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Zinc Regulation of Neural Stem Cell Proliferation and Antidepressant Efficacy

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ZINC REGULATION OF NEURAL STEM CELL PROLIFERATION AND ANTIDEPRESSANT EFFICACY

By

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ABSTRACT

Changes in zinc homeostasis are strongly associated with abnormal brain function and a variety of neurological and neuropsychiatric disorders, including depression. It is hypothesized that the neurogenic potential of chronic antidepressant administration contributes to its therapeutic effects in depression. Thus, the goal of this work was to determine the extent to which zinc is needed for antidepressant drug induction of neural stem cell proliferation and differentiation. Human NTERA-2/D1 (NT2) cell culture, an established in vitro model system to study neuronal development, was utilized. Zinc deficiency impaired NT2 cell proliferation measured by the number of Ki67-positive cells. Treatment with fluoxetine or lithium did not result in a significant increase in cell proliferation rate. However, six-day treatment with these antidepressants had a stimulatory effect on NT2 cell differentiation revealed by immunofluorescent detection of the neuron-specific marker TuJ1. Furthermore, zinc deficient cultures treated with fluoxetine or lithium appeared to have a decreased expression of this neuronal marker. Taken together, these results suggest that the essential trace element zinc is needed for neuronal stem cell proliferation and differentiation.
CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Zinc and its Functions

Zinc is an essential nutrient that affects many fundamental biological processes. Imbalances in zinc homeostasis, either deficiency or excess, can lead to a long list of severe detrimental effects. For instance, nutritional zinc deficiency causes alopecia, growth failure, skin lesions, and immune and reproductive dysfunction, among others. Being the second most abundant trace metal in the body after iron, zinc provides the structural integrity to a variety of proteins, including regulatory, structural and enzymatic (Choi and Koh, 1998). Further, zinc is considered to play a vital role in the regulation of nucleic acid synthesis and transcription that are necessary for cell replication and maturation. Specifically, zinc serves as an essential structural component of DNA and RNA polymerase and DNA-binding proteins that contain zinc-binding domains and are thus called zinc finger proteins. Zinc finger proteins act as transcription factors, bind to the specific sequences of DNA and, consequently, affect gene transcription and cell proliferation (Bulyk et al., 2001; Nakamura et al., 2004). Interestingly, zinc finger proteins are coded by up to 1% of the human genome (Frederickson et al., 2000).

Additionally, recent studies demonstrate that there is an association between zinc and normal brain function. Zinc is found at high concentrations in central nervous system (CNS), specifically, in neurons of the cortex and hippocampus (Nowak, 1998). It is reported that this trace metal is concentrated in the synaptic vesicles of a subset of glutamate releasing neurons and released during synaptic transmission. Zinc interacts with pre- or postsynaptic membrane receptors, and thus acts as a neuromodulator (Kay and Toth, 2008). Furthermore, zinc is found to modulate both excitatory and inhibitory amino acid neurotransmission (Barañano et al., 2001; Frederickson et al., 2000; Trevorg et al., 2004; Smart et al., 2004). In addition, zinc may regulate the activity of brain acetylcholinesterase, which is known to regulate behavioral processes. This membrane-bound enzyme is present on the postsynaptic membrane and important for cholinergic function as well as for cell survival, and neurite outgrowth (Whyte and Greenfield, 2003). Certain neurotoxic drugs or metals and free radicals can elevate the enzyme activity of acetylcholinesterase, but zinc administration has been found to improve the activity of this enzyme (Bhalla et al., 2007). Moreover, zinc is known as a complex antioxidant. It is a cofactor for superoxide dismutase
(SOD) and other enzymes that capture free radicals. Studies have shown that SOD has a protective action against neuronal death and brain injury (Greenlund et al., 1995; Uyama et al., 1992). It has been reported that zinc protects against H$_2$O$_2$-induced cytotoxicity, stabilizes cell membranes, and inhibits lipid peroxidation (Chung et al., 2005; Sidhu et al., 2005; Sidhu et al., 2006). Inhibiting lipid peroxidation can further regulate the membrane fluidity and ultimately normalize the activity of acetylcholinesterase (Bhalla et al., 2007).

Importantly, changes in zinc homeostasis have been linked to a wide variety of neurological and neuropsychiatric disorders, such as dementia, schizophrenia (Andrews, 1992), Alzheimer’s disease (Cuajungco and Lees, 1997b; Lovell et al., 2006), and Parkinson’s disease (Forsleff et al., 1999). Recent research demonstrates that alterations in blood and brain zinc concentration may be involved in the pathophysiology of depression (Nowak et al., 1999; Wojcik et al., 2006).

**Neural Stem Cells and Adult Neurogenesis**

**Neural Stem Cells (NSCs).** Neural stem cells are clonogenic and multipotent cells that have the capability of both self-renewal and giving rise to all different cell types of the CNS: neurons, astrocytes, and oligodendrocytes (Steindler and Pincus, 2002). The series of NSC developmental changes that result in the appearance of a new neuron is known as neurogenesis (Kempermann, 2002). Neural stem cell differentiation into glia is called gliogenesis. Recently, it has been largely accepted that NSCs are present in the adult mammalian brain and can serve as a continuous source for newly developed neurons throughout adulthood. Indeed, these cells have been isolated from the adult rodent brain, induced to proliferate in vitro, and subsequently developed into neurons in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) (Gage et al., 1995; Reynolds and Weiss, 1992; Richards et al., 1992).

The evidence for NSCs in adult brain potentially opens exiting prospects in the treatment of a wide range of disorders where neuronal tissue is diseased or damaged and can be replaced by new healthy neurons (Conti et al., 2003). Among these disorders are traumatic brain injury, stroke, Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease (Temple, 2001). Recently, it has been proposed that depression can be one of these disorders where the production of new neurons may also have a therapeutic effect (Jacobs et al., 2000).

There are several phases of neurogenesis: NSC proliferation (the process of cell replication or reproduction), survival, and differentiation into neurons. There are also other important aspects of
the neurogenesis such as neuritogenesis, synaptogenesis, generation of action potentials, and development of functional connections (Kempermann, 2002). Current work is focused on understanding the regulation of NSC proliferation and differentiation. To confirm cell proliferation, 5-bromo-2’-deoxyuridine (BrdU), a thymidine analog, is mainly used because it incorporates into the DNA during the S-phase (DNA synthesis phase) of the cell cycle and thus is a good marker for dividing cells. Additionally, cell proliferation can be determined by using the immunolabeling for Ki67. The Ki67 is a nuclear antigen expressed by proliferating cells in all active phases of the cell cycle (G1, S, G2, and M phase) and is absent in resting (G0) cells (Misiuta et al., 2006). However, the fact that cells can proliferate does not mean that they are NSCs. To confirm the NSC properties, cells have to be immunopositive for a specific marker, the intermediate filament protein nestin (Frederiksen and McKay, 1988). To confirm the efficient differentiation into neurons from NSC, cells have to be positive for the specific neuronal markers. For example, the neuron-specific class III beta-tubulin TuJ1 is highly expressed by the dopamine neurons (Park et al., 2005).

**Adult Neurogenesis.** The production of new neurons in adulthood is established to occur in nonlimbic brain areas, such as the subventricular zone (Kukekov et al., 1999; Palmer et al., 1995; Pincus et al., 1998) and limbic brain areas, such as the subgranular zone of the dentate gyrus of the hippocampus (Arsenijevic et al., 2001; Eriksson et al., 1998; Gage, 2002) and the olfactory bulb (Gritti et al., 2002; Peterson, 2002). Other studies indicate that other brain areas, such as the substantia nigra and the cerebral cortex, also seem to have neurogenic potential (Lie et al., 2002; Magavi et al., 2002). In another study, phenotypic analysis shows that neurogenesis dominated the dentate gyrus, whereas in the medial prefrontal cortex most newborn cells are glia (Czech et al., 2007). However, the evidence for the possible de-differentiation of glia to cells with stem-cell-like properties assumes that various brain regions also may be involved in generation of new neurons (Alvarez-Buylla et al. 2001; Laywell et al., 2000).

