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The Role of Trace Metals in Neuronal Gene Expression

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ABSTRACT

The trace elements iron (Fe), copper (Cu) and zinc (Zn) play important roles in neuronal differentiation, function, and survival. These metals have been implicated in neuropsychiatric and neurodegenerative disorders, such as Parkinson’s, Alzheimer’s, and Wilson’s diseases, that have been associated with changes in neuronal proliferation and differentiation, and are characterized by apoptotic neuronal death. The work described in this dissertation explores the molecular roles of Fe, Cu, and Zn, with a particular focus on gene expression during neuronal differentiation and apoptosis. The first part of this work uses both in vivo and in vitro models to explore the role of Fe in neuronal differentiation. This work showed that retinoic acid, a known molecular regulator of neuronal differentiation, may act through its ability to increase expression of ferritin H, a protein that plays a role in neuronal Fe utilization. The second part of this work examined the role of Cu in the molecular mechanisms responsible for neuronal apoptosis. This work showed that Cu toxicity, such as that seen in several neurodegenerative disorders, induces neuronal apoptosis that is dependent on the expression and nuclear translocation of the tumor suppressor protein p53. Because p53 acts as a DNA-binding transcription factor, follow-up work used oligonucleotide array to identify p53-target genes that regulate neuronal survival and apoptosis. Both pro-apoptotic genes (IGFBP-6 and c-jun) and anti-apoptotic genes (Hsp 70 and 27) were regulated by p53 under conditions of neuronal Cu-overload. We also showed that p53 trafficking was regulated by Zn. High concentrations of neuronal Zn prevented nuclear translocation of p53, inhibiting its functions in gene transcription and apoptosis. The final part of this work used subtracted cDNA libraries, differential hybridization, and high throughput gene expression profiling (microarray) to identify new molecular roles of Zn in the olfactory bulb, a region of the brain known to have high concentrations of Zn and an association with several neurological disorders. This work showed that Zn regulates a variety of bulb genes involved in both neuroplasticity and apoptosis, and suggests that previously reported positive effects of caloric restriction on neuronal survival and plasticity are abrogated by the development of Zn deficiency. In summary, this work has identified a number of novel molecular roles for the trace elements Fe, Cu, and Zn in neuronal differentiation and apoptosis, and suggests a role for these metals in neurological disorders associated with changes in neuronal survival.
BACKGROUND AND SIGNIFICANCE

The work in this manuscript was designed to evaluate the molecular mechanisms of trace metals in the central nervous system (CNS). Iron, copper and zinc are the most abundant metals in the CNS and their homeostatic balance is critical for neuronal viability. While many functions of these metals are recognized in the following review, our studies used specific neuronal models including differentiation, copper toxicity and zinc deficiency to elucidate the molecular roles of these metals at the level of gene and protein expression. By studying neuro-molecular expression, our goal was to characterize the mechanisms by which trace metals function in models of neuronal death and thereby assist in the development of therapeutic strategies.

Neuronal Iron

Iron Functions. The micronutrient iron is the most abundant transition metal found in the brain. Iron is involved in many neuronal processes including DNA, RNA and protein synthesis. Interestingly, neuronal iron is critical for the development of myelin and dendritic arborization. It also functions as a cofactor for the synthesis of the neurotransmitters, dopamine and serotonin (Youdim, 2000). Dietary iron deficiency is the most prevalent nutritional problem in the world today leading to alterations in cognitive development secondary to abnormalities in myelin and neurotransmitter synthesis (Beard, 2003). Within the brain, iron is most abundant in the basal ganglia (Beard et al., 1993) and its primary storage is found in oligodendrocytes (Connor and Menzies, 1996). Despite the necessity for iron in neuronal viability many studies have shown that free iron can be neurotoxic through the generation of reactive oxygen species (Ke and Ming, 2003). Neurodegenerative disorders, such as Parkinson’s disease have been correlated to iron toxicity (Berg et al., 2001). The maintenance of neuronal iron homeostasis is therefore critical for cellular integrity. Precision of iron regulation is carried out through an interplay of iron sensors (iron-regulatory proteins), transporter proteins (transferrin/transferrin receptors), and iron storage proteins (ferritin) (Sorond and Ratan, 2000).

Neuronal Ferritin. Ferritin is the major storage protein of neuronal iron and can bind up to 4,500 atoms of iron (Morris et al., 1992). Ferritin like iron is found predominantly in oligodendrocytes of the brain where it is thought to play a role in myelination (Morris et al.,
Interestingly, ferritin can be found in two separate assembled structures. The ferritin H (heavy) subunit has ferrioxidase activity allowing for rapid uptake and release of iron, therefore functioning in iron utilization. In contrast, the ferritin L (light) subunit is involved in the slow uptake of iron and long term storage (Cheepsunthorn et al., 1998). Findings by Connor, 1994 have shown that the ferritin H subunit in some cases is located in neurons suggesting that ferritin may function as a mechanism for rapid uptake of neuronal iron. Furthermore, ferritin is developmentally regulated in the brain (Levenson and Fitch, 2000) and functions in cellular differentiation (Sanyal et al., 1996).

**Neuronal Copper**

*Copper Functions.* Copper (Cu) is an essential micronutrient serving multiple physiological functions. The Cu ion is a cofactor in numerous enzymes including cytochrome c oxidase, involved in the electron transport chain and ATP production, lysyl oxidase required for collagen synthesis and maintenance of vascular integrity, superoxide dismutase a free radical scavenger that protects cells from oxidative stress, and dopamine β-hydroxylase which converts the neurotransmitter dopamine to norepinephrine. Many neuropeptides including NPY require Cu availability for modifications by the enzyme peptidyl α-amidating monooxygenase (PAM). Cu is also required for the function of ceruloplasmin which has ferrioxidase activity needed for iron metabolism in both the peripheral central nervous system (CNS). The homeostatic balance of Cu is critical for neuronal viability. Examples of neuronal proteins involved in Cu balance include metallothioneins, the P-type ATPase Wilson protein, and cytoplasmic Cu chaperones (Tapiero et al., 2003).

