at least 80 different mammalian enzymes that require zinc (1). Zinc is essential for the activity of nuclear enzymes DNA and RNA polymerases. Mitochondrial enzymes such as, pyruvate carboxylase are also zinc dependent. Lysosomes and golgi apparatus need zinc for α-D-mannosidase and peptidases. The cell membrane integrates zinc in its enzymes like carbonic anhydrase and phospholipase C (PL-C). Others enzymes include copper-zinc superoxide dismutase, alkaline phosphatase, lactate dehydrogenase, and carboxypeptidases. Many of the enzymes in intermediary metabolism including lactate dehydrogenase, and alcohol dehydrogenase, all require zinc. As mentioned in the main introduction, zinc is involved in the structural component of many proteins forming zinc fingers. These transcription factors and many others are responsible for the main regulatory functions of zinc.

While it appears that zinc rarely regulates the abundance of zinc finger protein, given the structural role of zinc in these proteins and the importance of their function in gene transcription, we have hypothesized that zinc deficiency regulates gene expression in the limbic system. Previously, our lab has custom-made subtracted cDNA libraries and used cDNA microarray chips to identify zinc-regulated genes in the olfactory bulb. The current work has shown that ZD

impairs the efficacy of fluoxetine. Thus, in this chapter I will describe work using these chips to begin to identify target genes that may play a role in this observation. Specifically, I have examined gene expression in the OB and hippocampus of rats treated chronically with fluoxetine and fed ZA or ZD diets. This will enable me to test the hypothesis that ZD interferes with fluoxetine-mediated molecular mechanisms. Testing this hypothesis will provide us with a preliminary set of target genes for future study throughout the limbic system including the hippocampus.

Materials and Methods

Animal Care. Two month old male Sprague-Dawley rats (n=16) (Charles Rivers Laboratories, Wilmington, MA) were individually housed in temperature controlled, rooms with a 12-hour light-dark cycle. Rats were fed a commercially prepared (Research Diets Inc., New Brunswick, NJ) ZA diet (30 ppm) for 3 days. After this baseline period, rats were fed zinc adequate (ZA, 30 ppm, n=8), or zinc deficient (ZD, 1 ppm, n=8) for a period of 3 weeks (Table 2-1).

Drug Administration. For each dietary treatment, 5 animals were given ad libitum access to deionized water. The remaining 3 animals were provided with the antidepressant fluoxetine (Sigma-Aldrich, St. Louis, MO) administered daily in drinking water for the duration of the 3 week dietary treatment. Water intake was monitored daily to permit the administration of 10 mg fluoxetine/kg/day.

mRNA Isolation. At the end of the 3 week diet and drug period, animals were euthanized using CO_2. Bilateral olfactory bulbs and hippocampus were immediately removed and homogenized in 1 mL Trizol solution (Gibco/ BRL, Gaithersburg, MD) to permit the isolation of intact total cellular RNA. Concentrations and purity were determined using spectrophotometry at A_{260}/A_{280}. Poly $(A)^+$ mRNA was isolated by oligo-dT cellulose mRNA isolation kit (Ivitrogen Life Technologies, Carlsbad, CA).

Microarray chips. Microarray chips were prepared by robotic spotting of approximately 1,280 cDNA clones isolated from an olfactory bulb cDNA library. To remove housekeeping genes and increase the representation of low abundance messages, the cDNA library was

prepared by subtraction of zinc-adequate and zinc-deficient bulb cDNA libraries. Chips were also spotted with GAPDH and β-actin that served as message controls. Each clone was arrayed on poly-lysine coated slides (CEL Associates, Santa Clara, CA) and printed in triplicate using a spot size of 130 μm.

Labeling of mRNA targets. The CyScribe cDNA Post Labeling Kit (Amersham Biosciences, Piscataway, NJ) was used to produce Cy3 and Cy5 labeled cDNA targets for the use of dual fluorescent color microarray hybridizations. Briefly, random nanomers and oligo (dT) primers were annealed to isolated mRNA (500 ng), followed by the addition of nucleotide mix and an amino allyl coupled dUTP for generation of first strand cDNA. Next, mRNA was removed from first strand cDNA to allow for amino allyl labeled cDNA binding to its complement on the microarray chip. This was accomplished with alkaline treatment (2.5 M NaOH and 2 M HEPES) which causes fragmentation of mRNA. QIAquick PCR Purification kit (Qiagen, Valencia, CA) was used to remove short mRNA oligomers (Figure 4.1). Modified cDNA were then coupled to respective CyDyes (Cy3 and Cy5 for separate cDNA groups) by the reaction of CyDye NHS-esters with incorporated amino allyl groups on the cDNA. 4M hydroxylamine was added after CyDye labeling to inactivate any unreacted CyDye NHS ester molecules and cDNA was purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA). All CyDye labeling was done in the dark to prevent direct exposure to fluorescent light and subsequent photodestruction of CyDye fluorophores.