It is estimated that the hippocampus of the adult rodent brain generates 9,000 new cells each day, or approximately 250,000 per month with about 50% of these cells identified as new granule neurons (Cameron and McKay, 2001). On the other hand, many of these newly born cells die between one and two weeks after they are born (Gould et al., 1999b). Cameron and McKay (2001) indicated that if these newly generated cells were to survive four weeks, the numbers of young granule neurons could be as large as 138,000, or 6% of the total granule cell population of 2.4
million (West et al., 1991). In humans, rates of neurogenesis are not as high as in rodents, but still significant. The functions of these young granule neurons have not been clearly identified. However, the large number of newly generated neuronal cells suggests an important role of adult neurogenesis in brain function. Moreover, recent experiments indicate that these cells participate in hippocampal-dependent memory (Shors et al., 2001). Also, there are observations that decreased neurogenesis is involved in the pathophysiology of neurological and mood disorders (Duman et al., 2001; Kempermann, 2002).

Notably, the rate of adult neurogenesis can be regulated by numerous factors. For example, studies in adult rodents indicate that neurogenesis decreases with age and can be reversed by exposure to an enriched environment and voluntary exercise (Peterson, 2002; van Praag et al., 1999). Furthermore, recent findings have shown that stress produces a significant damaging effect on neurogenesis due to activation of the hypothalamic-pituitary-adrenal axis (HPA) and elevation of glucocorticoid levels (Brown et al., 1999; Gould et al., 1997; Gould et al., 1998). Nonlimbic brain structures are not affected by stress to the same degree (Czeh et al., 2007). Corticosteroid reduction by adrenalectomy in aged rats appears to restore the rate of neurogenesis to the level of that seen in young adult animals, which supports the idea of the important role of adrenal steroids in the neurogenesis status throughout adulthood (Cameron and McKay, 1999). The exact mechanisms involved in the regulation of neurogenesis are still not completely understood, although certain trophic factors, such as brain-derived neurotrophic factor (BDNF), FGF2, and insulin-like growth factor 1 (IGF-1) are known to up-regulate neurogenesis (Aberg et al., 2000; Palmer et al., 1997; Yoshimura et al., 2001; Zigova et al., 1998). The expression of these trophic factors are increased by exercise (Carro et al., 2000; Neeper et al., 1996; van Praag et al., 1999) and inhibited by glucocorticoids (Hansson et al., 2000; Schaaf et al., 1998; Smith et al., 1995). Long-term administration of antidepressant therapies, including electroconvulsive therapy and antidepressant drugs, has also been shown to produce new neurons in the adult mammalian brain (Chen et al., 2000; Czeh et al., 2006; Perera et al., 2007; Santarelli et al., 2003).

Thus, understanding the mechanisms that are involved in the regulation of neurogenesis can contribute to the development of powerful therapeutic tools to help patients with neurobiological illnesses, including depression.
Adult Neurogenesis, Depression, and Antidepressants

Depression is a common devastating psychiatric disorder that affects millions of Americans each year (McIntyre and O'Donovan, 2004). It is a recurrent and potentially life-threatening illness that may lead to suicidal attempts, and is one of the major causes of mortality and morbidity (Woods, 2000). There are several neurotransmitter and neurohormonal pathways that have been implicated in the pathophysiology of depression, but the mechanisms underlying the pathogenesis of this complex illness remain unknown (Vaidya and Duman, 2001). According to the previous monoaminergic hypothesis, depression results from a deficiency of monoamine neurotransmitters, notably serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE). However, several studies have shown that monoamine depletion does not produce depressive symptoms in healthy individuals (Heninger et al., 1996; Vaidya and Duman, 2001). It has been suggested that this illness may be a result of dysregulation of the HPA, hippocampus, frontal cortex, nucleus accumbens, and amygdala. These limbic brain areas are involved in learning, motivation, responses to reward and aversive stimuli, eating, sleeping, and energy level, which are found to be impaired in patients with depression (Nestler et al., 2002). Furthermore, post mortem and brain imaging studies have shown the atrophy or volume loss of the prefrontal cortex and hippocampus of patients with mood disorders (Gurvits et al., 1996; Sheline et al., 1999; Sheline, 2000). These studies, together with reports that depression is often caused or worsened by stress or other aversive stimuli, have contributed to a new, neurotrophic hypothesis that posits that the pathogenesis of depression is likely to involve damage to hippocampal and cortical neurons. Impaired neurogenesis, including neuronal plasticity, has been proposed to play a critical role (Duman et al., 2004).

Among treatment approaches to depression is the administration of antidepressant therapies, including classic treatments such as lithium and electroconvulsive therapy (ECT), and recently developed antidepressant drugs (ADs). Most ADs normalize brain levels of monoamine neurotransmitters such as 5-HT, NE, and dopamine and down-regulate their postsynaptic receptors. However, the immediate increase in brain levels of these neurotransmitters is not correlated with the onset of AD clinical effects, which are often delayed by 3 to 4 weeks (Wong and Licinio, 2001). This delay may underlie slow cellular and molecular adaptations in the limbic structures of brain in response to ADs. Only recently have we learned that long-term AD treatment may block or even reverse depression-related neuronal damages via increased neurogenesis.
Different classes of AD drugs, including selective serotonin and norepinephrine reuptake inhibitors, were shown to induce/normalize cell proliferation in adult mammalian hippocampal dentate gyrus and medial prefrontal cortex and in neural cultures in vitro (Czeh et al., 2006; Lee et al., 2001; Malberg and Duman, 2003; Malberg et al., 2000; Manev et al., 2001; Santarelli et al., 2003). Phenotypic analysis revealed that the majority (70-77%) of newly generated cells in the dentate gyrus expressed the neuronal marker NeuN, whereas in the medial prefrontal cortex most newborn cells were glia (Czeh et al., 2007). Other studies have demonstrated up-regulation of cell proliferation in the hippocampus by ECT (Malberg et al., 2000; Perera et al., 2007), an effect seen with long-term lithium administration as well (Chen et al., 2000). Lithium treatment has been shown to stimulate proliferation of hippocampal neural progenitor cells both in vivo and in vitro (Hashimoto et al., 2003). Also, it was demonstrated that lithium induces progenitor cell proliferation in rat cerebellar granule and cerebral cortical cultures (Kim et al., 2004). Additionally, lithium has been shown to increase the viability and proliferation of human NT2 cells (Misiuta et al., 2006). Thus, an increase in proliferation seems to be a common cellular effect of all AD treatments.

Antidepressants have been shown to regulate hippocampal neurogenesis also via antiapoptotic pathways. In animal model of stress, administration of the AD fluoxetine (selective serotonin reuptake inhibitor, SSRI) significantly reduced apoptosis in the dentate gyrus and temporal cortex (Lee et al., 2001), while treatment with tianeptine (atypical AD) had an antiapoptotic effect both in psychosocially stressed and control animals (Lucassen et al., 2004). Another group of investigators found that low doses of ADs, such as amitriptyline or venlafaxine (both tricyclics), enhanced the intensity of Bcl-2 (B cell lymphoma protein-2) immunostaining in rodent hippocampal mossy fibers (Xu et al., 2003). The protein Bcl-2 is known to play a neuroprotective role and has been demonstrated to have antiapoptotic functions and promote neuronal regeneration in the mammalian CNS (Chen et al., 1997).

The exact molecular mechanisms of how ADs work have not yet been determined, but it is unquestionable that they are complex and need to be evaluated. There are several studies demonstrating that chronic, but not acute, AD treatment up-regulates the cyclic adenosine monophosphate (cAMP) signal-transduction cascade, increases activity and expression of cAMP response element-binding protein (CREB), and enhances expression of BDNF in the hippocampus (Nibuya et al., 1995; Nibuya et al., 1996; Thome et al., 2000, Xu et al., 2003). Up-regulation of
BDNF may contribute to neurotrophic effects of ADs, including neuronal generation and differentiation (Duman et al., 2000).

