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Zinc Deficiency Impairs Retinoic Acid-Induced Differentiation of Human Neurons

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ZINC DEFICIENCY IMPAIRS RETINOIC ACID-INDUCED DIFFERENTIATION OF HUMAN NEURONS

By

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ABSTRACT

Neurogenesis is the process of stem cell proliferation, survival, and differentiation. Recent research has confirmed the presence of ongoing neurogenesis throughout life in humans. This fact has led to vast interest in the mechanisms that underlie this process. Manipulation of adult neurogenesis has the potential to enhance the treatment of a multitude of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and depression as well as injury and stroke. Previous work has shown that the essential trace metal zinc regulates neuronal precursor proliferation and survival. Thus, this work is based on the central hypothesis that zinc is also needed for neuronal differentiation. Furthermore we proposed that transforming growth factor signaling may be involved in the zinc regulated mechanisms of differentiation. Zinc deficiency (ZD; 0.4μM) impaired the ability of neuronal precursor cells (NT2) to differentiate into mature neurons (NT2-N) when exposed to 2 wks of 10μM retinoic acid (RA), as measured by the early neuronal marker TuJ1. Additionally, we demonstrated a differential regulation of Transforming Growth Factor Beta (TGF-β) receptor isoforms type I (RI) and II (RII) under zinc deficient (0.4μM) conditions in NT2 cells undergoing RA-induced differentiation. Measurements of TGF-β RI and RII in zinc adequate (ZA; 2.5μM) differentiated NT2-N neurons showed that neither receptor isoform was expressed in these cells. TGF-β RI was up-regulated in NT2-N cells in response to ZD (0.4μM) however, while TGF-β RII remained down-regulated under ZD (0.4μM) conditions, as demonstrated via TGF-β RI and RII immunocytochemistry. These data confirmed that ZD (0.4μM) does impair RA-induced differentiation of human NT2 neuronal cells. There is also evidence that a differential regulation of the TGF-β receptor I and II isoforms may be involved in this mechanism, as the loss of RII expression in ZD (0.4μM) NT2-N cells could be responsible for a decline in TGF-β signaling in these cells and thus an attenuated cellular response to TGF-β responsive genes. This research suggests an important role for TGF-β and the trace metal zinc in regulating neuronal differentiation, and helps to improve understanding of adult neurogenesis in the human brain.

CHAPTER 1

INTRODUCTION

Neurogenesis

Neurogenesis is the process in which new neurons are generated in the brain through a balance of proliferation, survival and differentiation of neuronal stem cells. While the existence of adult stem cells in the central nervous system of rodents and other vertebrates, including primates has been recognized for some time, the first clear evidence that these cells also exist in specific regions of the adult human brain was only recently shown (Eriksson et al., 1998; Santarelli et al., 2003). A landmark study by Eriksson et al. has shed light on the ongoing process of neurogenesis in the adult human brain. These researchers identified proliferating cells in postmortem human brain tissue of cancer patients (n=6) through the use of bromodeoxyuridine (BrdU) labeling and showed for the first time that the adult human brain has cells that are capable of dividing throughout the entire lifespan in the hippocampus and the subventricular zone (SVZ) of the caudate nucleus.

Neurogenesis can be viewed as a manifold process involving proliferation of new cells, as well as migration, differentiation and survival of those cells. The continuous production of new neurons is of minimal use if those cells are unable to mature and integrate into the existing circuitry for proper functioning in the central nervous system (CNS). Thus Eriksson's observation that after asymmetric proliferation, these cells in the subgranular layer (SGL) of the dentate gyrus and subventricular zone (SVZ) of the lateral ventricles could differentiate into fully functional post-mitotic neurons was highly significant. This observation was demonstrated using labeling of cells with BrdU, glial fibrillary acidic protein (GFAP), NeuN, and calbindin or neuron specific enolase (NSE) antibodies. GFAP can be used to detect astroglia under immunofluorescence while NeuN, calbindin and NSE are neuronal markers. Eriksson showed that each of these antibodies were present in different BrdU labeled cells. Approximately 20% (22.0 ± 2.4%) of BrdU immunofluorescent cells in the dentate migrated into the granular cell layer (GCL) and expressed both BrdU and NeuN, while 22.7 ± 2.8% expressed BrdU and NSE,

and 7.9 ± 2.2% expressed BrdU and calbindin. In addition 20% (18.1 ± 1.8%) of cells in the dentate gyrus also exhibited double labeling for BrdU and GFAP (Eriksson et al., 1998).

The finding of adult neuronal differentiation and neurogenesis is significant due to the large number of known clinical conditions that would benefit from the ability to generate new neurons. This has particular relevance in the pursuit of improved understanding and potential treatment in a multitude of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease, but also following traumatic brain injury, stroke, and seizure disorders. Most recently, the stem cells in the dentate gyrus have been implicated in the mechanisms related to major depression, and it appears that these cells may even be needed for the action of antidepressant drugs.

Santarelli et al. employed sliding shields to specifically expose the neurogenic regions of the hippocampus in adult mice to irradiation, resulting in impaired neurogenesis as demonstrated by an 85% decrease in BrdU-positive cells in the SGZ. Mice were exposed to 5 Gy of irradiation on days 1, 4, and 8. Simultaneous with this procedure, mice were administered fluoxetine, an antidepressant drug belonging to the class of serotonin selective reuptake inhibitors (SSRIs), imipramine, belonging to the class of tricyclic antidepressant drugs, or a vehicle treatment. On day 27, mice received injections of BrdU and on day 28 mice were subjected to the novelty-suppressed feeding (NSF) test in which latency to feed in a novel environment is assessed to determine the efficacy of antidepressant therapy. Results of the experiment indicated a significant reduction ($p=0.02$) in latency to feed in sham mice, but not in irradiated mice in response to antidepressant administration. Thus irradiation and the consequent decline in cellular proliferation in the hippocampal SGZ blocked the behavioral effects of antidepressant therapy, suggesting a mechanistic requirement of adult neurogenesis for the efficacy of SSRI treatment in depression (Santarelli et al., 2003).

While cells of the SGL of the dentate migrate into the GCL and integrate themselves in hippocampal circuitry (Duan et al., 2007; Tanaka et al., 2004), we also know that proliferating cells of the SVZ migrate as well (Mendoza-Torreblanca et al., 2007). Lois et al. demonstrated that neural progenitor cells that proliferate in the subventricular zone of the lateral ventricle

migrate via the rostral migratory stream to the olfactory bulb, where they then differentiate into post-mitotic neurons (Lois et al. 1996). Van Praag et al. took this evidence of proliferating and differentiating neuronal cells in the adult human brain one step further by demonstrating that indeed these newly formed neurons have functional capabilities that allow them to integrate into the existing circuitry and act as post-mitotic cells. This was exhibited by synaptic exchange, cell morphology and action potentials observed in these cells following differentiation, which were characteristics of other mature cells in the same region of the brain (Van Praag 2002). Given these data, we are particularly interested in studying cells like those found in the dentate gyrus of the hippocampus, which may be implicated in learning, memory, and emotional control (Chudasama et al., 2008; Goeldner et al., 2008; Van Praag 2002). Identifying the underlying mechanisms of neuronal differentiation and survival therefore are critical steps in harnessing the medical potential of adult neurogenesis.