Fluorescent cDNA probes hybridization. Prior to hybridization microarray chips were pre-washed following a protocol specifically designed to enhance fluorescent binding of labeled cDNA targets (Figure 4.1). Briefly, slides were vigorously washed with 0.2% SDS at room temperature, immediately transferred to a prehybridization solution (5X SSC; 1% Bovine Serum Albumin; 1% SDS) at 55 ºC for 45 min., followed by 5-7 washes in dH_2O and 1 wash in isopropanol, and left to air dry. CyDye labeled cDNA were then placed over the array covered with 22x25 mm cover slip (Corning, Corning, NY) and placed in hybridization chamber (ArrayIt Brand products, TeleChem International Inc., Sunnyvale, CA). Microarray chips were hybridized for 18 hours at 42ºC then removed and washed per protocol (CyScribe Post-Labeling Kit). All microarray chips were air dried, placed in microscope slide box to prevent light exposure and stored at -80ºC. A total of three microrray chips were hybridized with mRNA from different

animals from each dietary and drug treatment, producing a total of 3 chips per brain region hippocampus and olfactory bulb (Table 4.1).

Table 4.1. Probes for microarray hybridization

	Hippocampus	Olfactory Bulb
Microarray chips	n = 3	n = 3
Cy3 labeled probe	Zinc adequate, fluoxetine treated	Zinc adequate, fluoxetine treated
Cy5 labeled probe	Zinc deficient, fluoxetine treated	Zinc deficient, fluoxetine treated

Data Analysis. After hybridization and air drying, labeled microarrays were scanned on a ScanArray 5000XL scanner (GSI Lumonics, Moorpark, CA) in the appropriate channels. Scanned microarrays were then analyzed by QuantArray Imaging software (GSI Lumonics, Moorpark, CA) and a grid was created to account for the locality of all spots, and used for locating, identifying and quantifying gene expression on subsequent hybridized microarrays. Fluorescent cDNA target microarrays were scanned at 535 nm for Cy3 and at 625 nm wavelength for Cy5. Background signal to noise ratio was quantified for each probe element. The ratio was corrected for the two signal intensities and calculated as the differential expression ratio between the two target cDNA populations. Data were then analyzed in Microsoft Excel following global normalization to account for overall intensity differences between the two fluorescent dyes and control the control message (β-actin). A 2-fold increase or decrease in expression ratios was used as the cut-off for differential expression.

Gene sequencing. cDNAs representing differentially regulated mRNA were sequenced following isolation and quantification of plasmid DNA using QIAquick PCR Purification kit. Sequencing was performed in the DNA sequencing facility, Biology Department at Florida State University. Sequencing was performed using an Applied Biosystems 3100 Genetic Analyzer with Capillary Electrophoresis. DNA sequencing chemistries are performed using Big-Dye terminator chemistry.