*Copper Toxicity.* Neurodegeneration is linked to Cu overload in multiple disorders. In research models of Parkinson’s disease Cu is linked to increased striatal Lewy bodies and α-synuclein with subsequent production of reactive oxygen species and neuronal death (Lee et al., 2003). In Alzheimer’s disease Cu is involved in the oligomerization of the Abeta protein which is required for the formation of senile plaques (Atwood et al., 2004). Cu is also responsible for the oxidation of the C-terminal methionine of the beta-amyloid protein which generates free radicals and causes neuronal death (Pogocki, 2003). Wilson’s disease (WD) is an autosomal recessive disorder that leads to the accumulation of Cu with subsequent cellular injury and tissue damage. The advancement of WD is characterized by Cu overload in multiple organ systems, however,
early signs of WD are related to hepatic and neurological dysfunction. Patients often present first with cognitive impairments and behavioral abnormalities (Dening, 1991). These neurological symptoms often progress to Parksinsonian-like disturbances with muscle rigidity, tremor and bradykinesia (Strand et al., 1998). The primary defect in WD is a mutation in the Cu-transporting ATP7B P-type ATPase (WD protein) (Stapelbroek et al., 2003). This protein is required for the trafficking of intracellular Cu to ceruloplasmin and bile. Therefore, a dysfunctional WD protein leads to a build up of Cu in the liver which spills over into the circulation as free redox-active Cu and is transported to the CNS causing neuronal damage. The WD protein is expressed in neurons (Cox, 1995; Saito et al., 1999). This would suggest another mechanism increasing Cu levels in the CNS with subsequent early clinical signs of neurological impairment.

Copper deficiency. Menkes disease is an inherited X-linked recessive disorder that is lethal in early childhood (Rossi et al., 2001). The symptoms of this disorder include altered brain development, mental retardation, and neurodegeneration with the lowest levels of Cu being found in the thalamus, brain stem, and cerebellum (Yoshimura et al., 1995). Cu is required for neurodevelopment and when the Menkes protein (ATP7A P-type ATPase) is dysfunctional there is an inability for gut epithelial cells to efflux Cu to the portal circulation leading to both liver and CNS deficiency (Rossi et al., 2001).

The p53 Gene. The work described here will discuss the role of the trace metals zinc and copper on the tumor suppressor protein p53. The human p53 gene is found on the short arm of chromosome 17, band 13, with a length of 20 kb (Zupanska et al., 2002). It contains 11 exons and codes for a 393 amino acid protein. The p53 protein has multiple domains with varying biological functions. The N-terminal domain is involved in the selection of transactivated genes and carries the site for Mdm2 binding which controls p53 stability. There is also a proline-rich region involved in protein-protein interactions and the mediation of apoptosis. The C-terminal domain is involved in p53 oligomerization and repair of damaged DNA. The DNA binding domain is centrally located and carries out the major function of p53 which is controlling the transcription of a multitude of genes involved in cell cycle arrest, DNA repair, and apoptosis (Candau et al., 1997; Momand et al., 2000; Venot et al., 1998; Courtois et al., 2004).

p53 Functions. The p53 protein is a tumor suppressor protein that functions as a guardian of the human genome. Through its role as a DNA-binding transcription factor it responds to DNA damage first by initiating cell cycle arrest and then DNA repair, however, when DN
irreparable, p53 activates programmed cell death or apoptosis (Shwartz and Rotter, 1998). This form of p53-induced apoptosis is critical to prevent cellular proliferation after DNA has been mutated. Many forms of pathological neuronal death are the result of p53-induced apoptosis. These include focal cerebral ischemia in rat pups where p53 proteins were elevated 3-fold in infarct regions compared to regions of neuronal viability (Renolleau et al., 1997). In a model for Alzheimer’s disease, using Abeta treatment on SK-N-BE neurons, the p53-antagonist alphapifithrin prevented cell death by 60% (Tamagno, et al., 2003). Increased p53 levels are seen in neuroblastoma cells undergoing apoptosis following treatment with 6-hydroxydopamine, a model for Parkinson’s disease (Puttonen et al., 2003). Furthermore, the withdrawal of nerve growth factor from PC12 cells leads to the activation of p53 and subsequent neuronal apoptosis (Vaghefi et al., 2004). Neuronal control of p53 expression is therefore critical for both cell viability and DNA integrity.

Increased expression of neuronal p53 induces a multitude of genes involved in neuronal apoptosis. Examples of genes regulated by p53 include p21, a cyclin dependent kinase which arrests cells in the G1 phase of the cell cycle allowing time for repair of damaged DNA (Gramantieri, 2003). p53 controls the expression of GADD45, which functions to halt DNA synthesis and acquire time for DNA repair mechanisms to operate (Maeda et al., 2002). One of the ways in which p53 regulates the apoptotic cascade is through trans-activation of Bax which activates caspases and subsequent cell death. Interestingly, the expression level of Bax is dependent on p53 trans-repression of the anti-apoptotic transcript, Bel-2 (Cheng et al., 2003). Other pro-apoptotic genes regulated by p53 include IGFBP-6 which binds and prevents neurotrophic support of insulin-like growth factors (Grellier et al., 2002), and the immediate early gene, c-jun, which has been shown to increase mitochondrial cytochrome c release leading to the activation of pro-apoptotic caspases (Whitfield et al., 2001).