NT-2/D1 EC Cell Line

The human embryonal carcinoma (EC) stem cell line, NTERA-2/clone D1 (NT2/D1) is an excellent model for studying neuronal precursor cells similar to those found during neurogenesis in the adult human brain. NT2/D1 cells were originally cloned from isolated EC cells from a human teratocarcinoma that was extracted from a young male patient and grown in a tumor in a null mouse. Andrews et al. have previously described in detail the origin and characteristics of these cells (Andrews et al., 1988). NT2 cells have demonstrated the ability to differentiate into a neuronal phenotype in vitro with exposure to 10μM retinoic acid for approximately 2-4 wks. Numerous studies have demonstrated this ability of NT2 precursor cells to differentiate into mature neuronal cells (NT2-N), thus mimicking processes involved in neurogenesis, including proliferation and differentiation (Jain et al., 2007; Serra et al., 2007; Van Landingham and Levenson, 2003)

Retinoic Acid-Induced Differentiation

Retinoic acid (RA) and its receptors are known to play an important role in CNS development by initiating cellular differentiation of neuronal precursors. RA has also been

shown to be required for the survival and maintenance of neurons. RA is derived from the diet indirectly through a series of transformations as a derivative of retinol, a form of vitamin A (Borghi et al., 2003; Misiuta et al., 2006). Retinol is obtained through the diet via carotenoids found in plant sources and retinyl esters from animal sources. Once ingested, retinol is transported through the blood attached to retinol binding protein 4 (RBP4) and is taken into the cytoplasm of target cells via the RBP4 membrane receptor, STRA6. Once inside cells, retinol binds to retinol binding protein 1 (RBP1) and can be metabolically converted first to retinaldehyde via retinol dehydrogenase 10 and then to RA via retinaldehyde dehydrogenase enzyme activity (Fierce et al., 2008; Isken et al., 2008; Maden 2007).

Two proteins located in the cytoplasm of target cells known as cellular retinoic acid binding proteins I and II (CRABP I, II), bind specifically to RA and CRABP II chaperones RA into the cell nucleus. RA has the ability to impart its signaling potential in both a paracrine and an autocrine manner (Borghi et al., 2003; Budhu and Noy, 2002). Following nuclear translocation RA can act as a transcription factor through ligand binding with 2 families of retinoid receptors, retinoic acid receptor (RAR) and retinoic X receptor (RXR), to regulate cellular differentiation. These nuclear retinoid receptors, which are typically localized in the nucleus regardless of ligand presence or absence, form RAR/RXR heterodimers and bind to specific DNA sequences (retinoic acid response elements, RARE) on the promoter region of target genes. The DNA binding domains (DBDs) of nuclear retinoid receptors are known to require zinc for proper functional activity. Zinc finger proteins are located within the structural conformation of the RAR and RXR receptors and are responsible for direct binding to DNA within retinoid responsive target genes (Holmbeck et al., 1998; Leon and Roth, 2000). RARE sequences are direct repeats (DR) of the motif PuG(G/T)TCA which is separated by either 1 (DR1), 2 (DR2), or 5 (DR5) base pairs. RAR/RXR heterodimers binding to either DR2 or DR5 RAREs act similarly in that the RXR partner occupies the 5' motif, while the RAR subunit resides on the 3' motif. RXR/RAR heterodimers bind to the DR1 RARE with reverse polarity, meaning that the RAR partner occupies the 5' motif and the RXR subunit is located on the 3' motif. This change in direction of bound receptor complex results in a switch of the activity associated with the complex from activation to repression of retinoid target genes. Additionally, in the absence of ligands, heterodimer complexes are bound to RAREs and repress transcription

in target genes. Upon ligand binding, the receptor complex activates the dissociation of corepressors and the recruitment of coactivators to the promoter region of the gene. Finally, coactivators are dissociated while a mediator complex forms, additional transcription factors are recruited to the promoter, and transcription begins, completing the signal transduction pathway initiated by RA. Following transcription and subsequent alterations in gene expression, RA leaves the nucleus and returns to the cytoplasm where it is catabolized by the CYP26 class of P450 enzymes. Both subunits of the RARE bound retinoid receptors also become degraded via the ubiquitin-proteasome pathway (Bastien and Rochette-Egly, 2004; Maden, 2007; Manglesdorf and Evans, 1995).

In vivo, RA plays an important role in initiating and influencing differentiation during periods of growth. A number of different cells and tissues are influenced towards differentiation in response to RA including alveolar cells in lung tissue (Sugimoto 2008), female germ cells (Suzuki 2008), retinal photoreceptors (Osakada 2008), inner ear sensory neurons (Martinez-Modenero 2008), and even myeloid leukemia cells (Si 2007; Love 2008).

Levels of mRNA transcription resulting from this signaling process have been measured in the dentate gyrus of the adult hippocampus of mice through both in vivo and in vitro experiments, indicating a specific role for RA-induced differentiation in neurogenesis (Wang 2005). Wang et al. explained that RA treatment of neurospheres developed from neural stem cells of the SVZ in rodents significantly increased the percentage of neurons ($p=0.0001$) while decreasing the percentage of astrocytes ($p=0.001$) present in culture, as well as significantly increasing the percentage of BrdU positive neuroblasts ($p<0.05$) in response to RA. Wang also concluded that cultured secondary neurospheres showed a shift towards neuronal morphology with evidence of neurite outgrowths following RA exposure for approximately 1 wk (Wang 2005).

In vitro models of cellular differentiation have also exploited the use of retinoic acid to cause differentiation in different cell types such as SH-SY5Y human dopaminergic neuroblastoma cells (Canstantinescu 2007), human promyelocytic leukemia HL-60 cells (Racanicchi 2008) and skeletal muscle stem cells (Invernici 2008). NT-2 cells have demonstrated

this property as well. In 1984, Peter W. Andrews documented the differentiation of NT2/D1 cells into post-mitotic neurons, when exposed to retinoic acid in culture. Characteristics of differentiation providing evidence for this observation included a loss of the immature EC cell morphology as well as a decline in the expression of a cell-surface antigen typically expressed by EC cells known as SSEA-3 and an increase in the expression of the neuronal markers tetanus toxin receptor and neurofilaments (Andrews 1984).

Zinc Deficiency

The micronutrient zinc is an essential trace metal necessary for proper human health. Zinc may be one important factor helping to regulate the process of neurogenesis in the adult human brain. Studies have implicated the role of zinc in development of a variety of tissues throughout the mammalian lifespan. Zinc is required for the formation and proper functioning of DNA and RNA polymerases (De and Campbell, 2007; Okuda et al., 2005; Schoenen and Wirth, 2006). These are critical proteins involved in carrying out the very processes that form and maintain life which have implications in reproduction, growth and development. Without adequate or viable proteins such as these, transcription and gene expression cannot occur. Other roles for zinc include enzymatic activity of superoxide dismutase, a long list of dehydrogenases including retinol and retinal dehydrogenases, lactate dehydrogenase, and malate dehydrogenase as well as alkaline phosphatase (Mariani et al., 2007). Additionally, zinc plays a role in immune functioning (Prasad, 1998), cell signaling, and neurotransmitter modulation. A study by Ketterman and Li demonstrated the presence of zinc ions in vesicles localized with the neurotransmitter glutamate in glutamatergic neurons. When these neurons fire, the zinc is released into the synapse along with the neurotransmitter (Ketterman and Li, 2008).