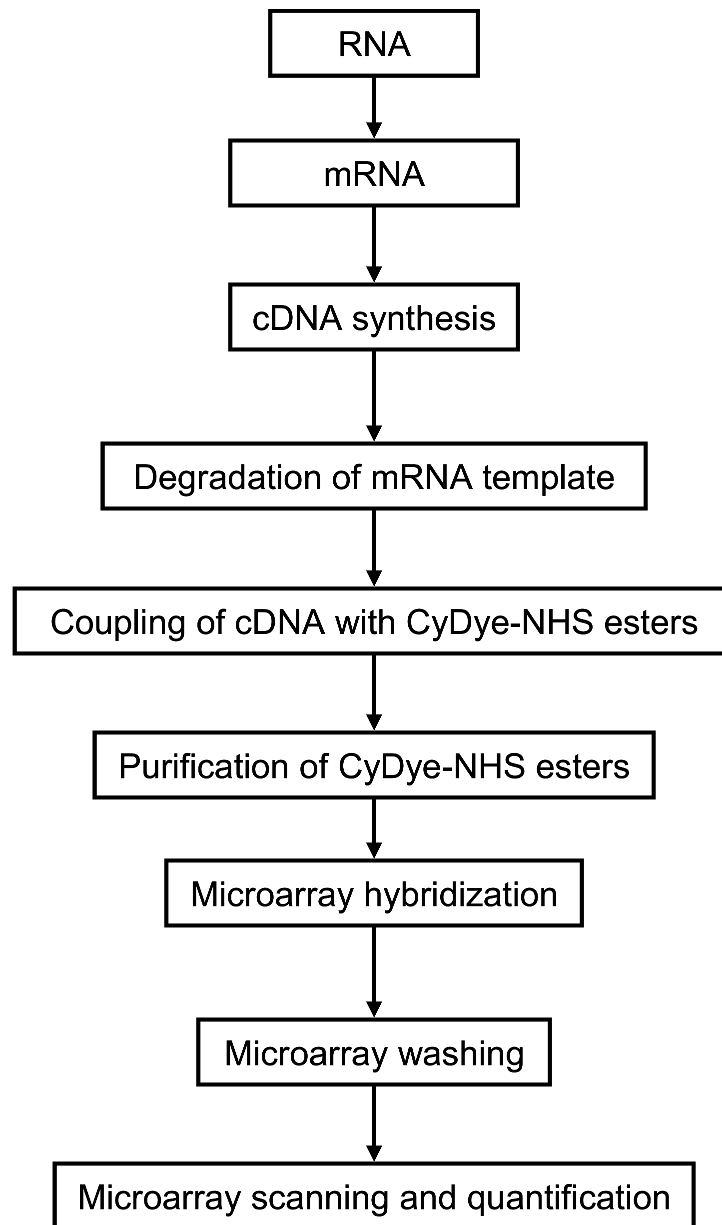

Figure 4.1. Flow diagram of microarray chip labeling and hybridization.

Results

Zinc regulation of fluoxetine-mediated hippocampal gene expression. There were a total of 9 genes that were down-regulated by ZD (Table 4.2). Of these, six are mitochondrial genes involved in ATP synthesis and two are implicated in neuronal proliferation and differentiation.

Zinc regulation of fluoxetine-mediated olfactory bulb gene expression. There were a total of 3 genes that were down regulated by ZD in the olfactory bulb (Table 4.3). Of these, two are involved in ATP synthesis and one is a zinc finger protein. All the genes identified in the OB, were also regulated in the hippocampus.

Table 4.2. ZD alterations in fluoxetine-responsive genes in the hippocampus.

GENE	MEAN RATIO[a]	SD	FUNCTION
ATP synthase	0.44	0.14	ATP synthesis
ATPase subunit 6 & 8	0.47	0.11	ATP synthesis/ apoptosis suppressor
BN/ SsNHs	0.47	0.10	Zinc finger protein
Cytochrome C Oxidase subunit I	0.46	0.14	ATP synthesis
Cytochrome C Oxidase subunit II	0.52	0.13	ATP synthesis
Cytochrome B	0.51	0.19	ATP synthesis
NADH dehydrogenase	0.56	0.10	ATP synthesis
β-carotene 15,15 dioxygenase	0.59	0.15	Differentiation
TGF-beta receptor	0.59	0.39	Proliferation and differentiation

[a] Ratio determined by Quantarray of triplicates after detection using Scanarray and normalizeation to β-actin.

Table 4.3. ZD alterations in fluoxetine-responsive genes in the olfactory bulb.

GENE	MEAN RATIO[a]	SD	FUNCTION
ATP synthase	0.47	0.13	ATP synthesis
BN/ SsNHs	0.33	0.06	Zinc finger protein
Cytochrome C Oxidase subunit I	0.35	0.12	ATP synthesis

[a] Ratio determined by Quantarray of triplicates after detection using Scanarray and normalizeation to β-actin.

Discussion

As discussed in previous sections, antidepressants such as fluoxetine not only enhance stem cell proliferation in the dentate gyrus, but are dependent on this mechanism for efficacy. We also know that zinc is a key modulator of gene expression. Thus, because we have shown that dietary zinc deficiency impairs stem cell proliferation and reduces the effectiveness of this SSRI in the Porsolt swim test, we hypothesized that zinc modulates fluoxetine-mediated gene expression in the hippocampus.