**Neuronal Zinc**

**Zinc Functions.** Like copper and iron the trace metal zinc (Zn) is vital to neuronal function. Zn is required as a cofactor for many enzymes including superoxide dismutase which catalyzes the removal of intracellular free radicals and metallothioneins which capture free radicals and prevent lipid peroxidation. The metalloenzymes, DNA and RNA polymerase, require Zn for the generation of nucleic acids. Furthermore, Zn is responsible for the conformation of a family of
transcription factors and nuclear receptors known as Zn-finger proteins which are involved in the regulation of gene transcription (Bulyk et al., 2001). The OB along with the hippocampus has the highest concentration of Zn in the brain (Ono and Cverian, 1999). Extensive electrophysiological studies have been performed to study the role of Zn in synaptic transmission. Zn is co-localized in a subset of glutamate releasing neurons and is involved in both inhibitory and excitatory neurotransmission in the OB (Trombley and Shepard, 1996). Interestingly, Zn in glutamatergic neurons causes increased levels of excitotoxic cell death following neuronal ischemia and seizure activity (Chen and Liao, 2003).

**Zinc Deficiency**  
Zn deficiency is a causative agent in multiple physiological abnormalities including growth retardation, diminished taste acuity, and hypogonadism (Prasad et al., 1985). Effects of cerebral Zn deficiency include decline in superoxide dismutase with increased oxidative stress and a decrease in the production of glutamate dehydrogenase which leads to improper glutamate metabolism and subsequent excitotoxic cell death (Constantinidis, 1991). Interestingly, human Zn deficiency has been linked to anorexia (Shay and Mangian, 2000). Studies have shown that dietary Zn deficiency in a rat model causes a reduction in food intake that begins within 3-5 days (Rains et al., 1998). Repletion of Zn in the food or water supply reverses this pattern of diminished food intake within 24 h (Shay et al., 1998). Zn supplementation in anorexic patients has been shown to enhance their the rate of recovery (Su and Birmingham, 2002). Regulation of appetite by neuronal centers in the appetite regulating network of the hypothalamus may be effected by dietary Zn deficiency. Furthermore, depression has been linked to anorexia and other neurodegenerative disorders such as Parkinson’s disease that are all correlated to a deficiency in Zn (Kaiser et al., 2000).

**Experimental Design**  
The following studies were specifically designed to study the role of trace metals in neuromolecular expression. Chapter one discusses the role of retinoic acid on the molecular expression of the iron-utilization protein, ferritin H. Data from this study was used to determine the involvement of iron in neuronal differentiation and the potential for ferritin H to prevent cell proliferation during development. In chapter 2 we evaluated the interactions of copper and zinc on neuronal viability. The role of p53 in metal-mediated mechanisms of neuronal cell death was analyzed as a potential target for intervention. To further elucidate the role of p53 in neuronal
apoptosis, data from chapter 3 were used to analyze the expression of p53-regulated genes. In chapter 4 we observed the effects of zinc deficiency on olfactory bulb gene expression. Previous studies suggest that Zn imbalances may lead to neuronal cell death with underlying molecular mechanisms.
CHAPTER 1

EFFECT OF RETINOIC ACID ON FERRITIN H EXPRESSION DURING BRAIN DEVELOPMENT AND NEURONAL DIFFERENTIATION

Introduction

The importance of adequate iron status during the development of the central nervous system (CNS) is well established. Iron is required for activity of several enzymes responsible for the synthesis and metabolism of neurotransmitters (Youdim, 2000) and lipid biosynthesis (Stangl and Kirchgessner, 1988). It is an essential co-factor for the heme-containing cytochromes b and c that participate in electron transport (Evans and Mackler, 1985), and is required for normal myelination throughout CNS development (de los Monteros et al., 2000; Connor, 1994). These functions, in part, help explain the observation that nutritional iron deficiency in early childhood results in cognitive impairment and behavioral abnormalities (Webb and Oski, 1973; Lozoff, 1988), which appear to be irreversible (Felt and Lozoff 1996). Thus, an understanding of the roles and regulation of iron and iron binding proteins in the brain is important.

Given the requirements for iron during brain development, it is not surprising that the iron-binding protein ferritin is developmentally regulated in both humans (Ozawa et al., 1994; Zecca et al., 2001) and rats (Focht et al., 1997; Levenson and Fitch, 2000). Ferritin, a heterogeneous association of heavy (H) and light (L) subunits coded by genes on separate chromosomes (Worwood et al., 2000), is involved in iron storage and utilization. The L subunit is the predominating isof orm in organs, such as liver and spleen, that participate in iron storage (Bomford et al., 1981; Cairo et al., 1991), while the H subunit is known to play a role in iron utilization (Arosio et al., 1978). Thus ferritin H is the primary subunit found in organs such as brain and heart that require high levels of iron availability and have high iron utilization (Connor, 1994; Cairo et al., 1991).

To date most of the work examining the regulation of ferritin in brain development has focused on oligodendrocytes, largely because 70% of brain iron is associated with myelin (de los Monteros et al., 2000). Myelination is critical to normal CNS development (Blissman et al.,
However, it is also clear that ferritin, particularly the H subunit, is important in neuronal iron metabolism. Ferritin H (but not L) is found in neuronal nuclei (Cheepsunthorn et al., 1998) and is highly enriched in synaptosomes (Ishimoto et al., 2000). Iron is required for the neuronal activity of tyrosine hydroxylase and tryptophan hydroxylase, the enzymes responsible for catecholamine and serotonin synthesis, and is needed at the synapse by the enzyme monoamine oxidase that catabolizes neurotransmitters (Youdim, 2000). Iron is also utilized by neurons to modulate the activity of dopamine D2 receptors (Youdim et al., 1989; Ben-Shachar et al., 1986). Furthermore, the delivery of iron to cultured neurons resulted in increased mitochondrial activity and neurite outgrowth (Bruinink et al., 1996).