Zinc also assists the activity of transcription factors through a unique ability to form molecules known as zinc finger (ZnF) proteins. ZnF proteins are responsible in many cases for allowing a transcription factor to actually bind to the promoter region of DNA in the nucleus of cells and direct the expression of genes and thus control of protein levels (Brayer and Segal, 2008; Juchi et al., 2001;). Aside from DNA binding, ZnF proteins are also thought to interact with RNA and function in protein-protein interactions. While several classes of ZnF proteins are

known to exist, by far the most common form is known as the classical ZnF, consisting of 2 Cysteine and 2 Histidine (C2H2) or 1 Cysteine and 3 Histidine (C1H3) residues centralized around a zinc ion with a short β hairpin and an α helix also included in the structure. The conformation of ZnF brought about by the interaction between these components allows the molecule the potential to form a finger-like structure capable of binding tightly with specific sequence domains (Gamsjaeger et al., 2007).

Zinc deficiency is very common among developing and developed countries, affecting nearly 2 billion people worldwide. Zinc deficiency is characterized by symptoms such as retarded growth, diarrhea, hair loss, delayed sexual maturation, a decrease in taste and appetite, weight loss, impaired wound healing, and mental lethargy (Prasad 2003). Dietary and supplemental zinc administration serves as an effective treatment for zinc deficiency, a potentially fatal condition if left untreated. Recommendations for daily zinc intake range from 2 mg/d in infants 0-6months of age, to 11 mg/d in adult males and 8-9 mg/d in adult females. Recommendations for pregnant and lactating women are increased to a range of 11-14 mg/d. Dietary sources of zinc include oysters, red meat, nuts, and fortified cereals.

Transforming Growth Factor Beta

Cellular differentiation can occur through a number of different mediators, including a superfamily group of second messenger signaling molecules known as Transforming Growth Factor-Beta (TGF-β). TGF-β is involved in regulation of cellular differentiation, via mediation of the cell cycle and cellular growth. TGF-β can act as an anti-inflammatory cytokine and has shown a neuroprotective effect under different conditions such as in Alzheimer's disease or following insult to the central nervous system (Battista et al., 2006; Ren et al., 1997).

There are 3 different isoforms of TGF-β, known as types I, II, and III. Types II and III appear to be most prevalent in neuronal and glial cells, while type I expression increases in response to acute injury. The 3 types of ligands also interact with 3 different isoforms of the TGF-β receptor, known as receptor types I, II, and III. Receptor type III, also known as betaglycan, is thought to assist in localizing ligands towards the cell membrane in order to

increase receptor-ligand binding affinity. Receptors type I and II have been identified extensively throughout the CNS and are thus the focus of the current investigations. They belong to a class of transmembrane protein serine/threonine kinase receptors and are specifically responsible for directing TGF-β signal transduction. These receptors are membrane bound with an extracellular ligand-binding region and an intracellular region consisting of the serine/threonine kinase activity (Böttner et al., 2000; Shi and Massagué, 2003).

The TGF-β signaling pathway is propagated through a class of proteins that act as transcription factors known as Small Mothers Against Decapentaplegic (Smad). There are three different classes of Smad proteins, each of which plays an important role in TGF-β signal transduction. The first class of Smads is called the receptor-regulated Smad's (R-Smads) and includes isoforms Smad1, Smad2, Smad3, Smad5 and Smad8. This class of proteins, specifically Smad2 and Smad3, interact directly with the transmembrane serine/threonine TGF-β receptors. Additional Smads included in classes 2 and 3 are observed downstream of these particular proteins. The second class of Smads is known as the common-mediator Smad (co-Smad) and includes only Smad4. This Smad forms heterodimeric complexes with the R-Smad in the cytoplasm of the cell following phosphorylation and activation of the R-Smad proteins by the TGF-β receptors. The third class of Smads, the antagonistic or inhibitory Smads (I-Smads), includes Smad6 and Smad7. The I-Smads can exhibit a control mechanism over expression of TGF-β responsive genes. This can be done by inhibiting the signaling pathway via preventing the binding of R-Smads to the membrane bound receptor, competing to bind with Smad4, or even by repressing TGF-β target genes before transcription begins (Böttner et al., 2000; Kitisin et al., 2007; Tsukazaki et al., 1998). The TGF-β signaling cascade that has been identified to facilitate responses to TGF-β ligand binding involves a combination of these Smad proteins, which work together to transmit the signal potential from cellular membranes to their nuclei, culminating in either gene expression or repression, as depicted in Table 1 below.

Table 1. Three classes of Smad proteins involved in TGF-beta signal transduction from the cell membrane to the nucleus

Classification	Smad Protein Member	TGF-β Signaling Function
Receptor-regulated (R-Smads)	Smad 2/3	Activated near cell membrane by TGF-β receptor-ligand complex and forms heterodimer in cytosol with co-Smad
Common-mediator (co-Smad)	Smad 4	Forms cytoplasmic heterodimeric complex with R-Smad and translocates to cell nucleus
Inhibitory (I-Smad)	Smad 6/7	Inhibits TGF-β signaling pathway as a control mechanism over expression of TGF-β responsive genes

The TGF-β signaling pathway begins specifically with ligand binding to the TGF-β type II receptor, which is located on the cell surface membrane. RII is autophosphorylated in the absence of TGF-β ligand. Upon ligand-receptor association, the type II receptor becomes constitutively activated, recruiting the nearby membrane-bound RI to form a complex via the phosphorylation of serine and threonine residues located in a conserved region known as the GS box and thus activating RI. Type I and type II TGF-β receptors work dependant of one another, and without one the other does not properly function. Under normal conditions, when both receptor isoforms are present, the two activated receptors, together with the bound ligand, form a heterodimeric complex at the cell membrane consisting of 2 copies of each of the two receptor isoforms. Following receptor activation, the signal travels from the receptor to the nucleus via the Smad protein cascade. This begins with the R-Smad, either Smad2 or Smad3, associating with the RI isoforms of the activated receptor complex where it becomes activated via phosphorylation. Next, the activated R-Smad binds to the co-Smad, Smad4, protein in the cytosol of the cell. Finally, this R-Smad/Co-Smad complex translocates to the nucleus of the cell, where it can act as a transcription factor to regulate gene expression in target cells (Böttner et al., 2000; Gomes et al., 2005; Kitisin et al., 2007).

Involvement of the TGF-β family has been noted in the development and maintenance of both the central and the peripheral nervous systems. A multitude of potential roles in regulating processes involved in neurogenesis have emerged. Functions of TGF-β noted in the literature include cell cycle control with roles in modulating proliferation and differentiation of various cell types, regulation of neuronal survival or death and possible anti-inflammatory roles in repair processes related to ischemic injury or neurodegenerative disease. TGf-β may also help to produce extracellular matrix proteins, as well as functioning in hematopoiesis, angiogenesis, chemotaxis, and the promotion of neurotrophic influences in concert with other known neurotrophic factors over neuronal cells (Böttner et al., 2000; Farkas et al., 2003; Peterziel et al., 2007; Ren et al, 1997).