Most recently, Pechnick et al. (2008) have demonstrated that the AD imipramine (tricyclic) down-regulates the expression of the cyclin-dependent kinase inhibitor p21, which inhibits cell cycle progression and thereby cellular proliferation.

Several studies indicate that the induction of adult neurogenesis by SSRIs may be via regulation of the 5-HT system and 5-HT receptors (Knobelman et al., 2001; McEwen, 2000). Indeed, SSRIs increase synaptic levels of 5-HT and 5-HT has been implicated in the generation of new neurons in the mammalian dentate gyrus (Brezun and Daszula, 2000; Gould, 1999). It is reported that the neurogenic action of SSRIs is mediated by activation of 5-HT$_{1A}$ subtype receptors (Santarelli et al., 2003). Also, activation of 5-HT$_7$ receptors has been associated with initiation of the intracellular signaling system, such as cAMP cascade (Duman et al., 2000). There is also the possibility that other receptors may underlie the AD response in humans.

Taken together, all these findings indicate that ADs down-regulate multiple types of receptors, activate intracellular cascades, and alter gene expression, which contribute to their neurogenic potential.

Importantly, hippocampal neurogenesis seems to be necessary for the therapeutic effect of ADs (Perera and Lisanby, 2000). Focal x-irradiation of mouse brain prevents antidepressant-induced neurogenesis in the subgranular zone of the dentate gyrus of the hippocampus and blocks responses to ADs in two behavioral paradigms, novelty suppressed feeding and chronic unpredictable stress (Santarelli et al., 2003).

**Depression, Antidepressants, and Zinc**

Interestingly, there is an association between depression and the trace metal zinc. High concentrations of zinc have been reported in neurons of cortex and hippocampus (Nowak, 1998), the brain areas that are thought to be involved in depression and where neurogenesis has been shown to occur. Recently, research has demonstrated that alterations in blood zinc concentrations are directly related to depression. For instance, lower serum zinc levels have been found in patients with major and postpartum depression compared to non-depressed controls (Maes et al., 1994a; Maes et al., 1997; Maes et al., 1999; Nowak et al., 1999; Wojcik et al., 2006). Notably, Maes et al. (1997) have found a significant inverse correlation between serum zinc and staging of
depression. Dietary zinc deprivation may influence brain zinc homeostasis, resulting in learning impairment, mental dysfunction, behavioral alteration, and increase of susceptibility to epileptic convulsions (Takeda, 2000). Our laboratory has found that dietary zinc deficiency in adult rats leads to depression- and anxiety-like behaviors, such as anorexia, anhedonia (reduced saccharin: water intake, p<0.001), and decreased exploratory behavior in a light-dark box test [p<0.05] (Tassabehji et al., 2008). Also, rats with low zinc status had a decreased cell proliferation in the dentate gyrus of the hippocampus (Somers et al., 2008). Furthermore, preliminary data from our laboratory have demonstrated a decreased proliferation in zinc deficient NTERA-2/D1 cell line [NT2 cells] (Somers et al., 2008). NT2 cell line derives from human embryonal carcinoma and is used as in vitro model system to study neuronal development because these cells are similar to neural stem cells and can proliferate and be differentiated into neurons (Misiuta et al., 2006).

Notably, there are a large number of patients who do not respond to current ADs (Nestler et al., 2002; Vaidya and Duman, 2001). Moreover, patients who display resistance to AD medications have lower serum zinc levels than treatment responsive patients (Hansen et al., 1983; Maes et al., 1997). Maes et al. (1997) suggest that lower serum zinc is a marker of treatment resistant depression. Furthermore, new studies have demonstrated the benefit of zinc supplementation in AD treatment. A zinc supplemented group of patients with major depression has shown a statistically significant improvement compared with a placebo group after six and twelve weeks of AD administration (Nowak et al., 2003). Using rodent models, our laboratory has confirmed that zinc deficiency limits the efficacy of ADs (Tassabeji et al., 2008). While there is some evidence that ADs up-regulate cell proliferation and neurogenesis, the role of zinc in this complicated process is not clear.

Given the association between zinc deficiency, neurogenesis, depression, and antidepressant action, combined with the evidence for zinc as one of the key elements in cell replication, the current work was designed to study and understand the zinc regulation of neural stem cell proliferation and AD efficacy. First, the role of zinc in the cell cycle was tested under conditions of mild and significant zinc deficiency in human cultured NT2 cells. Second, the effects of fluoxetine and lithium on the cell proliferation and differentiation were examined. Next, whether zinc is needed for these AD effects was tested in these cells. And finally, the abundance of the 5-HT1A receptors in NT2 cells was measured to test the hypothesis that SSRI antidepressants may act through the 5-HT1A receptor-mediated mechanisms.
CHAPTER 2
MATERIALS AND METHODS

Cell Cultures

Human NT2 cells, obtained from Invitrogen, were used for all experiments. Cells were grown in Dulbecco's Modification of Eagle's Medium supplemented with F-12 nutrient mixture (DMEM/F12) media with 10% cosmic calf serum, 1% antibiotic-antimycotic solution, and 0.1% gentamicin in a T-75 flask. Cells were maintained in a 37°C humidified chamber containing 5% CO₂. After reaching 100% confluency, the cell cultures were split and plated at 20-30% confluency for the following appropriate treatment into 6-well plates with glass coverslips for further immunocytochemistry and into 6-well plates without coverslips for cell counts. Cell cultures were settled overnight to permit attachment.

Creating Zinc Deficient State

For the first group of experiments, NT2 cells were treated with DMEM/F12 media containing the zinc chelator N, N, N’, N’-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) at concentrations up to 8 µM for 18-72 hours (n=4). Preliminary data in our laboratory have demonstrated these TPEN concentrations induce zinc deficiency in NT2 cells without killing all cells. For the second group of experiments, NT2 cells were treated with media containing zinc at concentrations of 1 µM or 0.4 µM for 18, 24, or 48 hours (n=4). Untreated cells in normal media (n=4) served as controls (2.5 µM zinc).

Antidepressant Treatment

TPEN treatment or zinc deficient media were used to produce two sets of zinc deficient cultures. Both sets were maintained until AD treatment. Antidepressants fluoxetine (Sigma Chemicals) and lithium chloride (Sigma) were added at concentrations of 1 µM and 1mM, respectively. Previous in vitro studies demonstrated that these concentrations are clinically applicable and comparable to therapeutic plasma concentrations (Edgar et al., 1999; Misiuta et al., 2006). The time periods for treatment with ADs were 24 and 48 hours. Untreated cells served as controls (n=4).
**Cell Count**

Cell count was used to determine cell survival at different time intervals. Six-well plates without coverslips were used to grow NT2 cells. Wells were scored with a grid pattern to permit the identification of the same fields at different time periods. Before treatment, fields were randomly selected and counted according to previously published methods (Levenson et al., 2004) [n=4]. After treatment, cells in chosen fields were recounted at appropriate time intervals.

**Immunocytochemistry**

Immunocytochemical labeling for Ki67 nuclear antigen is an accurate method for measuring cell proliferation (Misiuta et al., 2006) and was used in the current work to determine cells that were in all active phases of the cell cycle. NT2 cells were plated on glass coverslips in 6-well plates and treated as described above. Following treatment, media were aspirated from each well and cells were rinsed with phosphate buffered saline (PBS, pH 7.4) containing magnesium and calcium chloride (PBS+). The cells were then fixed with 3.7% paraformaldehyde in PBS at room temperature for 10 minutes, followed by several rinses with PBS without magnesium or calcium chloride (PBS-). After washing, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Following three additional washes with PBS-, coverslips were incubated in 2N HCl for 30 min. After washing in PBS- three times, cells were incubated with borate buffer at room temperature for 10 min. Cells were washed again three times in PBS-. After the last wash, bovine serum albumin (BSA, 10 mg/ml in PBS-) was added to cells for 15 min to prevent nonspecific binding of the antibody. After blocking with BSA, coverslips were incubated with the primary antibody in BSA (all antibodies description can be found in Table 2.1) for one hour at 37°C in a humidified chamber. The primary antibody was then removed from the coverslips, which, after several PBS- washes, were incubated with secondary antibody in BSA for one hour at 37°C. Cells were then washed three times with PBS- followed by incubation in 4',6-diamidino-2-phenylindole (DAPI, diluted 1:300 in distilled water) for 15 min at room temperature to permit visualization of cell nuclei. The coverslips were then rinsed several times in PBS and mounted on microscope slides with a commercially prepared anti-fade mounting medium (Gel Mount, Biomedia). To prevent photo-destruction, samples were protected from the light. Slides were observed and photographed using a Nikon Microphot-FX microscope equipped with epifluorescence. Exposure times for all photographs were held constant to permit comparison of intensities.
Immunofluorescence detection of neuron-specific class III beta-tubulin TuJ1 was performed as described above, using specific antibodies characterized in Table 2.1.