Microarray analysis, using mRNA isolated from fluoxetine-treated rats fed either a ZA or ZD diet, identified several potential gene targets for future study. The differentially regulated genes fell into two functional classes: 1) genes that code for mitochondrial proteins involved in electron transport and mitochondrial function, and 2) genes that code for proteins involved in cellular proliferation and differentiation.

Mitochondrial Gene Expression

Mitochondria are unique organelles that have their own circular DNA that is approximately 16,500 base pairs in length. While there are hundreds of proteins encoded by nuclear genes that are imported from the cytosol into the mitochondria, there are 13 proteins encoded by mitochondrial DNA (MtDNA). Mitochondrial DNA also codes for two rRNAs (16S and 12S rRNA), and tRNA for each of the 20 amino acids. Interestingly, in this study all of the mitochondrial proteins that were regulated by dietary zinc are protein subunits encoded by MtDNA, suggesting that zinc has a specific role in the expression of MtDNA. For example, mammalian cytochrome *c* oxidase has 13 subunits of which subunits 1-3 are mitochondrially encoded. Our work showed that the catalytic subunit 1, and subunit 2 were both down-regulated by zinc deficiency. However, we identified no changes in the nuclear-encoded subunits of mitochondrial cytochrome *c* oxidase. Similarly, we found that the gene that codes for subunits 6 and 8 of mitochondrial ATPase were down-regulated in ZD animals. Only subunits 6, 8 and 9 of this ATPase are derived from MtDNA. Furthermore, the genes for subunits 6 and 8 partially overlap and are translated in different reading frames.

Although the finding that genes involved in mitochondrial respiration may suggest that dietary zinc deficiency would disrupt energy metabolism, our previous work, where we carefully

measured energy expenditure by continuous monitoring of oxygen consumption and metabolic rate, found no significant differences between ZD and PF rats (22). In fact, all of the changes in metabolic rate could be explained by the reduction in food intake and body weight. Thus, it appears that the zinc-mediated changes in hippocampal gene expression that we report likely do not result in alterations in metabolic rate. Given our data on the role of zinc in stem cells in this region of the brain, we have hypothesized that zinc deficiency is acting on the mitochondria of fluoxetine-treated rats to impair neurogenesis, induce apoptosis, and reduce the efficacy of this SSRI.

The down-regulation of mitochondrial genes by zinc deficiency in fluoxetine-treated rats is consistent with our current understanding of the role of mitochondria in depression. For example, a recent report utilizing muscle biopsy and mitochondrial analysis provided evidence for mitochondrial dysfunction in patients with major depressive disorder. There was reduced mitochondrial enzyme activity and impaired ATP production compared to controls (248). While it is impossible at this time to say if these differences would also be seen in hippocampal mitochondria of depressed patients, it is interesting to speculate that zinc deficiency-induced mitochondrial dysfunction may lead to depression-like behaviors seen in the current work. Furthermore, monoamine oxidase inhibitors that act as antidepressants have been reported to improve mitochondrial efficiency and protect against mitochondrial insults (249). This suggests that zinc deficiency may prevent fluoxetine-mediated improvement in mitochondrial function.

The finding that zinc is involved in fluoxetine-mediated mitochondrial gene expression is also consistent with previous work showing that the mitochondria play a critical role in neurogenesis and synaptic plasticity (250). Not only do mitochondrial proteins increase during brain development (251), mitochondrial mass increases 3-fold during neurogenesis. Furthermore, mitochondrial proteins such as ATP synthase, and cytochrome *c* oxidase are needed for neurite out-growth and synaptogenesis (252). DMSO-induced differentiation also increased ATPase in activity neuroblastoma cells (253). While the effect of zinc deficiency on ATPase in the central nervous system has not been previously tested, zinc supplementation has been shown to increase ATPase activity by 20% in the hippocampus (254) and peripheral nerves of guinea pigs (255). This can be explained by the presence of a heavy-metal-associated repeat domain on the NH terminal of ATPase. Binding of zinc to this domain leads to conformational changes and increased activity (256). Thus it appears that not only does zinc deficiency decrease ATPase

mRNA, as shown in the current study, but may also regulate this enzyme by impairment of the catalytic activity.