The knowledge of adult neurogenesis in specific regions of the brain raises questions of how such a process is regulated. This work aimed to identify mechanisms underlying neuronal differentiation, a critical component in the process of neurogenesis. Based on a thorough review

of current literature and preliminary experiments, we hypothesized that zinc deficiency impairs RA-induced differentiation of human neurons via the manipulation and consequent disruption of TGF-β signaling.

CHAPTER 2

METHODS

Human Neuron Cultures

Human NT2 teratocarcinoma cells (Ntera2/D1 cell line) were obtained from Stratgene (La Jolla, CA). Cells were plated at approximately 30% confluency in T-75 flasks and given fresh media every 3 days. Once reaching approximately 100% confluency, cells were split back at a ratio of 1:20. Cells were maintained in a 37˚C humidified chamber that contained 5% CO_2 and were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with F-12 nutrient mixture (D-MEM/F12, Life Technologies, Gaithersburg, MD), 10% cosmic calf serum, (Hyclone Laboratories, Logan, UT), 0.1% antibiotic-antimycotic solution (Sigma Chemicals).

Induction of Neuronal Differentiation with Retinoic Acid

Aggregate in vitro cultures of neuronal precursor cells were induced to differentiate via the addition of 10 μM retinoic acid (RA, Sigma Chemicals, St, Louis, MO) into post-mitotic central nervous system neurons (NT2-N). This process was described previously by Pleasure et al. (Pleasure, 1992). Briefly, NT2 cultures were exposed to 10μM RA for approximately 3 wks. After 3 wks treatment with RA cells were suspended and trypsinized, then re-plated onto sterile, plastic, tissue grade 6-well plates coated with Matrigel Matrix (BD Biosciences) to support attachment and treated for 1 additional wk with RA. RA treatment was then stopped and cells were instead treated with the mitotic inhibitors 5-fluoro-2"-deoxyuridine (10 μM), cytosine β-D-arabinofuranoside (10 μM) and uridine (10 μM) for 7-10 days. The total duration of treatment was approximately 5 wks. RA and RA-containing culture stocks were protected from light to prevent photo-destruction. Cells were maintained in a 37˚C humidified chamber that contained 5% CO_2 and were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with F-12 nutrient mixture (D-MEM/F12, Life Technologies, Gaithersburg, MD), 10% cosmic calf serum, (Hyclone Laboratories, Logan, UT), 0.1% antibiotic-antimycotic solution (Sigma

Chemicals). Figure 1 depicts the process of RA-induced differentiation from NT2 precursors to NT2-N neuronal cells.

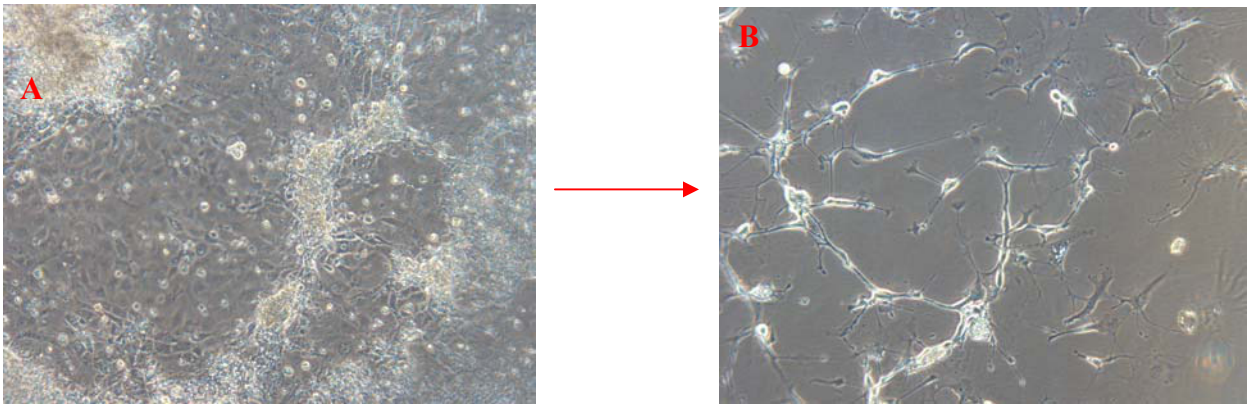

Figure 1. Retinoic acid-induced differentiation of NT2 cells. Neuronal differentiation of NT2 precursor cells (A) to NT2-N mature neurons (B) induced by the addition of 10μM retinoic acid for approximately 4 wks

Zinc Deficiency

To study the effects of zinc deficiency on RA-induced differentiation of NT2 cells, RA treated NT2 cells were grown in sterile, plastic, tissue grade 6-well plates coated with Matrigel Matrix (BD Biosciences) to support attachment. Cells were grown in 1 of 3 different concentrations of zinc obtained by chelexing the serum before adding it to DMEM media and then adding back specific amounts of zinc, to mimic various severities of a zinc-deficient state. Cells were treated with zinc adequate (n=8; 2.5 μM Zn), moderately zinc deficient (n=8; 1.0 μM Zn) or severely zinc deficient (n=8; 0.4 μM Zn) media. Cells were collected at times 0, followed by 1-wk, 2-wks, 3-wks post-RA treatment at each level of zinc.

Immunocytochemistry of TuJ1

To examine the effect of zinc deficiency on RA-induced neuronal differentiation, immunocytochemistry was used to monitor the appearance of the neuronal marker TuJ1. After treatment cells were washed with phosphate buffered saline (PBS, pH 7.4) and fixed with 3.7% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS for 10 minutes at room temperature. Cells were then permeabilized with 0.2% triton X-100 in PBS. Following 3 additional washes with PBS, coverslips were incubated overnight at 4˚C with a commercially prepared primary antibody including a mouse monoclonal antibody for β-tubulin type III, TuJ1 (Covance), at a concentration of 1:500 in 1% bovine serum albumin (BSA). After 3 washes in PBS, cells were incubated with a fluorescently labeled secondary antibody (overnight at 4˚C). This was a donkey anti-mouse antibody conjugated to the fluorescent dye cyanine 3 (Cy3) at a concentration of 1:250 in 1% bovine serum albumin. After 3 PBS washes (5 min each, room temperature) the cells were incubated in 4', 6-diamidino-2-phenylindole (DAPI) for 20-minutes to stain for nuclear morphology analysis. The cells were then rinsed in PBS and mounted onto microscope slides with a commercially prepared anti-fade mounting medium (FluorSave Reagent, Calbiochem-Novabiochem, La Jolla, CA). Cells were then examined and photographed using a Nikon Microphot-FX equipped with epifluorescence. Exposure times were held constant to permit comparisons between treatment groups.

Immunocytochemistry of TGF-beta Receptor Types I and II

The same immunocytochemistry protocol described above was employed to determine the effect of zinc deficiency on TGF-beta receptor expression using a rabbit polyclonal antibody for TGF-β RI (Santa Cruz Biotechnology) and TGF-β RII (Santa Cruz Biotechnology). The same procedure was repeated, with the exception of omitting the cell membrane permeability step involving the use of 0.2% triton X-100 in PBS. This allowed specific observation of membrane vs. nuclear localization of TGF-beta RI and RII receptor expression. Antibodies were used at a concentration of 1:500 in 1% bovine serum albumin. Cells were then incubated with fluorescently labeled secondary antibodies (overnight at 4˚C); donkey anti-rabbit antibody conjugated to the fluorescent dye cyanine 3 (Cy3) at a concentration of 1:250 in 1% bovine serum albumin. Immunocytochemistry was used to detect both receptor isoforms at time 0 and 3-wks, following treatment with retinoic acid as well as the 3 zinc conditions listed above. Cells were then examined and photographed using a Nikon Microphot-FX equipped with epifluorescence. Exposure times were held constant to permit comparisons between treatment groups.