Immunocytochemistry was also used to determine 5HT1A receptor abundance and localization in NT2 cells. All steps of this procedure were performed as described above excluding the step with permeabilization with 0.2% Triton X-100 in PBS for 5 minutes and following steps with three washes with PBS- and incubation in 2N HCl for 30 min.

Table 2.1. Antibody (Ab) properties for Immunocytochemistry in NT2 Cells

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Rabbit anti-human IgG (1:100)</td>
<td>Cy3 conjugated goat anti-rabbit IgG (1:250)</td>
<td>Biomeda</td>
</tr>
<tr>
<td>SR-1A (H-119)</td>
<td>Rabbit anti-human IgG (1:250)</td>
<td>Cy3 conjugated goat anti-rabbit IgG (1:250)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TuJ1</td>
<td>Mouse anti-human IgG (1:500)</td>
<td>Cy3 conjugated donkey anti-mouse (1:250)</td>
<td>Covance</td>
</tr>
<tr>
<td>Nuclear DAPI</td>
<td>DAPI (1:300)</td>
<td></td>
<td>Sigma Chemicals</td>
</tr>
</tbody>
</table>
CHAPTER 3
RESULTS

Zinc Regulation of Neural Stem Cell Number and Proliferation

Effect of Zinc Deficiency on Cell Number. Addition of the zinc chelator TPEN (3 µM) to NT2 cells resulted in a significant decrease in cell number after 72 hours of treatment, p<0.05 (Figure 3.1). Treatment with 8 µM TPEN resulted in cell death in this time frame. NT2 cells treated with zinc-deficient media, containing zinc at concentrations 1 µM or 0.4 µM, showed a significant decrease in cell number after 48 hours of treatment, p<0.05 or 0.005, respectively (Figure 3.2).

Effect of Zinc Deficiency on Cell Proliferation. NT2 cell proliferation was measured by immunocytochemical labeling for Ki67 nuclear antigen (cell proliferation marker). Immunocytochemistry revealed that treatment with 6 µM TPEN for 18 hours or zinc deficient (0.4 µM Zn) media for 48 hours decreased the nuclear antigen Ki67 abundance in NT2 cells (Figure 3.3 and 3.4), suggesting that cellular proliferation was inhibited by arresting the cell cycle in these cells. To determine the relative abundance of Ki67, the number of Ki67-positive cells was compared with the total cell number determined by DAPI nuclear staining, p<0.005 (Figure 3.5).

Antidepressant Effects on Neural Stem Cell Number and Proliferation

The 24-hour exposure of NT2 cells to 1 µM fluoxetine did not alter cell number. Treatment with 1 µM fluoxetine for 48 hours resulted in only a modest increase in NT2 cell number compared with controls and did not reach statistical significance (Figure 3.6). NT2 cell proliferation was measured by immunocytochemical labeling for Ki67 nuclear antigen (Figure 3.7 and 3.8). After 48 hours, the number of Ki67-positive cells in the fluoxetine- or lithium-treated and the untreated NT2 cultures was not significantly different (Figure 3.9).

Immunocytochemistry using specific antibodies for serotonin receptor, 5HT1A, did not detect any visible staining of this receptor in NT2 cells (data are not shown).
Effect of Zinc Deficiency on Antidepressant Efficacy

Following 48-hour treatment, cell number was determined in zinc adequate and deficient NT2 cells treated with 1 µM fluoxetine or 1 mM lithium chloride (Figure 3.10). Two-way ANOVA with Bonferroni post hoc tests revealed a significant effect of zinc (p<0.0001; F = 58.57), but not drugs (p=0.2636; F=1.44).

Proliferating cell number was determined in zinc adequate and zinc deficient NT2 cells treated with fluoxetine or lithium chloride for 48 hours (Figure 3.11) as was measured by immunolabeling for Ki67 nuclear antigen. Two-way ANOVA with Bonferroni post hoc tests revealed a significant effect of both zinc (p<0.0001; F=69.15) and drugs (p=0.0031; F=7.40). The Ki67-staining intensity was visibly lower in zinc deficient cells (Figure 3.12).

Antidepressant Effect on Neural Stem Cell Differentiation

After six days of treatment with 1 µM fluoxetine or 1 mM lithium chloride, immunocytochemistry was performed to determine the effect of these ADs on expression of neuron-specific class III beta-tubulin TuJ1. There was a significant increase in expression of this neuronal marker compared with controls, indicating that both fluoxetine and lithium chloride induced neuronal differentiation in zinc adequate NT2 cells (Figure 3.13).

Zinc deficient cultures treated with fluoxetine appeared to have a decrease in expression of neuronal marker TuJ1, indicating that zinc deficiency impaired NT2 neuronal differentiation induced by AD treatment (Figure 3.14).
Figure 3.1. Effect of zinc deficiency (TPEN treatment) on NT2 cell number. Zinc deficiency was induced by treatment with zinc chelator TPEN at concentrations 3 µM and 8 µM. The addition of 3 µM TPEN to cells resulted in a significant decrease in cell number after 72 hours of treatment. Treatment with 8 µM TPEN resulted in cell death after 24 hours of treatment. Bars indicate mean ± SEM. * p<0.05.
Figure 3.2. Effect of zinc deficiency (zinc deficient media) on NT2 cell number. Zinc deficiency was induced by treatment with zinc-deficient media containing zinc (Zn) at concentrations 1 µM or 0.4 µM. Bars indicate mean ± SEM. * p<0.05; ** p<0.005.
Figure 3.3. Effect of TPEN treatment on NT2 cell proliferation. Immunocytochemistry was used to identify newly proliferating cells (red, Ki67-labeling). Treatment with 6 µM TPEN (ZD) for 18 hours decreased Ki67-labeling compared with control (ZA). Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.4. Effect of zinc deficient media on NT2 cell proliferation. Immunocytochemistry was used to identify all cells (blue, DAPI staining) and newly proliferating cells (red, Ki67-labeling). Treatment with zinc deficient media (ZD [0.4 µM Zn]) for 48 hours decreased the Ki67-labeling compared with control cells (ZA [2.5 µM Zn]). Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.5. Effect of zinc deficiency on the NT2 cell proliferation. Cell proliferation was measured by immunocytochemical labeling for Ki67 nuclear antigen after 48 hours of treatment. Number of Ki67-positive cells was compared with total cell number determined by DAPI nuclear staining. ZA, zinc adequate state (2.5 µM Zn); ZD, zinc deficient state (0.4 µM Zn). Bars indicate mean ± SEM. ** p<0.005.
Figure 3.6. Effect of fluoxetine on NT2 cell number. Treatment with 1 µM fluoxetine (FLX) did not alter cell number. Bars indicate mean ± SEM.
**Figure 3.7.** Effect of fluoxetine on NT2 cell proliferation. Immunocytochemistry was used to identify all cells (blue, DAPI nuclear staining) and newly proliferating cells (red, Ki67-labeling) in control and fluoxetine-treated (+ FLX) cells after 48 hours of treatment. Number of Ki67-positive cells was compared to total cell number. Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.8. Effect of lithium chloride on NT2 cell proliferation. Immunocytochemistry was used to identify all cells (blue, DAPI staining) and newly proliferating cells (red, Ki67-labeling) in control and lithium-treated (+ Li) cells after 48 hours of treatment. Number of Ki67-positive cells was compared to total cell number. Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.9. Effects of fluoxetine and lithium treatment on NT2 cell proliferation. Cell proliferation was measured by immunolabeling for Ki67 nuclear antigen. Number of Ki67-positive cells was compared to total cell number determined by DAPI nuclear staining. Bars indicate mean ± SEM.
**Figure 3.10.** Effect of zinc deficiency on cell number in NT2 cell cultures treated with fluoxetine (FLX) or lithium chloride (Li) for 48 hours. ZA, zinc adequate state (2.5 µM Zn); ZD, zinc deficient state (0.4 µM Zn). **p<0.005, significantly different from ZA. Bars indicate mean ± SEM.**
Figure 3.11. Effect of zinc deficiency on cell proliferation in NT2 cell cultures treated with fluoxetine (FLX) or lithium chloride (Li) for 48 hours. Cell proliferation was measured by immunolabeling for Ki67 nuclear antigen. Number of Ki67-positive cells was compared to total cell number determined by DAPI nuclear staining. ZA, zinc adequate state (2.5 µM Zn); ZD, zinc deficient state (0.4 µM Zn). Bars indicate mean ± SEM. ** p<0.005, significantly different from ZA.
Fluoxetine (FLX) or lithium chloride (Li) for 48 hours. Immunocytochemistry was used to identify zinc deficient media (ZD [0.4 µM Zn]) for 24 hours decreased the Ki67-labeling compared with zinc adequate cells (ZA [2.5 µM Zn]). Photomicrographs are representative of images from dishes at a 20X magnification.