Given the high demands for iron during development and the probable role for ferritin H in neuronal iron utilization during this period, it is not surprising that ferritin H mRNA (Levenson and Fitch, 2000) and protein (Connor, 1994) abundance increase after the early postnatal period. The molecular mechanisms responsible for this pattern of regulation are not known. Previous work has shown that thyroid hormone plays a role in the developmental pattern of ferritin H mRNA expression in brain (Levenson and Fitch, 2000), but did not explore the critical issue of the regulation of ferritin in neuronal differentiation and development.

In this work we test the hypothesis that retinoic acid (RA) plays a role in the regulation of ferritin H in brain development and neuronal differentiation. The vitamin A metabolite, retinoic acid (RA), is involved in the regulation of CNS development (Maden and Holder, 1992; Kornyei et al., 1998; Ross et al., 2000). This action is mediated by the of nuclear RA receptors RAR (α, β, γ isoforms) and RXR (α, β, γ isoforms) (Stunnenberg, 1993; Glass et al., 2000). These Zn-finger proteins are part of the type II class of nuclear steroid hormone receptors (Stunnenberg, 1993). In the presence of RA, they form heterodimers, either with each other or with other members of the type II class such as vitamin D receptors (VD3R) or thyroid hormone receptor (T3R) to bind RA response elements (RARE) in the promoter regions of RA-responsive genes (Glass et al., 1991). In this way RA has been shown to direct the gene expression responsible for embryogenesis and growth (De Luca, 1991; Ross, 2000) and regulate the differentiation of a wide of a variety of cell types including neurons (Moasser et al., 1995; Rohwedel et al., 1999).

Given the importance of iron in CNS development and neuronal differentiation, this work explored the possible role of RA in the developmental regulation of ferritin H in vivo and tested the hypotheses that RA contributes to the observed developmental increases in ferritin H mRNA.
Clearly neuronal differentiation is part of the developmental process. Thus, an in vitro model was used to directly test the hypothesis that ferritin H is regulated by RA-induced neuronal differentiation and to explore the specific time frame of both ferritin H mRNA and protein expression following exposure to RA.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (n=3) were subcutaneously injected with 1 µg/g body weight all-trans retinoic acid (RA, Sigma Chemicals, St. Louis, MO) on postnatal day 1 (Veness-Meehan et al., 2000; Song and Levenson, 1997). Control rats were injected with equal volumes of the vehicle alone (10% ethanol in normal saline). On postnatal day 2, rats were decapitated and brains were immediately removed for the isolation of total cellular RNA.

**Neuronal Culture**

The human neuroblastoma cell line, NTera-2 (NT2, St ratagene, La Jolla, CA) was cultured in Dulbecco’s Medium Essential Medium with F-12 nutrient mixture (DMEM/F-12; Life Technologies, Gaithersburg, MD), 10% cosmic calf serum (Hyclone Laboratories, Logan, UT), and 0.1% antibiotic-antimycotic solution (Sigma Chemicals, St. Louis, MO). Cultures were maintained in a 37 °C humidified chamber containing 5% CO2. NT2 cells were induced to differentiate into post-mitotic neurons (NT2-N) with 10 µM RA (Pleasure et al., 1992). After 4 weeks, post-mitotic neurons were mechanically dislodged and transferred to 6-well plates with media containing mitotic inhibitors 5-fluoro-2'-deoxyuridine (10 µM) cytosine β-D-arabinofuranoside (1 µM) and uridine (10 µM). To determine the acute effects of RA, NT2 cells were treated with 10 µM RA for 0, 24, and 72 h. Cultures treated with RA were protected from light to avoid photodestruction of RA.

**Northern Analysis**

Total cellular RNA, isolated by Trizol extraction with chlorofrom (Life Technologies, Gaithersburg, MD) from rat brain and cultured neurons, was fractionated by size on a 1% agarose gel containing formaldehyde (0.66 M). Equal loading and integrity of the RNA was confirmed by visualization of ethidium bromide staining under UV light. The RNA was then transferred to a nylon mem brane (GeneScreen; NEN, Boston, MA) by capillary blotting overnight and UV crosslinked to the membrane. The nylon membrane was then hybridized for
18 h at 65° C with random primed 32P-labeled cDNA probes for ferritin H mRNA (Levenson and Fitch, 2000) and 28S rRNA (loading control). After three 65 ° C washes with 40 mM sodium phosphate buffer with 1 mM EDTA and 1% SDS, pH 7.2. All blots were then exposed to Kodak X-OMAT AR film at -80° C overnight. The relative amounts of bound cDNA probe were determined using computer evaluated densitometry (Quantity One Quantification Program) created by Protein and DNA Imaging (PDI, Boston, MA) and expressed as a function of 28S rRNA abundance.

**Immunocytochemistry**

To determine cellular localization and abundance of ferritin H protein, post-mitotic neurons and NT2 precursor cells were plated on sterile 22 mm 2 glass coverslips (n=6 for each condition) (No.1, Corning Glassworks, Corning, NY). After treatment with RA, cells were washed 3 times with phosphate buffered saline (PBS) and then fixed by incubation with 3.7% paraformaldehyde for 10 min. Cells were then permeabilized with 0.2 % Triton X-100 in PBS for 5 min. After 3 PBS washes, cells were incubated for 2 h at 37 ° C with a commercially prepared rabbit polyclonal IgG antibody specific for human ferritin (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution in 10 mg/ml bovine serum albumin (BSA). Cells were then incubated (2 h, 37 ° C) with a goat anti-rabbit secondary antibody conjugated to the fluorescent dye cyanine 3 (Cy3) (Jackson ImmunoResearch Laboratories, Westgrove, PA). Coverslips were mounted with a commercially prepared mounting medium (Fluorsave Reagent, Calbiochem-Novabiochem, La Jolla, CA) and examined using a Nikon Microphot-FX microscope equipped with epifluorescence. All images were collected using the same exposure time and digitized to permit quantification of pixel density.