CHAPTER 3

RESULTS

Retinoic Acid Induces Differentiation

Immunofluorescent expression of the TuJ1 antibody in NT2 precursor cells was not observed. Evidence supporting the lack of TuJ1 expression was documented via 4', 6-diamidino-2-phenylindole (DAPI) staining for the identification of cellular nuclei in culture (data not shown). An abundance of nuclei were expressed, while TuJ1 was not evident (Figure 2A). A comparison of TuJ1 expression in NT2 with that of mature neuronal NT2-N cells following 2 weeks of retinoic-acid (RA) induced differentiation under zinc adequate conditions confirmed an abundant expression of TuJ1 in differentiated cells (Figure 2B). One wk of RA treatment in zinc adequate cells was sufficient to induce expression of TuJ1 (data not shown). Figure 2 depicts TuJ1 immunofluorescent staining in NT2 and NT2-N cells cultured under zinc adequate conditions. Neuronal cell morphology was also detected at 2 weeks of RA-induced differentiation under zinc adequate conditions via decreased cell body size and the appearance of neurite elongation. Figure 3 shows a magnified photograph of a growth cone in an NT2-N neuron grown in a zinc adequate culture, further supporting the presence of neuronal differentiation and phenotype.

Zinc Deficiency Impairs Neuronal Differentiation

Two weeks of RA-induced differentiation under zinc deficient conditions yielded a significant decrease in the intensity of TuJ1 staining under microscopic observation compared to that noted in the zinc adequate differentiated NT2-N cells (Figure 2B). In addition, morphological changes indicating the presence of mature neurons in culture was not observed in NT2-N cells grown in zinc deficient conditions. Furthermore, 1 wk of RA treatment in zinc deficient cells was sufficient to show signs of impairment (data not shown). Cells grown in a milder concentration of zinc deficiency (1.0μM) also showed a decrease in TuJ1 expression in response to RA, although this observation was not as extreme as that noted in the severely zinc

deficient (0.4μM) state (data not shown). Figure 2 depicts TuJ1 immunofluorescent staining in NT2-N cells cultured under zinc deficient conditions, as compared to that expressed in the zinc adequate NT2 and NT2-N cultures.

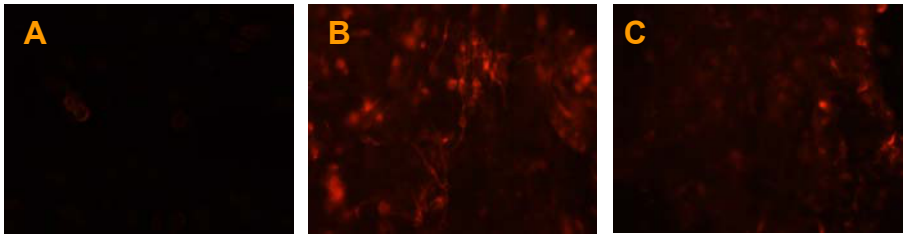

Figure 2. Effect of zinc on TuJ1 expression during retinoic acid-induced NT2 differentiation. TuJ1 immunofluorescent staining at 20x magnification (A, B, C). TuJ1 control NT2 cells under ZA (2.5μM) conditions with no exposure to RA (A). TuJ1 immunofluorescent staining in NT2-N cells following 2 wks exposure to 10μM RA under ZA (2.5μM) and ZD (0.4μM) conditions (B, C).

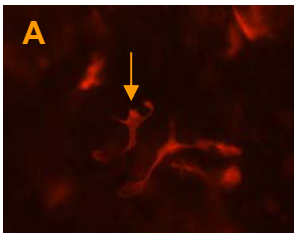

Figure 3. Retinoic acid-induced growth cone formation in NT2 cells. TuJ1 immunofluorescent staining at 40x magnification; NT2-N morphology under ZA (2.5μM) conditions following 2 wks treatment with 10μM RA identified by cell body size, neurite outgrowth, and presence of growth cone (indicated by arrow) mediating movement (A).

Expression of TGF-beta Receptors During Differentiation

Transforming Growth Factor-beta Receptor I. Immunocytochemistry of TGF-β RI immunoreactivity revealed that undifferentiated NT-2 cells expressed this receptor isoform on the plasma membrane (Fig 4A). Under zinc adequate conditions, differentiation of NT-2 precursor cells into post-mitotic neurons using 10uM RA resulted not only in morphological changes consistent with neuronal differentiation, but also reduced expression of RI (Fig 4B). However, under conditions of zinc restriction, RI expression was not reduced by RA treatment (Fig 4C).

Transforming Growth Factor-beta Receptor II. Like the TGF-β RI isoform, the TGF-β RII isoform was also identified on the plasma membranes of NT2 neuronal precursor cells (Fig 5A). Differentiation of cells with RA also reduced the immunoreactivity of this receptor isoform (Fig 5 B). However, in contrast to RI, zinc restriction, did not alter this pattern such that RA treated cells had reduced RII immunoreactivity in both zinc adequate and zinc deficient conditions (Fig 5 B, C). Immunocytochemical localization of TGF-β RII in permeabilized cells suggested that reduction in plasma membrane localization of this receptor isoform was the result of zinc reduced protein synthesis.

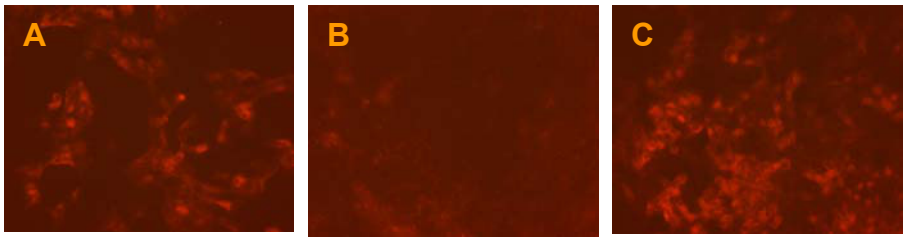

Figure 4. Effect of zinc on TGF-β RI expression during retinoic acid-induced NT2 differentiation. TGF-β RI immunofluorescent staining at 20x magnification (A, B, C). TβRI control NT2 cells under ZA (2.5μM) conditions with no exposure to RA (A). TβRI immunofluorescent staining in NT2-N cells following 2 wks exposure to 10μM RA under ZA (2.5μM) and ZD (0.4μM) conditions (B, C).

Figure 5. Effect of zinc on TGF-β RII expression during retinoic acid-induced NT2 differentiation. TGF-β RII immunofluorescent staining at 20x magnification (A, B, C). TβRII control NT2 cells at under ZA (2.5μM) conditions with no exposure to RA (A). TβRII immunofluorescent staining of NT2-N cells following 2 wks exposure to 10μM RA under ZA (2.5μM) and ZD (0.4μM) conditions (B, C).