**Figure 3.12.** Effects of zinc deficiency on cell proliferation in NT2 cell cultures treated with fluoxetine (FLX) or lithium chloride (Li) for 48 hours. Immunocytochemistry was used to identify all cells (blue, DAPI staining) and newly proliferating cells (red, Ki67-labeling). Treatment with zinc deficient media (ZD [0.4 µM Zn]) for 24 hours decreased the Ki67-labeling compared with zinc adequate cells (ZA [2.5 µM Zn]). Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.13. Effects of fluoxetine and lithium treatment on NT2 cells’ differentiation. Following six days of treatment with 1 μM fluoxetine (FLX) or 1 mM lithium chloride (Li), neuronal differentiation was determined by expression of the neuron-specific class III beta-tubulin TuJ1 (red). Nuclear morphology was determined by DAPI staining (blue). Photomicrographs are representative of images from dishes at a 20X magnification.
Figure 3.14. Effect of zinc deficiency on NT2 cell differentiation induced by fluoxetine and lithium treatment. Following six days of treatment with 1 μM fluoxetine (FLX) or 1 mM lithium chloride (Li), neuronal differentiation was determined by expression of the neuron-specific class III beta-tubulin TuJ1 (red) in zinc adequate (ZA) and zinc deficient (ZD) cells. Nuclear morphology was determined by DAPI staining (blue). Photomicrographs are representative of images from dishes at a 20X magnification.
Zinc and Neural Stem Cell Proliferation

According to the neurotrophic hypothesis, neuronal damage and/or decreased neurogenesis in brain limbic structures, such as hippocampus and cortex, may result in depression (Duman et al., 2004). Numerous studies have demonstrated that ADs may block or reverse these damaging effects (Czeh et al., 2006; Lee et al., 2001; Malberg and Duman, 2003; Malberg et al., 2000; Manev et al., 2001; Santarelli et al., 2003). Importantly, depression-related brain areas, such as cerebral cortex, hippocampus, and amygdala, are found to have high concentrations of zinc (Frederickson and Danscher, 1990; Frederickson et al., 2000; Nowak, 1998). Furthermore, a significant negative correlation between severity of depression and serum zinc levels has been observed (Maes et al., 1994a; Wojcik et al., 2006). As discussed in chapter 1, zinc is vital for numerous biological functions and it is not surprising that imbalances in homeostasis of this mineral have been linked with an impairment of neuronal function. Data on high concentrations of zinc in the brain regions that have neurogenic potential, combined with the evidence for the important role of zinc in neuronal function and ubiquitous nature of this mineral, suggest that zinc has a defining role in NSC proliferation. Thus, the current work examined the rate of NSC proliferation under the condition of zinc deficiency.

Being an excellent in vitro model for studying human NSC, NT2 cell culture was used in all our in vitro experiments. To create a zinc deficient state, NT2 cells were treated with either the zinc chelator TPEN, or zinc deficient media. While TPEN was used in earlier experiments, later, TPEN treatment was substituted by zinc deficient media in order to accomplish an effective, noninvasive technique to deplete zinc from the cells.

Zinc deficiency significantly decreased both cell number and proliferation of the NT2 cells. This is consistent with earlier reports from our laboratory that have shown that dietary zinc deficiency in rats results in decreased cell proliferation in the dentate gyrus of the hippocampus. Other research has also shown that zinc is necessary for the optimal replication in many other cell types, including peripheral blood mononuclear cells (Wiessgarten et al., 2002), hepatocytes (Cherian and Apostolova, 2000), aortic endothelial (Fanzo et al., 2002), and prostate epithelial
cells (Yan et al., 2008). On the other hand, zinc deficient human bronchial epithelial cells have not exhibited changes in the cell cycle process. Moreover, these cells have shown a delay at G2/M phases during cell cycle progression if they received zinc supplementation (Shih et al., 2008, Wong et al., 2008). Additionally, it is reported that dietary zinc deficiency induces esophageal cell proliferation in rodents (Fong et al., 1998; Fong and Magee, 1999; Guy et al., 2007). Thus, it appears that the ubiquitous nature of this mineral can lead to differential effects in different types of cells.

It is known that zinc deficiency may cause disorders such as skin lesions, reproductive dysfunction, growth retardation and various other diseases characterized by cell proliferation and growth failure. Also, for many years, zinc has been known as a cytoprotective agent and used to treat various epithelial abnormalities like wound healing or ulcerative colon disease. There are many possible proposed physiological and molecular mechanisms of action of intracellular zinc, but the exact mechanisms of how zinc influences cell proliferation are still unclear. According to the literature, zinc plays a vital role in nucleic acid synthesis and transcription that are necessary for cell replication. It is established that zinc serves as an essential structural component of DNA and RNA polymerase and zinc finger proteins. Zinc finger proteins act as transcription factors, bind to the specific sequences of DNA, modulate gene transcription and thus can affect cell proliferation (Bulyk et al., 2001; Nakamura et al., 2004). Thus, in this project, zinc deficiency in NT2 cells might lead to structural and functional impairment of DNA and RNA polymerases and transcription factors and ultimately to a decrease in NT2 cell proliferation.

Antidepressants, Cell Proliferation, and Differentiation

Evidence in the literature indicates that adult neurogenesis may be inhibited in depression, may be regulated by antidepressant (AD) treatment, and that this regulation may contribute to the therapeutic effects of ADs. In the animal model of stress, AD treatment has been shown to reverse a decrease in cell proliferation in the dentate gyrus of the hippocampus and the medial prefrontal cortex (Czeh et al., 2006; Lee et al., 2001). Increased cell proliferation induced by ADs has coincided with improvement in depression-like behavior in rats (Santarelli et al., 2003).

To test whether ADs stimulate NSC proliferation, two drugs have been tested in this project, fluoxetine and lithium chloride. Fluoxetine is a commonly prescribed member of the class of selective serotonin reuptake inhibitors (SSRI). SSRIs block the reuptake of 5-HT at the 5-HT
transporters, thus increasing 5-HT extracellular levels in brain. SSRIs have fewer side effects compared with other classes of ADs and are regarded as first-line pharmacotherapy for major depressive disorder (MDD) and other mood disorders (Robinson, 2007). Lithium is also widely used as a mood-stabilizing drug for the treatment and prophylaxis of mania and depression (Chuang et al., 2002; Manji and Lenox, 2000). Lithium is known to increase the catabolism of dopamine and uptake of norepinephrine (Wolf et al., 1989), affect nerve exitation, synaptic transmission, and neural metabolism (Ghoshdastidar, 1999).