Changes in mitochondrial gene expression may result in apoptotic cell death in the hippocampus. The mitochondrion has been previously implicated in the pathways involved in the initiation of programmed cell death (257-259). For example, it has been shown that zinc deficiency induces apoptosis in T-cells of the immune system (260), skin keratinocytes (261), airway epithelial cells (262), hepatocytes, glioma cells, kidney cells, monocytes, fibroblasts, and testicular cells (263). Previous work has also shown that zinc chelation with TPEN increased cytochrome *c* release into the cytosol leading to apoptotic cell death (264, 265). Interestingly, antidepressant drugs appear to have the opposite effect on cytochrome *c*. Fluoxetine inhibited opening of the mitochondrial permeability transition pore and cytochrome *c* release, protecting cells from apoptotic death (266). The work in Chapter 2 showed that zinc deficiency reduces the number of proliferating stem cells in the SGZ of the dentate gyrus. While it is likely this is the result of reduced cellular proliferation, based on the microarray data reported here it is also reasonable to hypothesize that the reduced number of Ki67-positive cells may be the result of zinc deficiency-induced increases in stem cell apoptosis. Future work will be needed to examine the role of zinc in apoptosis in the SGZ and GCL of the dentate gyrus, as well as other regions of the brain that may be susceptible to zinc deficiency-induced damage.

Proliferation and Differentiation Genes

Given the large number of zinc finger proteins and their role in gene expression, it is reasonable to hypothesize a role for zinc in cellular proliferation and differentiation. In the developing brain a number of zinc finger proteins have been shown to play an essential role in these processes. For example, cellular nucleic acid binding protein (CNBP), a zinc finger protein, is needed for mouse forebrain development. CNBP-null mice had a significant reduction in cellular proliferation in anterior regions of embryos at the stages of gastrulation and neural-folding (267). Zac1 (268) and LMO4 (269), proteins that contain zinc fingers formed by different zinc-binding motifs, also regulate central nervous system proliferation during development.

While zinc is clearly needed for the structure and function of zinc finger proteins, there is very little reported evidence for zinc finger proteins that are regulated by zinc at the level of transcription. Thus, we found it interesting that mRNA for the zinc finger protein BN/SsNHs was

found to be reduced by ZD in both the OB and the hippocampus of fluoxetine-treated rats. The functional significance of BN/SsNHs mRNA regulation is confounded by the fact that BN/SsNHs has not been fully characterized. Furthermore, we do not know whether this decrease is the result of decreases in transcription or a reduction in mRNA stability. However, we do believe that the reduction in mRNA is specific to zinc. Previous work from our lab has shown that 2 weeks of zinc deficiency, without fluoxetine treatment, reduced BN/SsNHs mRNA in the OB. Furthermore, PF animals whose caloric intake was matched to that of the ZD animals, did not have a change in BN/SsNH mRNA, suggesting that zinc restriction, not caloric restriction, was responsible for the decrease in mRNA.

Once stem cells have proliferated in the SGZ, they migrate in the GCL where they differentiate into functional neurons or supporting glial cells. Several early studies suggested that dietary zinc deficiency impaired neuronal differentiation in the cerebellar cortex (56, 270), but did not explore possible mechanisms responsible for this. The current work suggests that zinc regulation of the enzyme β-carotene 15, 15-dioxygenase may play a role in zinc modulation of fluoxetine-mediated neuronal differentiation. This enzyme is responsible for the metabolism of β-carotene to vitamin A. It has been isolated from brain tissue (271) and cleaves β-carotene into retinal, which is in turn converted to retinoic acid (RA) (271-273). RA is a key transcriptional regulator of neuronal differentiation. In fact, recent work has shown that RA is essential for neuronal differentiation of stem cells in the dentate gyrus (274). RA depletion did not alter stem cell proliferation, but did impair survival of these cells (274). Based on these data, we hypothesize that in fluoxetine-treated rats, zinc deficiency alters stem cell survival and differentiation via reductions in β-carotene 15, 15-dioxygenase. Future studies will be needed to determine if fluoxetine independent of ZD increases expression of this enzyme. This work is important in light of a previous study showing that 6 weeks of a low zinc diet (3 ppm) did not impair intestinal β-carotene 15,15-dioxygenase (275). Thus it is possible that hippocampal expression of this enzyme is regulated differently from intestine, or that zinc deficiency specifically impairs fluoxetine-mediated increases in β-carotene 15, 15-dioxygenase.