CHAPTER 4

DISCUSSION

Zinc Deficiency Impairs Neuronal Differentiation

To test the hypothesis that zinc deficiency impairs retinoic acid-induced neuronal differentiation, we used the neuronal marker TuJ1. NT2 neuronal precursors were examined during the process of differentiation under zinc adequate and zinc deficient conditions. TuJ1, or Neuronal Class III β-tubulin is an essential protein required in the formation and stability of microtubules within cells and has been used as a neuronal marker even in early stage differentiation and cell fate commitment (Lee, 2005). Microtubules play roles in cytoskeleton structure, intracellular transportation and mitosis as well as assisting in the control of axonal elongation, a critical step in the process of neuronal differentiation (Erturk et al., 2007). Examination of TuJ1 antibody expression in NT2 neuronal precursor cells revealed that these cells do not express this neuronal marker. This is consistent with the undifferentiated, stem cell-like character of these cells.

In zinc adequate cells, robust TuJ1 expression, as well as the appearance of distinct morphological features such as small neuronal cell bodies with neurite extensions and the presence of growth cones, was clearly observed the longer the cells were exposed to RA. One week of RA treatment was sufficient to induce expression of TuJ1. This is consistent with previous observations that this protein marker is induced early in the process of cell fate commitment and cellular differentiation.

In contrast, when NT2 cells were exposed to RA under zinc deficient conditions TuJ1 expression and morphological indications of differentiation were impaired. RA-induced differentiation of NT2 cells under the lowest of the 3 experimental zinc concentrations (0.4μM) used showed the most marked decrease in both the amount and the intensity of TuJ1 expression. Similar to the zinc adequate cells, 1 wk of RA treatment under zinc deficient conditions was sufficient to show signs of impairment. These results suggest that zinc is indeed required for RA-

mediated neuronal differentiation. It is interesting to note that while severe zinc deficiency most effectively impaired neuronal differentiation, moderate zinc restrictions clearly inhibited the differentiation process as well. This suggests that developing neurons may be particularly susceptible to the effects of zinc deficiency. This would be true both during embryonic development as well as in the neurogenesis that takes place during the early postnatal period. Also of note is the fact that neurogenesis takes place throughout the life span in the hippocampus (Eriksson et al., 1998). Furthermore, it is well known that this region of the brain is the most susceptible to dietary zinc deficiency (Takeda et al., 2007). Thus, future work will be needed to determine the optimal amount of dietary zinc for normal neurogenesis both during development and in adulthood.

One possible mechanism that could be governing the observation of impaired neuronal differentiation in response to zinc deficiency is directly related to RA signal transduction under conditions lacking adequate zinc. The normal RA signal transduction pathway was described in the introduction of this work. Nuclear retinoid receptors require zinc for proper conformation and function. A number of studies have begun to identify the structural components of these receptors. Of particular interest to this research is the DNA binding domain (DBD) of the retinoid receptor (RXR) which is composed of 2 zinc finger motifs that are critical for folding of the domain and its ability to bind directly to the major groove of DNA. Binding occurs at retinoic acid response elements (RARE) located on the promoter region of retinoid targeted genes such as SOX3 (Mojsin et al., 2006), IGF binding proteins, Notch associated proteins, and HOX proteins, all of which have been shown to play a role in development and differentiation (Freemantle et al., 2002).

Wang et al. demonstrated that disruption of the RA mediated pathway leads to a deregulation and decline in the status of healthy viable neurons. Specifically, the blockade of RA signal via a dominant-negative (dn) retinoid receptor lacking necessary transactivation domains was obtained by transfecting P2 mouse SVZ cells with the dn retinoid receptors and a GFP reporter to distinguish cells containing the altered receptors. The experiment revealed changes in morphology of SVC cells, including shorter process extension, and more nestin immunoexpression suggesting the presence of an immature neuronal phenotype. This evidence

demonstrated a decline in neuronal differentiation in response to the loss of RA signaling. Additionally, these researchers observed a decrease in the length of migration to the olfactory bulb from the SVZ in this experiment, suggesting a possible role for RA in the regulation of cellular migration in neurogenesis (Wang et al., 2005).

Based on the current experiments as well as observations from the literature it is plausible to suggest that zinc deficiency impairs RA-induced neuronal differentiation because a lack of zinc compromises the structural stability of retinoid receptors required for RA signal transduction. Specifically, the inadequate zinc disrupts the ability of the RXR receptor to fold into the proper conformation at the DBD, leading to a loss of function. Additionally, the lack of zinc finger proteins required for DNA binding truncates the transcriptional activity of RA signaling. This inhibition of RA signaling would not only impair neuronal differentiation, but ultimately neuronal migration.

TGF-beta Modulation of Impaired Neuronal Differentiation Under Zinc Deficient Conditions

While there are likely to be a number of molecular mechanisms such as RAR/RXR-mediated gene expression that are disrupted by zinc deficiency during neuronal differentiation, the second phase of this work explored the possibility that zinc restriction alters TGF-β signaling in differentiating neurons. Figure 6, below, summarizes the expression of TuJ1 and TGF-β RI and RII as well as morphological characteristics of NT2 neuronal precursor cells, compared to mature NT2-N neuronal cells exposed to RA, under zinc adequate and zinc deficient conditions. The NT2 neuronal precursor cells, under zinc adequate conditions, showed no expression of TuJ1, however, robust expression of both TGF-β RI and RII was clearly observed, suggesting that both receptor isoforms are present in immature neuronal stem cells in vitro. Upon RA-induced differentiation of these cells under zinc adequate conditions, expression of both receptor isoforms RI and RII was down-regulated with almost no evidence of immunofluorescent staining for either receptor, suggesting that neither TGF-β RI nor RII is present in mature neuronal NT2-N cells in vitro. In contrast, TuJ1 became up-regulated and was clearly expressed in the zinc adequate NT2-N cells. Morphological characteristics of mature neurons also became evident

under these conditions, including decreased cell body size, increased neurite outgrowth, and the appearance of growth cones within these cells.

When NT2 cells were exposed to RA under zinc deficient conditions, TuJ1 was down-regulated with decreased expression, indicating the observed impairment of neuronal differentiation. Additionally, morphological characteristics of mature neurons were no longer evident under zinc deficient conditions. There also appeared to be a differential regulation of the 2 TGF-β isoforms taking place. TGF-β RI was up-regulated in zinc deficient NT2-N cells indicating impaired differentiation and the appearance of a mostly immature phenotype within the culture. Contrary to this observation, expression of TGF-β RII in zinc deficient NT2-N cells remained down-regulated as seen in the zinc adequate NT2-N cells, suggesting that RII may be differentially regulated from RI in response to zinc deficiency. Thus it appears that TGF-β RI, but not RII, is regulated by zinc. It is possible that under zinc deficient conditions, NT2 cells are partially differentiating with some characteristics of differentiated cells, such as decreased TGF-β RII expression, and other characteristics of undifferentiated cells including low levels of TuJ1 and increased expression of TGF-β RI. Alternatively, it appears from these data that both TGF-β RI and RII are needed for the response to RA. In zinc deficient conditions, TGF-β RII synthesis is reduced, suggesting that this may be playing a role in the inability of zinc deficient cells to differentiate when exposed to RA.