**Iron Uptake**

The iron sensitive fluorescent indicator calcein-AM (Molecular Probes, Inc., Eugene, OR) was used to evaluate the effects of RA on the labile iron pool (LIP) in NT2 cells according to the method of Pinero et al 2000. Cells were plated at a density of 6.0 X 10^4 cells/cm^2 in 24-well plates, allowed to adhere for 24 h, and treated with RA for 0, 24 and 72 h. Additional wells were treated with 0, 1, 5, and 10 µM iron as ferrous sulfate (n=9) for 18 h (longer time resulted in neuronal death). Following RA or iron treatment, cells were incubated in media containing 0.25 µM calcein-AM for 20 min at 37 ° C. After incubation, cells were washed twice with DME/F12 media to remove unincorporated calcein. The fluorescence in each well was
determined from the mean of 6 measurements at 486 nm (20 ms each) by fluorometry (FluoroCount, Packard Biosciences, Shelton, CT).

**Statistical Comparisons**

Data were first analyzed by ANOVA followed by a post hoc Tukey’s Multiple Comparison Test (GraphPad Prizm, San Diego, CA). Where only two groups were compared, differences in means were determined by *t*-test. Means were considered statistically different at *p*<0.05.

**Results**

**Retinoic Acid Regulation of Ferritin H mRNA**

Early neonatal administration of RA resulted in a 4-fold increase in whole brain ferritin H mRNA abundance (*p*<0.001, Figure 1-1) after 24 h. Treatment of cultured NT2 cells with 10 µM RA halted the cellular proliferation and induced the differentiation of these cells into a neuronal phenotype characterized by neurite outgrowth (Figure 1-2). Immunocytochemistry before and after RA-induced differentiation showed that ferritin synthesis is clearly affected by the differentiation state. Undifferentiated cells expressed very low levels of ferritin (Figure 1-2A). Differentiation to the NT-2N phenotype increased ferritin immunoreactivity 4-fold, with high levels of ferritin found in the cell bodies, neurites and nuclei of post-mitotic neurons (*p*<0.01, Figure 1-2B). These increases after differentiation were accompanied by a 2-fold increase in ferritin H mRNA (*p*<0.01, Figure 1-3).

To determine whether neuronal differentiation is required for the increased synthesis of ferritin H mRNA or if RA regulates ferritin H more directly, cultured NT2 neuronal precursor cells were treated with RA for 72 h. RA treatment resulted in an increase in ferritin H mRNA that appeared to begin within 24 h of treatment. By 72 h, ferritin H mRNA levels were elevated 1.8-fold (*p*<0.05, Figure 1-4).

Figure 1-5 shows that like ferritin H mRNA, ferritin immunolocalization at 0, 24 and 72 h after RA began to accumulate in RA-treated cells after 24 h (1.2-fold, *p*<0.05). Figure 1-5B shows that at 24 h there was an increase in ferritin around the nuclei of RA-treated cells consistent with active ferritin synthesis and golgi transport at this time point. After 72 h, the ferritin immunoreactivity was further increased (1.4-fold, *p*<0.01) and widely dispersed throughout the cells including cell nuclei (Figure 1-5C).
Role of Iron in Ferritin H Expression

In undifferentiated NT-2 cells, the labile iron pool (LIP), as measured by a decrease in calcien fluorescence, was significantly increased 72, but not 24 h, after treatment with RA (p<0.05, Figure 1-6). For comparison purposes, cells were also treated with 1, 5 and 10 µM iron. As expected, treatment with increasing concentration of iron resulted in a consistent decrease in calcien fluorescence indicating an increase in the LIP (Figure 1-6, inset).

Discussion

The in vivo work reported here suggests that RA plays a role in the regulation of brain ferritin H mRNA during postnatal development, a period which is characterized by CNS growth and cellular differentiation. Differentiation has been shown to increase ferritin in a variety of cell types. RA-induced differentiation of the promyelocytic cell HL-60 increased cellular ferritin (Fibach et al., 1985; Cayre et al., 1987), while RA-treatment of U-937 cells resulted in differentiation into a monocyte-like cell and a 2-fold increase in ferritin (Fibach et al., 1985). Transient transfection of Friend erythroleukemic cells with the ferritin H promoter attached to a reporter gene showed that the increases in ferritin during differentiation were due to increases in ferritin H transcription (Coccia et al., 1995). Thus, ferritin has been used as a marker for cellular differentiation (Cayre et al., 1987).

Given that CNS development is characterized by a rapid increase in oligodendrocyte differentiation (Blissman et al., 1996) and myelination, and ferritin H gene expression increases during oligodendrocyte differentiation (Sanyal et al., 1996), it is likely that most of the 4-fold increase reported here after RA administration to neonatal rats is the result of increases in ferritin H mRNA in oligodendrocytes. Astrocytes also express ferritin (Gunnersen et al., 2000) and RA has been shown to induce differentiation in astrocytes derived from a stem cell line (Wohl and Weiss, 1998). However, it is also clear that RA induces neuronal differentiation (Berrard et al., 1993; Libonati et al., 2000; McCaffery and Dräger, 2000; Leypold et al., 2001). Thus, we hypothesized that RA-induced neuronal differentiation would result in an increase in ferritin H.