Another finding of the current work is the observation that the hippocampal receptor for transforming growth factor-beta (TGFR) mRNA is decreased by zinc deficiency in fluoxetine-treated animals. This is a potentially significant finding for understanding the data reported in this report, because TGF-β, an anti-inflammatory cytokine, has been shown to participate in

cellular proliferation, differentiation (276, 277), survival (278, 279) and migration (280). It has also been implicated in axon growth and synaptogenesis (281). TGF-β is found in all areas of the CNS including the cortex, hippocampus, striatum, brainstem and cerebellum. Both TGF-β and its receptor are expressed in microglia (282, 283), astrocytes (282, 284-286) and neurons (284). In the hippocampus, TGF-β is found in pyramidal neurons and dentate granule cells as well as astrocytes.

The effects of TGFR are mediated by binding to TGF-β (I, II, or III), and activation of SMAD proteins by phosphorylation of SMAD 2/3 and SMAD 4. SMAD 2/3-4 complexes along with Co-SMAD proteins are then translocated into the nucleus (287) where they regulate downstream genes (288). TGF-β/TGFR association and these downstream pathways regulate astrocyte proliferation and differentiation. TGF-β inhibits astrocyte proliferation (285) by arresting cells at the G1/S phase of the cell cycle (289). It also increases astrocyte differentiation and migration (279, 280). In contrast, the proliferation of ramified microglia is inhibited by TGF-β (283). The finding that zinc deficiency reduced TGFR in the hippocampus of fluoxetine-treated rats suggests that differentiation and migration in the dentate gyrus may be altered. Future work will be needed to address the role of zinc in this process by using markers for astrocytes and other microglia in the dentate gyrus.

While the role of TGF-β in neuronal precursor proliferation and differentiation is complex and not fully understood, there is evidence that this growth factor participates in the regulation of neurogenesis by increasing neuronal differentiation (287, 290). TGF-β concentrations in the dentate gyrus have recently been correlated with stem cell proliferation in the SVZ (291). Immunoneutralization of TGFR reduced BrdU labeling in the dentate. This was also true in primary cultures of neuronal stem cells (291). Thus, the finding that zinc deficiency reduced TGFR mRNA in the hippocampus is consistent with our earlier finding that the number of Ki67-positive stem cells in the dentate is reduced by zinc deficiency. Immunoneutralization of TGF-β in primary cultures of cortical neurons reduced the neuroprotective factor Bcl-2 and led to neuronal apoptosis (279). Thus it appears that the decreases in TGFR that we report here may be consistent with our hypothesis that zinc deficiency increases apoptosis in the dentate gyrus. It should also be noted that TFGR mRNA is differentially regulated in the hippocampus, but not in the OB. Thus, while TGFR mRNA is expressed in the OB, it appears that the zinc regulation my

be specific to the hippocampus. Future work will be needed to determine the role of zinc and fluoxetine in the regulation of this important receptor.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The preceding chapters have outlined a series of experiments designed to examine the effect of zinc deficiency on stem cell proliferation in the adult rat dentate gyrus. Not only does this work show that zinc deficiency impairs stem cell proliferation in this important region of the brain, but has also linked these findings to behavioral outcomes that are consistent with depression and anxiety. Interestingly, the work not only causally linked zinc deficiency with depression-like behaviors, but also suggested that dietary restriction of this essential nutrient may impair the efficacy of the antidepressant drug fluoxetine. We believe that these data warrant future clinical investigations on the use of zinc supplements as an adjunct to antidepressant therapy, particularly in patients who are refractory to pharmacological treatment.

The final chapter of this work was designed to identify some possible mechanisms that may be at work in the interactions between zinc and fluoxetine. By altering the diets of fluoxetine-treated rats, we were able to identify a number of genes that may help to provide a link between dietary zinc and neurogenesis. Figure 5.1 is a diagram depicting a possible sequence of events, based on the data collected here, that could lead to reduced stem cell proliferation, reduced neurogenesis, and the behavior outcomes that we report. In this model zinc deficiency alters gene expression in the cells of the SVZ of the dentate gyrus. While we in no way claim to have identified all of the molecular players in this process, we identified two genes, one that codes for the enzyme β-carotene 15, 15-dioxygenase, and one that codes for the TGF-β receptor that appear to be involved in stem cell proliferation and neuronal differentiation. Furthermore, we identified a larger group of mitochondrially encoded genes that may, along with the nuclear encoded TGF-β receptor, mediate apoptotic death in the dentate. Together these mechanisms, leading to reduced stem cell proliferation, impaired neuronal differentiation, and increased apoptosis, would result in reduced neurogenesis. Impairment of neurogenesis would not only be expected to increase the incidence of depression and depression-related symptoms

such as anxiety, but because antidepressant drugs require neurogenesis in the dentate for their activity, would impair the efficacy of these pharmacological treatments (Figure 5.1).