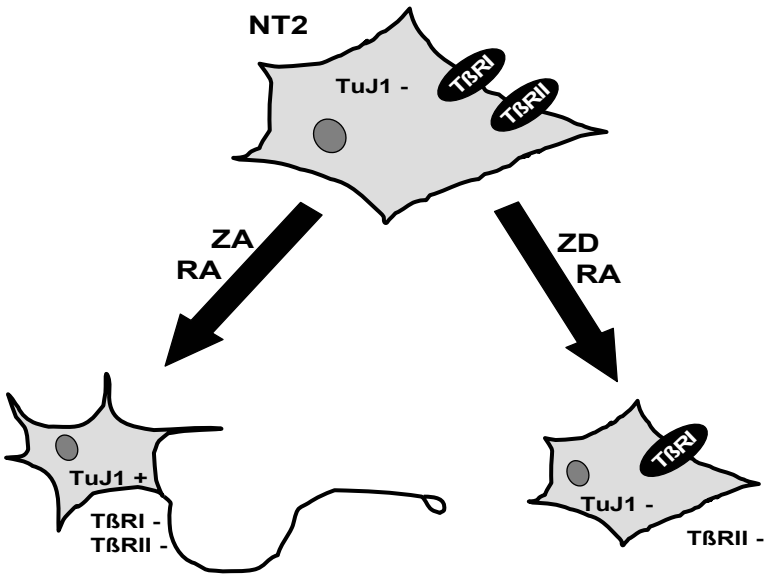

Figure 6. Effect of zinc on mechanisms of differentiation. Schematic summarizing the expression of TuJ1 and the differential expression of TGF-β receptor isoform types I and II. NT2 control cells do not express TuJ1, but do express both RI and RII under ZA (2.5μM) conditions with no exposure to RA. NT2-N cells express TuJ1, but do not express RI or RII under ZA (2.5μM) conditions following 2 wks of exposure to 10μM RA. NT2-N cells do not express TuJ1 and express RI, but not RII under ZD (0.4μM) conditions following 2 wks exposure to 10μM RA.

Consistent with this work, other researchers have also seen differential regulation of these 2 TGF-β receptor isoforms. A loss of TGF-β signaling has been identified by the down-regulation of TGF-β RII in various cell types. A study by Quan et al. exposed cultured human skin fibroblasts to UV irradiation and discovered that the TGF-β/Smad signaling pathway was reduced by means of a 60% decline in TGF-β RII mRNA expression. The same group had previously shown a similar decrease of TGF-β RII mRNA and protein expression combined with an increase in the inhibitory Smad7 protein in mink lung epithelial cells in response to UV irradiation as well. TGF-β RI expression was not altered in this scenario, providing another example of dissimilar regulation of RI and RII under cellular insult and impairment (Quan et al. 2004). Additionally, a study by Tesseur et al. looked at the role of TGF-β RII in Alzheimer's disease. A comparison of midfrontal cortical gray matter extracts from patients with Alzheimer's disease, Parkinson's disease, Pick's disease and various other forms of dementia revealed decreased levels of TGF-β RII in Alzheimer's diseased patients, but not in the other conditions (Tesseur et al., 2006).

The data collected through this work also conflicts with some studies that suggest a lack of these TGF-β receptors in neuronal precursors with an increase in expression, especially of the type II receptor, identified in a more mature neuronal phenotype. An experiment by Ren et al. found this to be true, indicating that the TGF-β type II receptor is not seen in NT2 precursors, but rather induced expression occurs with the process of differentiation in these cells. (Ren et al., 1997). A number of reasons could explain these differences in observations of TGF-β receptor expression in NT2 cells including potential discrepancies in culture conditions, serum batch differences and even the passage number of the NT2 cell line being utilized. Additionally, this work employed the use of Matrigel Matrix to form a basement membrane to support attachment of cells in culture. Matrigel also supplies a number of growth factors within the solution to enhance survival of neurons in culture, thus closely mimicking the in vivo microenvironment without the addition of astrocytes into the cell culture. The study by Ren et al. used poly-D-lysine to support attachment of cells, but did not utilize Matrigel and thus likely did not expose cultured cells to the same environment exploited in this work.

Once we saw that zinc deficiency reduced the plasma membrane RII immunoreactivity, we wanted to determine if this was the result of decreased protein transport to the membrane, or decreased protein synthesis. To answer this question a follow-up experiment was designed to examine intracellular RII synthesis and localization. Prior to primary antibody application, zinc adequate and zinc deficient cells were permeabilized with Triton X-100 to allow immunolocalization of intracellular RII stores. This approach enabled us to conclude that zinc deficiency reduces RII synthesis. It is not yet known if this reduction in synthesis is due to zinc requirements for gene expression and/or protein translation. Future work will be needed to examine the effect of zinc on RII mRNA abundance.

Multiple studies have demonstrated a role for TGF-β in an anti-proliferative/pro-differentiation model of cell cycle control (Lim, 2007; Lu, 2005). Lu et al. identified that hippocampal cells showed a significant decrease in BrdU expression when exposed to medium containing BDNF (50-100 ng/ml; $p<0.05$; $p<0.001$) or TGFβ-2 (1-10 ng/ml; $p<0.01$) indicating a regulatory role of cell cycle through the inhibition of cellular proliferation. The promotion of cell fate commitment and cellular differentiation was simultaneously shown via significantly increased neurite growth with exposure to both BDNF and TGFβ-2 ($p<0.05$). The specific down-regulation of TGF-β RII observed in NT2-N neuronal cells in this work would theoretically lead to a loss of TGF-β signaling in cells, since both RI and RII are required in a dependant manor for activation and propagation of TGF-β transcription to occur. Perhaps when these cells become impaired by their zinc deficient state, they prioritize their role in cell cycle control in an attempt to help govern the cell's decision between survival and death mechanisms. They might decrease signaling and in effect place preference on cell proliferation and survival over cell fate commitment and differentiation under the impaired intracellular conditions.

The TGF-β RI and RII promoter regions have been shown to contain a number of different regulatory elements. While both RI and RII promoters include some similar regions, such as Sp1 sites, RII contains additional binding sites including one referred to as a CCAAT-box (Lu et al. 2004). Toe et al, described a potential role for the zinc finger protein, Krox-26, in regulating gene expression through sequences associated with the CCAAT-box region in multiple tissues. This may suggest a second reason why we would see a decrease in TGF-β RII

expression in zinc deficient NT2-N cells, while RI remains up-regulated. In addition, it is conceivable that once the cell determines that it will block the TGF-β signal responsive to zinc deficiency, it is simply most efficient to down-regulate expression of the RII receptor isoform, which binds directly with TGF-β ligand in the first step of the signaling pathway. This could be another explanation for the differential regulation between RI and RII in the zinc deficient NT2-N cells; i.e. if the cell can effectively block signaling via the down-regulation of one receptor, why waste the energy needed to turn on TGF-β RI.

There is another place in the signaling pathway of TGF-β which could be affected by zinc deficiency. Tsukazaki et al. recently identified a novel molecule that is critically involved in the proper functioning of the TGF-β pathway and in assuring that the correct cellular outcome is induced. The molecule, called Smad Anchor for Receptor Activation (SARA), is primarily responsible for sub-cellular localization of the R-Smad proteins and thus facilitates their interactions with the membrane-bound TGF-β receptor complex. A series of experiments were utilized to identify the presence, characteristics, and interactions of SARA in reference to the TGF-β signaling pathway. Of particular relevance to this research was the finding that the SARA molecule contains a double zinc finger domain, known as FYVE, that is responsible for specifically recruiting Smad2 to the cell membrane and fostering its association with the TGF-β RI portion of the receptor complex. These researchers also found that a deletion of the FYVE zinc finger domain from SARA resulted in the misplacement of the Smad2 protein and a corresponding inhibition of TGF-β transcriptional activity within target cells (Tsukasaki et al., 1998).