As hypothesized, differentiation of NT2 cells into post-mitotic NT-2N neurons resulted in a significant increase in ferritin H that was accompanied by an increase in ferritin H mRNA. While we do not know if this increase was the result of increased transcription or increased mRNA stability, several lines of evidence suggest that increased transcription is at work. First,
ferritin mRNA is stored in cells as a highly stable inactive message that is rapidly translated when cellular iron increases (Munro, 1993). Thus, increases in ferritin mRNA levels appear to largely reflect transcriptional increases (Sanyal et al., 1996). Furthermore, RA-induced differentiation increases ferritin H transcription in other cell types (Cocca et al., 1995).

An RA-mediated increase in ferritin H transcription is also consistent with the fact that RA is known to regulate a wide variety of genes involved in cellular differentiation via genomic retinoic acid response elements (RARE) (De Luca, 1991; Ross, 2000). The RARE consensus sequence (A/G)G(T/G)TCA aligned in a direct repeat with a 5 base pair nucleotide spacer confers RA responsiveness (Glass et al.; Ross et al., 2000). While NT2 cells express RAR and RXR nuclear receptors (Kitareewan et al., 1999; Cheung et al., 2000), examination of the approximately 1 kb of the ferritin H promoter that has been published (Yachou et al., 1991; GenBank Accession M73680) revealed a only a single sequence of AGGTCA starting at position -707. This sequence did not appear in a direct repeat and would therefore not be expected to confer RA responsiveness. While this analysis does not rule out RARE sequences farther upstream, it does raise the possibility that the effect of RA on ferritin H expression is via an RARE-independent mechanism.

There is evidence to suggest that the RARE-independent mechanism may be mediated by tumor necrosis factor alpha (TNF-a). TNF-a increases ferritin H transcription through a promoter element located 4.8 kb from the start site that represents a binding site for the NF-κB/Rel family of transcription factors (Kwak et al., 1995). These trans-acting factors appear to be required for neuronal differentiation (Feng and Porter, 1990). RA has been shown to enhance the transcriptional effects of TNF-a in differentiating keratinocytes (Janssens et al., 1999). Thus, the possibility that RA increases ferritin H mRNA via TNF-a and NF-κB/Rel transcription factors in differentiating neurons should be considered in future work.

While it is possible that ferritin H mRNA is induced via an RARE-independent mechanism such as described above, we also needed to test the possibility that differentiation, rather than RA, induces increases in ferritin. Furthermore, the possibility that RA-induced changes in the labile iron pool increased ferritin H. To begin to distinguish between these possible mechanisms, we tested whether RA increases ferritin H prior to differentiation and whether labile iron is increased in this time frame. The finding that the initiation of ferritin synthesis occurs after only 24 h of RA treatment strongly suggests that RA is regulating neuronal
ferritin H synthesis independently of cellular differentiation. This finding is interesting in light of an earlier hypothesis that ferritin may in fact serve as a regulator of cellular differentiation (Sanyal et al., 1996). Furthermore, it appears that ferritin expression is induced prior to the accumulation of labile iron, suggesting that the RA-mediated regulation of ferritin H is independent of iron. This is consistent with the finding that TNF-α regulates ferritin H independently of iron in human myoblasts (Miller et al., 1991) and that the regulation of ferritin during the RA-induced differentiation of U937 promonocytic cells is independent of iron (Iturralde et al., 1992).
Figure 1-1. Effect of exogenous retinoic acid on neonatal rat brain ferritin H mRNA. Rats were injected with 1 µg/g body weight with all-trans retinoic acid (RA) or vehicle. Bars represent ferritin H mRNA abundance measured by Northern analysis and expressed as a function of 28S rRNA abundance, mean±SD (n=3). Photo inset shows a representative Northern from each treatment group. *Significantly different from vehicle-treated controls at p<0.01.
**Figure 1-2.** Effect of retinoic acid-induced neuronal differentiation on ferritin H immunoreactivity. Photomicrographs show ferritin abundance in cultured NT2 cells before (-RA) and after (+RA) 4 weeks of treatment with retinoic acid to induce terminal differentiation into post-mitotic neurons (NT-2N). Photos are representative of n=6 dishes in each treatment group. Scale bar = 25 µm.
Figure 1-3. Effect of retinoic acid-induced neuronal differentiation on ferritin H mRNA. Ferritin mRNA abundance was measured by Northern analysis in cultured NT2 cells before and after 4 weeks of treatment with retinoic acid (RA) to induce terminal differentiation into postmitotic neurons (NT-2N). Bars represent ferritin H mRNA abundance expressed as a function of 28S rRNA abundance, mean±SD (n=3). Photo inset shows a representative Northern from each differentiation state. *Significantly different from undifferentiated NT-2 cells at p<0.01.
Figure 1-4. Effect of retinoic acid on ferritin H mRNA in undifferentiated NT2 cells. Ferritin mRNA abundance was measured by Northern analysis in cultured NT2 cells treated with 10 µM retinoic acid for 0, 24 and 72 hours. Bars represent ferritin H mRNA abundance expressed as a function of 28S rRNA abundance, mean ±SD (n=3). Photo inset shows representative Northern from each time point. *Significantly different from controls (0h) at p <0.05.
Figure 1-5. Effect of retinoic acid on ferritin H immunoreactivity in undifferentiated NT2 cells. Photos show ferritin abundance in cultured Ntera 2 with 10 retinoic acid for 0, 24 and 72 hours and are representative of n=6 dishes as each time point. Scale bar = 25 µm.
Figure 1-6. Effect of retinoic acid on the labile iron pool in NT2 cells. The labile iron pool was measured by calcien fluorescence (photo counts) in cells treated with retinoic acid for 0, 24 and 72 hours (mean±SD, n=6 for each time point). As shown in the inset of iron-treated cells, a decrease in fluorescence represents an increase in the cellular pool of labile iron. *Significantly different from controls at p<0.05.
CHAPTER 2