Using immature rat cerebellar granule cell culture, Manev et al. (2001) have demonstrated that the 48-hour exposure to the AD fluoxetine, as well as to clomipramine and imipramine (the latter two are members of tricyclics), produces stimulatory effect on cell proliferation in older cultures (10 days in vitro), but triggers an inhibitory effect in younger cultures (3 days in vitro).

In the current project, NT2 cells were treated with fluoxetine and lithium at concentrations that were clinically applicable and comparable to therapeutic plasma concentrations (Edgar et al., 1999; Manev et al., 2001; Misiuta et al., 2006). Immunostaining for the cell proliferation marker Ki67, together with cell count, was used. Multiple experiments testing cell count showed that the 24-hour treatment with fluoxetine was ineffective. The 48-hour exposure to fluoxetine resulted in a modest increase in total NT2 cell number compared to controls, but this increase did not reach statistical significance. In order to determine the effect of fluoxetine on cell proliferation, the number of Ki67-labeled cells was compared to total cell number determined by DAPI nuclear staining, which allowed us to determine the proliferation ratio or percentage. Fluoxetine treatment for 48 hours resulted in an increase in the relative number of Ki67-labeled cells, but this did not reach statistical significance. In contrast to these data, other researchers have found that 48-hour exposure to fluoxetine has a stimulatory effect on proliferation of neural precursor cells in primary cultures from rat cerebellum (Manev et al., 2001).

The lack of proliferative increases in the current work can have several explanations. First, fluoxetine might cause only a minimal increase in NT2 proliferation rate that actually would be significant physiologically. Second, it might be necessary to treat NT2 cells for over 48 hours to see a statistical increase. The 48-hour treatment with lithium chloride also did not result in significant increase in cell proliferation compared with control group, but data indicated a trend toward an increase. These results seem to be discordant with findings in an earlier in vitro study, which reported that after only one day of lithium treatment there were significantly more NT2
proliferating cells than in the untreated group (Misiuta et al., 2006). However, the same study found that the total NT2 cell number did not increase after one day of lithium exposure despite the increased cell proliferation. These data are consistent with the current work and suggest that cell proliferation may be accompanied by cell loss after lithium treatment resulting in an increase in cell turnover, but no increase in cell number.

The next step in this work was to study the influence of ADs on NT2 cells’ differentiation. After six days of treatment with 1 µM fluoxetine and 1 mM lithium chloride, immunocytochemistry was performed to determine the effect of these ADs on expression of neuron-specific class III beta-tubulin TuJ1. We found a significant increase in the expression of this neuronal marker, indicating that fluoxetine and lithium chloride induce neuronal differentiation in zinc adequate NT2 cells. This finding is consistent with previous data showing that chronic treatment with fluoxetine promotes neuronal differentiation of progenitor cells isolated from cultured cerebellar granule cells (Zusso et al., 2008; Zusso et al., 2004; Wang et al., 2008). In addition, studies have also demonstrated that ADs influence the maturation of immature neurons and their functional integration. The observation of the dendritic morphology of the mice newborn hippocampal granule cells has shown that after chronic fluoxetine treatment, a larger fraction of these cells possess tertiary dendrites and display more complex dendritic arborization (Wang et al., 2008).

Given the growing evidence for involvement of 5-HT₁A receptors in the neurogenic and behavioral effects of SSRIs, the abundance of the 5-HT₁A receptors in NT2 cells was measured to test the hypothesis that fluoxetine may act through the 5-HT₁A receptor-mediated mechanism in these cells. However, immunocytochemistry with specific antibodies for the 5HT₁A receptor did not detect any visible staining of this receptor in NT2 cells. This finding suggests that NT2 cells are undifferentiated cells and they may not have the ability to express the 5HT₁A receptor yet. Other receptors may also be implicated in SSRI action as well.

**Zinc and Antidepressant Effects**

As discussed in chapter 1, significantly lower serum zinc levels have been found in treatment-resistant patients compared with normal controls and non-treatment-resistant patients (Hansen et al., 1983; Maes et al., 1997). Moreover, data from our laboratory have demonstrated that zinc deficiency leads to depression-like behavior in adult rats and limits the efficacy of antidepressant
drugs (Tassabehji et al., 2008). Furthermore, patients with major depression who received supplementation with 25 mg of zinc have shown a statistically significant improvement from the AD medication compared with the placebo group after 6 and 12 weeks of AD treatment (Nowak et al., 2003). In addition, it has been found that zinc supplementation potentiates the therapeutic effect of the AD imipramine in rats in a chronic unpredictable stress model of depression (Ossowska et al., 2004).

Our in vitro findings in the current work have also demonstrated that zinc depletion impairs the effect of ADs, particularly fluoxetine and lithium chloride. After six days of fluoxetine or lithium treatment, immunofluorescent detection of neuron-specific class III beta-tubulin TuJ1 in NT2 cells was performed to determine the effect of these ADs on neuronal differentiation of these cells. We found a significant increase in expression of this neuronal marker. However, zinc deficient cultures treated with fluoxetine or lithium chloride appeared to have a decrease in the expression of neuronal marker TuJ1, indicating that zinc deficiency impaired NT2 neuronal differentiation induced by these ADs. Furthermore, zinc deficient NT2 cells treated with fluoxetine or lithium chloride showed a significant decrease in the total and Ki67-positive cell number compared with zinc adequate cells treated with the same doses of these ADs. Interestingly, zinc deficient cultures treated with fluoxetine or lithium chloride had higher proliferating cell number compared with zinc deficient cultures that were not exposed to these ADs, but this was not statistically significant. More research is needed to evaluate the AD protective role against zinc deficiency.

Our results indicate that both zinc and ADs contribute to an increased neurogenesis with zinc being one of the important structural and regulatory instruments of the AD action. Evidence for the necessity of neurogenesis for the therapeutic effect of ADs as well as low serum zinc levels in treatment-resistant form of depression and benefits of zinc co-administration with ADs supports this suggestion.

Possible Mechanisms

The in vitro findings reported here demonstrate that zinc plays an important role in regulation of neural stem cell proliferation and the AD effect on differentiation. Although the molecular mechanisms responsible for the action of zinc and AD drugs were not explored in the current project, this report does raise the possibility that there are a number of different mechanisms that
could be at work. For example, both zinc and the ADs may be acting through the zinc-regulated transcription factor p53. Additionally, postsynaptic receptors for the neurotransmitters, such as glutamate, 5-HT, and dopamine, may play a role, as well as second messenger systems and neurotrophic factors.

**p53 Mechanism.** Recent studies suggest that one of the numerous transcription factors that receive zinc ions from metallothioneins is the p53 tumor suppressor protein (Apostolova et al., 2000; Shimoda et al., 2003). p53 is a zinc finger protein that is known as a major regulator of cell proliferation, DNA repair, and cell death and promotes various target genes involved in DNA repair, cell cycle arrest, and apoptosis (Donehower and Bradley, 1993). This protein up-regulates the growth arrest DNA damage protein (Gadd45) that binds to proliferating cell nuclear antigen (PCNA) and inhibits DNA synthesis (Smith et al., 1994). Importantly, the activation, stabilization and binding of p53 to specific sequences of DNA depend on zinc binding (Hainaut and Mann, 2001; Meplan et al., 2000a; Meplan et al., 2000b). Zinc deficiency has been found to increase p53 mRNA and p53 protein in the human hepatoblastoma [HepG2] (Reaves et al., 2000) and prostate epithelial cells (Yan et al., 2008), demonstrating that zinc up-regulates the expression of this important gene. Moreover, our laboratory data have also demonstrated an elevated p53 protein in zinc deficient NT2 cells (unpublished data). Furthermore, it is reported that the activated p53 promotes transcription of p21 gene, which is known to inhibit the G2/M cell cycle progression by inactivating cyclin-dependent kinases (Koutsodontis, 2005; Stewart and Pietenpol, 2001). On the other hand, zinc deficiency in the HepG2 cell line has been found to inhibit the transcriptional process of p21 protein (Wong et al., 2007). These data suggest that zinc deficiency may lead to a decreased ability of p53 to bind to the promoter region on p21. Also, changes in zinc concentration may alter p53 degradation and posttranslational modification, which may decrease the effect of this protein on downstream targets (Fanzo et al., 2001).