A loss of TGF-β signaling for any number of reasons has been shown not only to shift the balance of cell cycle regulation among proliferation, differentiation, and survival or death mechanisms, but also to trigger spontaneous formation of a variety of different types of cancerous tumors and may also be implicated in the age-dependant formation of β-amyloid plaques in the brain tissue of a rodent model of Alzheimer's disease as well as in an in vitro neuroblastoma cell line. There is an onset of neurodegeneration including a decline in neuronal cells, synaptic functioning and dendritic stability and increased deposition of β-amyloid peptide in rodent brains deficient in TGF-β RII (Das and Golde, 2006). These findings reiterate the

necessity for understanding the processes underlying adult neurogenesis and the ways in which it can go awry.

CHAPTER 5

CONCLUSION

Future Directions and Experiments

An important line of questioning to pursue for future investigation in this area of research could be the determination of specific serum vs. cellular zinc levels both in vitro and in vivo. Determining potential differences between peripherally circulating zinc and that which crosses the blood-brain barrier to directly affect the microenvironment of the brain and central nervous system could likewise be of great benefit in one day determining the clinical relevance of this research. While we have developed a working model using moderate and severe concentrations of zinc deficiency (1.0μM; 0.4μM) in vitro, we do not know how this relates to in vivo situations. One way of studying this question could include examining levels of zinc in brain cells. This may provide a baseline idea of the amount of zinc circulating in the brain during zinc adequacy as well as zinc deficiency to obtain a more precise idea of biological relevance in terms of zinc requirements.

Studies designed to look at the mechanisms underlying differentiation of neurons under multiple concentrations of zinc are also important. As this work suggests, the expression of TGF-β RI and RII may be involved in such a mechanism. Additional work to elucidate this role is necessary. This may be done by determining a list of genes that are up-regulated under zinc deficient impairment of RA-induced differentiation in NT2 cells. Comparing this to genes that may be regulated under zinc adequate conditions could show discrepancies and changes in molecular targeting of these cells. Using cDNA micro array technology, genes specific to TGF-β could be identified and analyzed. This type of experiment could also help to clarify whether the impairment of differentiation in zinc deficient neuronal cells can be contributed to a process of death such as apoptosis occurring in cells that attempt to differentiate or simply a failure of cells to begin maturing at all. A combination of the two is also possible.

Furthermore, a study designed to evaluate the specific role of TGF-β RII in differentiation would be useful. One way of doing this would be to employ a model using a TGF-β RII knockout (KO) construct in NT2 cells to determine if this is sufficient to impair differentiation in these cells. This could be done through the immunoneuralization of TGF-β RII. Secondly, overexpression of RII in zinc deficient cells to determine whether this would rescue their ability to differentiate would also be a valuable experiment.

Ultimately, the results of this research contribute to the overall knowledge of mechanisms governing adult neurogenesis throughout the human lifespan and may help improve understanding and development of therapeutic approaches to dealing with increasingly prevalent neurodegenerative disorders. Acknowledging the role of zinc in the impairment of neuronal differentiation is important and could be useful in targeting synergistic methods of nutrient and drug treatments in conditions such as depression and perhaps even Alzheimer's disease. Additional research investigating the multiple aspects of neurogenesis in the adult brain is of great interest among the scientific and medical communities. This research may help pave the way to a greater comprehension and application of neuronal differentiation, a key step in functional adult neurogenesis.

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BIOGRAPHICAL SKETCH

Shannon D. Gower-Winter

EDUCATION:

M.S. in Clinical Nutrition, College of Human Sciences, Florida State University, Expected Spring 2008

B.S. in Food and Nutrition, Dietetics with an Emphasis in Sports Nutrition**,** College of Human Sciences, Florida State University, Fall 2005

Clinical Nutrition Internship, Florida State University, May 2006-August 2006 and August 2007-November 2007

- Performed nutrition assessments and medical charting for patients in a nursing home setting and in a mental health hospital
- Participated in patient interaction and interviewing for dietary needs
- Offered individualized nutrition counseling for clients at the Leon County WIC Program and for college students at the Thagard Student Health Center at Florida State University
- Prepared and delivered nutritional lesson plans and activities to elementary age students in the Woodville Elementary After School Program

Relevant Coursework, Florida State University, Spring 2006-Spring 2007

- PSY 4930 – "Research Methods in Neuroscience," – learned skills pertaining to rodent care, rodent ovariectomy surgery, estrous cycle determination via swabs, and rodent brain tissue slicing and mounting
- PCB 5137 – "Advanced Cell Biology," – gained in-depth understanding of molecular and cellular mechanisms
- HUN 5242 - "Carbohydrates, Fats and Proteins," – gained in-depth understanding of macronutrient metabolism
- HUN 5243 – "Vitamins and Minerals," – gained in-depth understanding of micronutrient metabolism at the molecular and genetic levels
- HUN 5938 – "Medical Nutrition Therapy," – gained practical skills and knowledge in the assessment, diagnosis, and treatment of nutrition related diseases and health concerns for patient care
- HUN 5938 – "Nutrition Counseling" – gained skills and knowledge for counseling patients towards sound nutritional health

TEACHING SKILLS AND EXPERIENCE:

- HUN 1201 – "The Science of Nutrition," Florida State University, Fall 2006-Fall 2007
  - Prepared and delivered 28, 75 minute lectures to classes of 20-35 students
  - Administered, graded, and recorded all quizzes and examinations for 2 classes of 20-35 students
  - Administered final grades and student logistics for 2 classes of 20-35 students
  - Guided students in the understanding of nutritional science and dietary assessment
- PET 3322 – "Anatomy and Physiology 1 Laboratory," Florida State University, Summer 2007

    - Prepared and delivered 12 lectures in laboratory setting
    - Taught students using human anatomical models
    - Prepared, administered, graded, and recorded 10 quizzes and 3 examinations for approximately 65 students
- Society for Neuroscience, Brain Awareness Week 2007, Florida State University Chapter
    - Prepared and delivered lecture to educate high school students about important brain related issues

CURRENT RESERCH INTERESTS AND EXPERIENCE:

- The role of zinc in neuronal stem cell differentiation
- The role of zinc and TGF-beta in human neuronal stem cells
- Zinc mediated mechanisms of neuronal differentiation
- Mammalian cell culture methods
    - Growth, differentiation and maintenance of NT2 human neuronal stem cells
- Immunocytochemistry
    - Primary and secondary antibody staining of NT2 human neuronal stem cells

HONORS, AWARDS AND MEMBERSHIPS:

- Omicron Pi Chapter of Kappa Omicron Nu Human Sciences National Honor Society member Spring 2007-Present
- Florida State University Teaching Assistantship Fall 2006-Present
- Florida Bright Futures Academic Scholarship Fall 2000-Fall 2005
- American Dietetic Association, member Fall 2004-Present