ZINC INHIBITS THE NUCLEAR TRANSLOCATION OF THE TUMOR SUPPRESSOR PROTEIN p53 AND PROTECTS CULTURED HUMAN NEURONS FROM COPPER-INDUCED NEUROTOXICITY

Introduction

Zinc (Zn) is essential for normal neuronal function. In addition to its role as a cofactor in dozens of catalytic enzymes, Zn regulates neuronal gene expression and neurotransmission (Trung-Tran et al., 2000; Wood et al., 2000). Approximately 10% of brain Zn is sequestered in synaptic vesicles as free or chelatable Zn (Frederickson et al., 1983). Upon stimulation or injury, these “zincergic” neurons release free Zn into the synaptic cleft resulting in extracellular Zn concentrations of 10^-300 \mu M (Choi and Koh, 1998; Li et al., 2001). Furthermore, it has been estimated that Zn concentrations in the vesicles may reach concentrations as high as 1.4 mM (Frederickson et al., 2000).

Given the high concentrations of Zn that can accumulate in neuronal vesicles and be released into the synaptic cleft between pre- and post-synaptic neurons, it is not surprising that the rapid release of free vesicular Zn may lead to postsynaptic neuronal damage or death (Canzoniero et al., 1999; Kim et al., 1998; Sensi et al., 1999). A number of mechanisms are thought to contribute to this damage. Most recently Zn has been shown to inhibit NAD^+ and ATP production leading to cell death (Sheline et al., 2000). Furthermore, Zn has been implicated in the formation of reactive oxygen species that damage cell membranes (Sensi et al., 1999), and the release of mitochondrial cytochrome c that can activate the apoptotic cascade (Fiskum et al., 2000; Manev et al., 1997; Sensi et al., 1998).

In contrast, other reports suggest that high levels of Zn (500-1000 \mu M) prevent cellular apoptosis in a variety of cell types, including neurons (Fraker et al., 1997; Libonati et al., 2000). Zn has been shown to transiently inhibit glucocorticoid-induced apoptosis of thymocytes (Fraker et al., 1997) and hypoxia-induced apoptosis of cultured human neurons (Libonati et al., 2000). This appears to be a unique function of Zn because other trace metals,
such as copper and cadmium, do not inhibit apoptosis (Fraker et al., 1997). The mechanisms
by which Zn prevents apoptosis in neurons and other cells is not known, but may include the
antioxidant role of Zn (Powell, 2000). In neurons, Zn may also inhibit the excitotoxic
effects of glutamate that occur at postsynaptic NMDA channels (Kubota et al., 2000).

The trace metal copper (Cu) is also involved in cellular damage and death (Strand et
al., 1998; Narayanan et al., 2001). Cu has been shown to interact with high affinity Cu
-binding sites on nuclear DNA (Kim et al., 1997; Sagripanti et al., 1991) causing double
-stranded DNA scission (Tkeshelashvili et al., 1991). In hepatocytes, this DNA damage leads the
to induction of the tumor suppressor protein p53 (Narayanan et al., 2001). This
transcription factor regulates a variety of down-stream genes involved in cell cycle
termination (Meplan et al., 1999; Schmidt-Katerner et al., 2000), and the initiation of cell
death via the apoptotic cascade (Liang et al., 1999). Thus, Cu induces hepatocyte apoptosis
(Narayanan et al., 2001; Strand et al., 1998).

The possible role of Cu in neuronal death has not been fully tested. However, the
autosomal recessive disorder, Wilson’s Disease, characterized by copper accumulation,
shows that neurons can clearly be damaged by Cu in vivo (Berg et al., 2000; Saito et al.,
1999; Sarkar et al., 2000). Furthermore, Cu has been postulated to be involved in the
neuronal degeneration associated with both Parkinson’s Disease (Kim et al., 1997) and
Alzheimer’s Disease (Waggoner et al., 1999). It is not known, however, if Cu induces
neuronal apoptosis or if the metal simply triggers necrotic death in this cell type.
Furthermore, the role of p53 in the Cu-mediated neuronal death has not been evaluated.

Thus, we have developed a number of hypotheses. First, while we know that Cu will
result in neuronal damage and death, we hypothesized that Cu will induce apoptosis in
cultured human post-mitotic neurons. Second, we hypothesized that the addition of high
concentrations of Zn will prevent Cu-induced neuronal apoptosis. Finally, the mechanisms
responsible for the inhibition of apoptosis by Zn have not been fully explored. Our model of
Cu and Zn treatment provided an ideal opportunity to test, in neurons, the hypothesis that Zn
regulation of the tumor suppressor protein p53 is part of the anti-apoptotic mechanism that
has been attributed to Zn.
Materials and Methods

Neuronal Culture

The human neuroblastoma cell line, NTERA-2 (NT2) (Stratagene, La Jolla, CA) was cultured in Dulbecco’s Medium Essential Medium supplemented with F -12 nutrient mixture (DMEM/F-12; Life Technologies, Gaithersburg, MD), 10% cosmic calf serum, (Hyclone Laboratories, Logan, UT), and 0.1% antibiotic -antimycotic solution (Sigma Chemicals, St Louis, MO). Cultures were induced to differentiate into post-mitotic neurons (NT2-N) by the addition of 10 µM retinoic acid (RA) (Sigma Chemicals, St. Louis, MO). Cultures were protected from light to avoid photodestruction of RA and maintained in a 37° C humidified chamber containing 5% CO₂. After 4 weeks of RA treatment, neurons were mechanically dislodged and transferred to either 6-well or 24-well plates at which time mitotic inhibitors 5-fluoro-2’-deoxyuridine (10 µM), cytosine β-D-arabinofuranoside (1 µM) and uridine (10 µM), were added.