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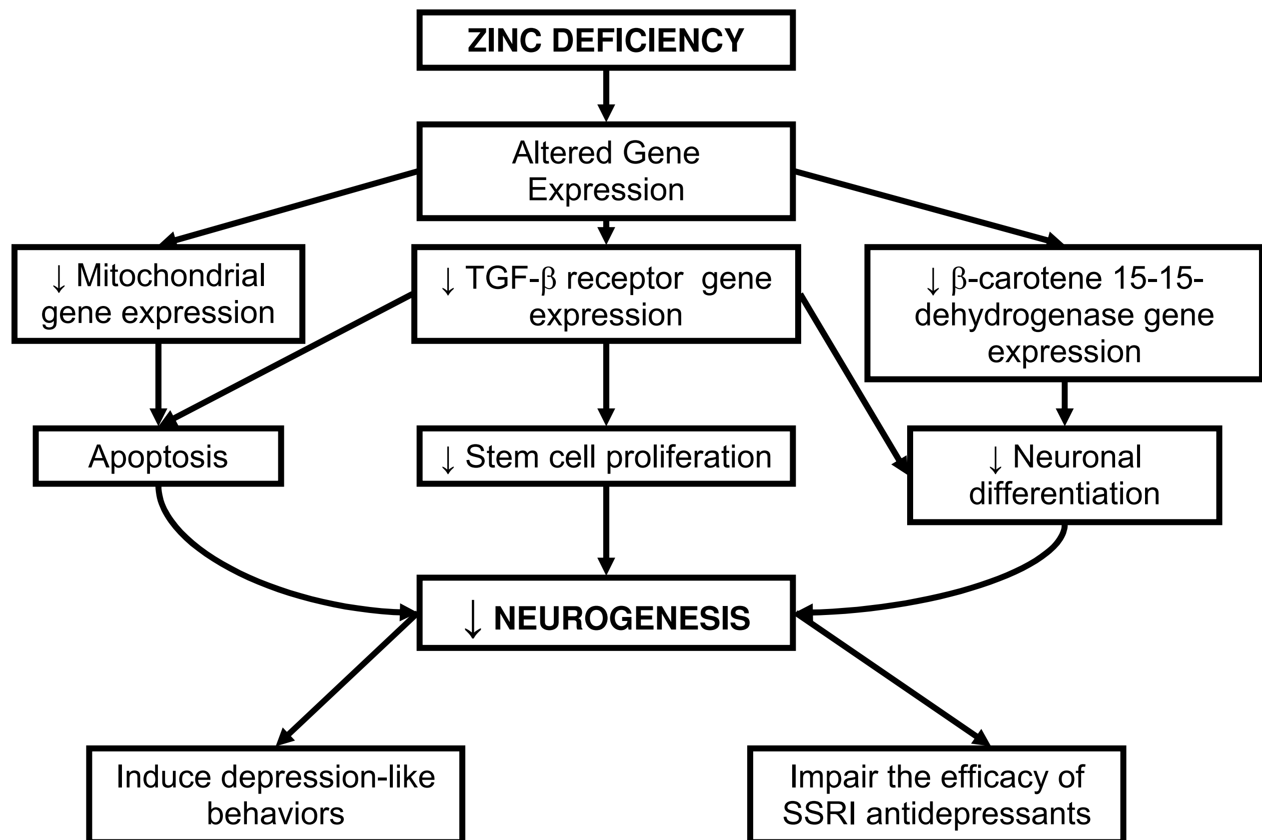

Figure 5.1. Summary of dissertation findings.

Future Directions

Future work will be needed to fully understand the role of zinc in stem cell proliferation and the development of depression. For example:

1. The current work employed a diet that was severely deficient in zinc. The rationale for this level of zinc was to ensure that if there were effects, our data would be robust enough to identify them. However, it is rare that adult humans who are free of genetic disorders of zinc metabolism would suffer from this severe level of deficiency. On the other hand, moderate or mild zinc deficiency is thought to be a common world-wide problem. Thus future studies should test the effects of more moderate levels of dietary zinc deficiency (e.g. 5-10 ppm) on stem cell proliferation, neurogenesis, symptoms of depression and antidepressant efficacy. Furthermore, because humans who are zinc deficient are likely to suffer from chronic zinc restriction, other studies would examine the effect of long-term (3-6 months) moderate zinc deficiency on these indices of hippocampal function.
2. Fluoxetine and other antidepressants work via stem cell proliferation. Future work should test the hypothesis that zinc deficiency impairs fluoxetine-mediated stem cell proliferation. This work could also be extended to include the effect of zinc in other conditions that we have previously been shown to enhance stem cell proliferation, such as voluntary exercise and environmental enrichment.
3. The work described here explored the interactions between zinc and the SSRI fluoxetine. This should be expanded to examine the role of zinc in the efficacy of other antidepressant drugs such as noradrenergic reuptake inhibitors and monoamine oxidase inhibitors. Furthermore, because it appears that zinc deficiency induces behaviors that are consistent with anxiety, the effect of zinc on the efficacy of anxiolytic agents should be examined.
4. Immunodetection of Ki67 permits the identification of cells that are undergoing proliferation. This enabled us to examine the effect of zinc deficiency on stem cell proliferation. However, it did not permit us to examine the effect of zinc on stem cell survival. This could be accomplished by injecting rats with BrdU for several days, followed by the development of dietary zinc deficiency. Immunolocalization of BrdU in zinc deficient and zinc adequate animals at 4-6 weeks later.

5. Several lines of evidence collected in the course of this work suggest that zinc deficiency may induce apoptotic mechanisms in the dentate gyrus. Thus, TUNEL labeling or other methods should be used to test this hypothesis directly.

6. Other evidence suggests that zinc may also regulate cellular differentiation in the GCL of the dentate. Thus, future studies should employ a variety of cell-specific markers such as GFAP, TuJ1, and nestin in the long-term model of zinc deficiency described in # 4.

7. Clearly the role and regulation of the genes identified by microarray will require follow-up studies. This will enable us to not only better understand the role of zinc in hippocampal gene expression, but also in fluoxetine-mediated mechanisms.

8. The microarray study described here enabled us to screen a limited number of genes for regulation by zinc deficiency in fluoxetine-treated rats. While this work enabled us to identify some targets of zinc action, future studies should use a more comprehensive cDNA chip to address this question.

9. Because depression has been shown to reduce hippocampal volume in animals and depressed human patients, future work should quantify hippocampal volume in ZA, ZD, PF, and ZS rats.

10. The forced swim test used here enabled us to test the efficacy of a SSRI. We found that in ZA animals it was effective but was not effective in ZD. Because this antidepressant functions by increasing serotonin levels in the synaptic cleft, future studies should measure the amount of serotonin in the synaptic cleft in ZD compared to ZA. This can be done using either microdialysis or micropunch.

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BIOGRAPHICAL SKETCH

Nadine Tassabehji was born in Beirut, Lebanon. During the civil war her family moved to Saudi Arabia where she attended The Continental School in Jeddah, Saudi Arabia. In 1995, she graduated from high school and moved to the United Kingdom where she joined the Malvern Girls' College in Worcestershire. After two years, she completed her A-levels in chemistry, biology and math and AS level in French. For her undergraduate studies, Nadine went back to Lebanon and attended the American University of Beirut where she received her Bachelor degree in Dietetics and Food Technology. At AUB she was involved in many activities such as the Environment club and in 1998 she was elected as a member in the Student Representative Committee. At Florida State University Nadine completed a Masters degree in Nutrition in 2003 under the supervision of Dr. Cathy W. Levenson. Her thesis work examined the role of copper in the regulation of the transcriptional factor p53. After completion of her master's degree (2003), Nadine continued working in Dr. Levenson's laboratory on her doctoral dissertation. At FSU, she had the opportunity to teach HUN1201 Science of Nutrition course. Nadine Tassabehji is a member in the American Society of Nutritional Sciences, North American AUB Alumni Association, American Dietetic Association, the Lebanese Dietetic Association and the Sigma Xi Society. Over the course of her graduate studies Nadine has received several honors and awards including; College of Human Sciences Dissertation Research Grant, Fall 2005, Gerber Foundation Predoctoral Fellowship of American Society for Nutritional Sciences in 2005, Susan E. Lucas Fellowship in Fall 2004, Robinson Endowed Fellowship in 2004 and 2005, Anne-Marie Erdman Scholarship 2002-2004, Elsie Thomas Miller Scholarship in 2003, Hallie Deaton Choate Scholarship in 2002. She has been married to Tarek A. Zeidan since 2002. After her graduation, she will pursue her career in research and continue being involved in teaching by working in academia.