Interestingly, research shows that elevated cellular zinc levels as well as decreased zinc concentrations may impair p53 functions. Using normal human bronchial epithelial (NHBE) and aortic endothelial cells, Fanzo et al. (2001; 2002) have demonstrated that both zinc deficiency and zinc supplementation modulate expression of the p53 gene and p53-related genes. This group has found a significant increase in p53 mRNA and p53 nuclear protein abundance in both zinc depleted and zinc supplemented conditions as compared with control cells. In addition, zinc depleted cells had higher Gadd45 mRNA abundance, but p21 mRNA levels were not affected.
These data suggest that an increase in p53 induced by zinc deficiency leads to activation of Gadd45 at the transcriptional level, its binding to PCNA, and ultimately to cell cycle arrest in the G2 phase (Fanzo et al., 2001). Furthermore, other investigators have also shown that high zinc status in NHBE cells lead to up-regulation of p53 expression, following an increase in p21, and G2/M delay or blockage during the cell cycle progression (Shih et al., 2008; Wong et al., 2008). Interestingly, folding of the DNA-binding finger of p53 requires an environment where free zinc concentration is low (Butler and Loh, 2007). Together these findings indicate that expression of the p53 gene and downstream targets of p53 may be very sensitive to alterations in zinc status, and mechanisms of action may depend on physiological zinc concentrations (Fanzo et al., 2001).

Recently, Pechnick et al. (2008) have found that antidepressant imipramine down-regulates the expression of the cyclin-dependent kinase inhibitor p21 that restrains cell cycle progression. Moreover, this group raises the possibility that suppression of p21 is a shared common mechanism of the AD action.

**Glutamate Receptors.** Zinc is highly concentrated in the synaptic vesicles of about half of glutamatergic neurons (Frederickson and Danscher, 1990). It has been also localized with GABA, glycine, and ATP currents and thus can affect both excitatory (glutamatergic, glycine) and inhibitory (GABA-ergic) neurotransmission pathways (Barañano et al., 2001; Frederickson et al., 2000; Hosie et al., 2003; Smart et al., 2004; Trevorg et al., 2004). Moreover, some authors are considering zinc as a neuromodulator, which is based on several criteria, including binding to postsynaptic receptors (Barañano et al., 2001; Kay and Toth, 2008). For example, zinc has been found to inhibit the activity of glutamate receptor subtype, N-methyl-D-aspartate (NMDA) receptor (Herin and Aizenman, 2004).

Interestingly, data show that systemic activation of NMDA receptors decreases proliferation of granule cells, whereas blockade of NMDA receptors rapidly increases the cell birth in the adult rat dentate gyrus (Cameron et al., 1995). In addition, administration of NMDA receptor antagonists enhances the birth of neurons and increases the overall density of neurons in the granule cell layer. Taken together, these data suggest that a zinc deficient state may result in elevated NMDA activity, which ultimately may contribute to a decrease in cell proliferation. Recent clinical data have shown that the reduction in the potency of zinc to inhibit NMDA receptors in the hippocampus may be involved in the psychopathology of suicide attempts (Nowak et al., 2005).
**Serotonin Receptors.** Although the mechanisms of action of ADs are still not fully elucidated, several studies indicate that their induction of adult neurogenesis may be via regulation of the 5-HT system and 5-HT receptors (Knobelman et al., 2001; McEwen, 2000). Brain 5-HT levels are directly linked with mood and anxiety-related behaviors (Argyropoulos et al., 2004; Guyton and Hall, 1996). It has been demonstrated that platelet 5-HT concentrations are significantly lower in suicidal than in non-suicidal patients or in healthy controls (Marcinko et al., 2007). Furthermore, 5-HT has been found to increase the generation of new neurons in the mammalian dentate gyrus (Gould, 1999). Literature describes seven families of 5-HT receptors (5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6, 5-HT7), and at least 15 subpopulations have been cloned (Glennon, 1995). Among members of this large receptor family, the 5-HT1A receptor has been strongly implicated in the modulation of mood, memory, and anxiety-related behaviors and regulation of neurogenesis (Gould, 1999). It is reported that the highest density of this receptor subtype among other limbic areas is in the hippocampus (Shih et al., 1995). The 5-HT1A receptor system has also been linked with regulation of the HPA and with modulating the release of stress hormones, adrenocorticotropic hormone (ACTH) and corticosterone (Carrasco and Van de Kar, 2003). Moreover, administration of a 5-HT1A antagonist, WAY 100,635, blocked the basal rate of neurogenesis (Jacobs et al., 1998).

Treatment with fluoxetine, together with a 5-HT1A-selective agonist 8-OH-DPAT (8-hydroxy-2-dipropylaminotetralin), caused a doubling of BrdU-labeled hippocampal cells in wild-type mice, but was not effective in mice lacking 5-HT1A receptor (Santarelli et al., 2003). In another study, repeated treatment with several classes of ADs has been shown to up-regulate the 5-HT1A receptor function in the hippocampus (Haddjeri et al., 1998). Although the 5-HT receptors have been shown to be active in vivo, particularly in response to SSRIs, this does not appear to be a mechanism in vitro. While zinc deficiency impairs the proliferation or survival of NT2 cells, we have shown that these cells do not express the major 5-HT receptor, 5-HT1A. Still it is possible that they express other 5-HT subtypes. Several studies have demonstrated that the 5-HT1B receptors are also activated by fluoxetine and contribute to AD effects (Knobelman et al., 2001).

**Dopamine Receptors.** Dopamine is believed to play an important role in the pathophysiology of depression and the therapeutic effects of ADs (Dunlop and Nemeroff, 2007). Indeed, depressed patients have demonstrated a functional deficiency of synaptic dopamine (Meyer et al., 2006), while animal behavioral models with altered dopamine functioning have shown depressive
behaviors (Willner, 1997). Pretreatment with a dopamine agonist has prevented dopamine depletion in the caudate nucleus and nucleus accumbens and “learned helplessness” behavior of the animals. Interestingly, the most recent research has found a link between the dopamine system and neurogenesis. For example, Yang et al. (2008) have demonstrated that the dopaminergic system promotes cell proliferation in the SVZ and the dentate gyrus of the hippocampus through dopamine D2 receptor and ciliary neurotrophic factor (CNTF). Furthermore, administration of the selective dopamine D2/D3 receptor antagonist, sulpiride, to depressed patients who have been previously treated successfully with SSRIs causes a substantial reinstatement of depressed mood, while administration of placebo in the control group improves subjective well-being. These data suggest that sensitization of D2-like dopamine receptors may represent a central pathway of the clinical action of SSRIs (Willner et al., 2005).

**Second Messenger Systems.** Postreceptor adaptations of long-term AD treatment have been linked with activation of the cAMP cascade via several intracellular pathways, including increased coupling of Gs (the stimulatory GTP-binding protein) with adenylyl cyclase and elevated levels of cAMP-dependent protein kinase (PKA). Studies have confirmed that AD medication increases activation of cAMP-dependent PKA activity (Nestler et al., 1989; Popoli et al., 2000). Activation of cAMP cascade by ADs results in increased function and expression of cAMP response element binding protein (CREB) and up-regulation of BDNF (Nibuya et al., 1995; Nibuya et al., 1996; Thome et al., 2000; Xu et al., 2003). It is suggested that CREB also can be regulated by Ca2+-dependent or microtubule-associated protein (MAP) kinases (Duman et al., 2000). It is reported that activation of the cAMP pathway or incubation with BDNF induces neuronal differentiation and neurite outgrowth of progenitor cells in vitro (Palmer et al., 1997). Additionally, CREB and BDNF increase synaptic plasticity and neuronal survival of the adult nervous system (Finkbeiner, 2000; Duman et al., 2000). BDNF was found to increase the number of newly generated neurons in the adult olfactory bulb if infused intraventricularly (Zigova et al., 1998) or to produce AD-like effects in animal models of depression if infused into the hippocampus (Shirayama et al., 2002). Interestingly, there is a study that has found a negative correlation between serum BDNF and the severity of depression symptoms, and that ADs may increase BDNF in depressed patients (Shimizu et al., 2003). Therefore, up-regulation of BDNF may contribute to neurotrophic effects of ADs, including stimulation of NSC cell proliferation and neuronal differentiation.
**Lithium.** Lithium is known to affect multiple cellular pathways. One of the suggested mechanisms of its action is regulation of the phosphoinositide signaling pathway (Bhalla et al., 2006). Lithium has been shown to inhibit enzymes, such as inositol monophosphatase and inositol polyphosphate-1-phosphase, thereby decreasing free inositol (York et al., 1995). Levels of free inositol are thought to be important for the production of various second messengers, including diacylglycerol (DAG) and inositol-1,4,5-triphosphate (Gould and Manji, 2002). Inositol-1,4,5-triphosphate increases intracellular calcium (Soderling, 2000), while DAG activates an enzyme protein kinase C, which directly affects cellular systems and many growth regulating properties in immature cells, and has additional cell specific responses in individual mature cells (Kanashiro and Khalil, 1998). The effect of lithium may also occur through a relative depletion of myoinositol (Kofman et al., 1993; Tricklebank et al., 1991). It is suggested that the levels of myoinositol are involved in triggering changes in gene expression and neuroplasticity (Gould and Manji, 2002). Lithium has been shown to increase the mRNA levels of G proteins, cAMP levels, and expression of CREB and BDNF (see Kim et al., 2004 for references).

Zinc co-administration with lithium has been beneficial in the behavior patterns of animals showing improved short-term memory and cognitive functions (Bhalla et al., 2007). Zinc supplementation in lithium-treated rats significantly improved acetylcholinesterase activity as well as the status of neurotransmitters such as dopamine and 5-HT. These data, together with the evidence for the neurogenic effects of these neurotransmitters (Gould, 1999; Yang et al., 2008), tend to suggest that improvement in the behavioral patterns in rodent models may also be caused by an increased granule cell proliferation via zinc-induced improvement of the neurotransmitters’ status or direct neurogenic action of zinc. But the rate of neurogenesis has not been studied in these rats. Memory problems often seen in depression have been linked to impairment of hippocampal neurogenesis (Kempermann, 2002; Shors et al., 2001), and zinc co-supplementation may up-regulate the generation of new neurons and improve the short-term memory and cognitive functions of animals in study performed by Bhalla et al. (2007).

Observations indicate that zinc and ADs can share their molecular targets to exert their neurogenic effects. For example, chronic zinc administration has been found to increase levels of BDNF mRNA in the rodent cerebral cortex or hippocampus (Nowak et al., 2004; Nowak et al., 2005); the same effect has been induced by chronic AD treatment (Nibuya et al., 1995; Nibuya et al., 1996; Shimizu et al., 2003; Thome et al., 2000; Xu et al., 2003). Also, both zinc and the AD
imipramine have been found to down-regulate the expression of the cyclin-dependent kinase inhibitor p21, which inhibits cell cycle progression and thereby cellular proliferation (Pechnick et al., 2008; Wong et al., 2008). Furthermore, in vitro studies have demonstrated that tricyclics have effects similar to zinc, such as blocking the NMDA receptors (Reynolds and Mille, 1988). Moreover, low, ineffective doses of imipramine (tricyclic) and citalopram (SSRI) have been shown effective if administered together with low doses of zinc (Szewczyk et al., 2002). Further, chronic treatment with imipramine increases the ability of zinc to inhibit the NMDA receptors in the cerebral cortex (Szewczyk et al., 2001). Interestingly, chronic citalopram or imipramine administration to rats has been found to significantly increase the hippocampus/brain region zinc concentration ratio (Nowak and Schlegel-Zawadzka, 1999), while serum zinc levels have been improved after chronic treatment with citalopram but not with imipramine (Nowak and Schlegel-Zawadzka, 1999), fluoxetine, or the tricyclic, trazodone (Maes et al., 1997).

Conclusions and Future Studies:

1. The current work shows that zinc deficiency impairs the growth and proliferation of NT2 cells. While the mechanisms that are responsible for this are not clear, we have proposed a number of possible factors, such as the transcription factor p53, the neurotrophic factor BDNF, and a variety of second messenger and postsynaptic receptor signaling mechanisms that may be regulated by zinc deficiency. Future work will be needed to explore the role of these mechanisms in vitro and in vivo.

2. The current work used the cell proliferation marker Ki67 to assess the number of proliferating cells. The advantage of this approach is that we were able to identify cells in all active phases of the cell cycle. The disadvantage is that we do not know which phases of the cell cycle are inhibited by zinc deficiency. Future work will be needed to explore these mechanisms and determine how zinc is acting in the cell cycle in vitro and in vivo.

3. We know from previous work that zinc deficiency induces cell death. Thus, while the current work was focused on understanding the role of zinc in AD-induced cell proliferation and differentiation, future studies will be needed to explore the role of apoptotic mechanisms in zinc deficient cells treated with ADs.

4. While the antidepressants fluoxetine and lithium may induce small increases in NT2 proliferation, a major finding of this work is that their most significant effect is the
induction of neuronal differentiation. In fact, our preliminary data, not reported here, show that these agents are more powerful at inducing differentiation, as measured by TuJ1 expression, than the classic stimulator of neuronal differentiation, retinoic acid (RA). The mechanisms responsible for this AD-induced differentiation are not known, but are likely to be a significant part of AD efficacy. Future work will be needed to identify these mechanisms and determine the degree to which these drugs act through RA-dependent pathways, and to what extent they are zinc dependent. This information could lead to the development of more efficacious AD therapies.

5. Neurogenesis requires cellular proliferation, differentiation, migration, and integration into the existing CNS circuitry via synaptic plasticity. This work has suggested that future work should explore zinc and AD-regulated mechanisms of proliferation and differentiation. However, it is possible that AD drugs also regulate stem cell migration as well as dendritic plasticity. Future work will be needed in vivo to determine the extent to which zinc and ADs regulate these mechanisms.
REFERENCES


**BIOGRAPHICAL SKETCH**

Tatyana Mullin was born in the small town Rodniki in central Russia. In 1988, she graduated from high school and moved to the larger city Ivanovo, Russia where she entered the A.S. Bubnov Ivanovo State Medical Academy. After six years of medical school, she received her Medical Doctorate and went on to continue her medical training as a Clinical Therapy Resident, which she completed in two years. Then, Tatyana went on to pursue her Ultrasound Diagnosis Training, which allowed her to get a position as an Ultrasound Diagnosis staff Physician at the Ivanovo Oblast Oncology Center Radiology Division in 1996. During her first two years of practice, she also worked part-time as an Internal Medicine Physician. In 2001 while Tatyana was on vacation in Turkey, she met her future husband who was a physician and lived in Ann Arbor, MI, U.S.A. Later, in 2002, she got married and moved to Ann Arbor. It was a great and also a very challenging time in her life. In 2003, she and her husband Vildan moved to Florida, where he started his new practice. While working in Russia, Tatyana developed a great deal of interest in the field of nutrition. After she moved to U.S.A., she wanted to develop her career as a Registered Dietitian to continue the promotion of the healthy eating and lifestyle as valuable input in the disease prevention, improving a prognosis of illness, and well being. Thus, in 2006 Tatyana entered the Graduate Program at the Department of Nutrition, Food, and Exercise Sciences at Florida State University. At FSU, she had the opportunity to teach Exercise Physiology Laboratory course. Tatyana is a member of the American Dietetic Association since 2007. Over the course of her graduate studies Tatyana has been recognized by receiving the university Florence Smith McAllister and Pao-Sen Chi Memorial Scholarships in 2007. After her graduation, she will pursue her career in nutrition as a Registered Dietitian.