Treatments

In an attempt to mimic CNS Cu concentrations that can occur in Wilson’s disease (Cuthbert et al., 1995; Scheinberg et al., 1979), post-mitotic NT2-N neurons (n=3) were treated with 0, 50, 100, and 200 µM Cu as cupric sulfate for 18 h. Cu concentrations were measured in washed cell lysates by graphite furnace atomic absorption spectroscopy (Zeeman 5100; Perkin Elmer, Norwalk CT) and normalized to cellular protein concentrations. To test the interactions of Cu and Zn, neurons treated with 100 µM Cu were also treated with 100, 500 and 700 µM Zn (as zinc sulfate). Cu and Zn concentrations were measured for each condition.

Cell Viability

Cell viability was evaluated using the vital dye neutral red (Sigma Chemicals, St. Louis, MO) which is incorporated by live cells. Cells were treated with Cu (100 µM), Zn at 100 µM and 700 µM, or a combination of Cu and Zn. Untreated cells served as controls. At 0, 6, 12, 18, and 24 hours cells (n=3 dishes for each time point and treatment in 2 separate experiments for a total of n=6) were incubated for 5 min in neutral red dissolved in sterile tris buffered saline (TBS, 3.3 mg/mL). Viable cells were counted using a hemocytometer under light microscopy. Differences in viability were determined to be statistically significant at p<0.05 using GraphPad Prizm statistical software (San Diego, CA).
Nuclear Morphology and Annexin V staining

Nuclear morphology and Annexin V was evaluated to test the hypothesis that Cu induces neuronal death via apoptosis. Annexin V staining was performed according to a the CLONTECH ApoAlert Annexin V protocol (Palo Alto, CA). Cells were plated on glass coverslips in 24-well plates and allowed to adhere overnight. Following 6 h of Cu treatment, cells were briefly washed with 150 µl of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5mM CaCl$_2$) in PBS (1:1 dilution). Cells were then incubated in the dark for 15 minutes with 2.5 µl of annexin V-FITC conjugate (40 µg/ml in PBS) and 5 µl of propidium iodide (50 µg/ml) in 100 µl of binding buffer per well (n=6 in 2 separate experiments). Untreated cells were used as controls. Following incubation, cells were washed 3 X with phosphate buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde. Additional cells were incubated with 4’, 6 diamino -2-phenylindole (DAPI; Sigma) for 10 min to allow visualization of nuclear morphology (n=6 in 2 separate experiments). Coverslips were mounted with commercially prepared mounting medium (FluorSave Reagent, Calbiochem-Novabiochem, La Jolla, CA) and examined using a Nikon Microphot –FX microscope equipped with epifluorescence.

Northern Analysis

NT2-N cells in 6-well plates (n=3 wells for each treatment) were incubated in media supplemented with Cu or Cu and Zn as previously described. After 4 h, total cellular RNA was collected from control (untreated) and treated neurons using TriZol Reagent (Life Technologies/Gibco BRL, Gaithersburg, MD). RNA collected from each treatment was pooled and fractionated by size on a 1% agarose gel that contained formaldehyde (0.66 M). Equal loading and integrity of the RNA was confirmed by visualization of ethidium bromide staining under UV light. The RNA was then transferred to a nylon membrane (GeneScreen; NEN, Boston, MA) by capillary blotting overnight. The RNA was UV crosslinked to the membrane. The membrane was hybridized for 18 h at 65 °C with random primed $^{32}$P-labeled cDNA probes for the tumor suppressor protein p53 and 28S rRNA (loading control). Blots were then exposed to Kodak X-OMAT AR film at -80°C. Relative amounts of bound cDNA probe were determined using computer evaluated densitometry (Quantity One Quantification Program) which was created by Protein and DNA Imaging (PDI, Boston, MA) and expressed as a function of 28S rRNA abundance.
**Western Analysis**

NT2-N neurons were grown in 75 mm² flasks (n=2 for each condition) and treated with Cu (100 µM, 12 h) or Cu and 700 µM Zn (12 h). Untreated cells served as controls. Cells were dislodged and collected in serum-free media at 4°C. After centrifugation at 1500 x g for 5 min at 4°C, cells were resuspended in ice-cold buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT) with freshly added protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO). Following incubation at 4°C (15 min), NP-40 solution was added at a final concentration of 0.6%. Samples were then vortexed and centrifuged (16,000 x g, 1 min, 4°C). Pellets containing nuclei were resuspended in ice-cold buffer containing 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1mM EDTA, 1 mM DTT with freshly added protease inhibitor cocktail. Samples were continuously agitated at 4°C for 15 min and centrifuged at 16,000 x g for 15 min at 4°C. The supernatant containing nuclear proteins was collected and concentrated. Total protein concentration was determined using the BCA method (Pierce, Rockford, IL).

Nuclear protein (20 µg) was added to an equal volume of sample buffer (200 mM Tris, pH 6.8, 1% SDS, 30% glycerol, 7.5% mercaptoethanol, 0.1% bromophenol blue), heated to 95°C for 5 min, and subjected to SDS-PAGE using a 10% polyacrylamide gel. Samples were then transferred to a nitrocellulose membrane on ice and the membrane was blocked (TBS with 5% nonfat dry milk, 0.1% Tween 20) for 15 min at room temperature. A mouse anti-human antibody for wild-type p53 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:250 in blocking solution and incubated overnight with continuous agitation at 4°C. Chemiluminescence was used to detect p53 following the manufacturer’s protocol (ECL Western Blotting Analysis System, Amersham Pharmacia Biotech, Piscataway, NJ). Nuclear p53 was detected using Kodak Biomax MS film for high sensitivity (Kodak, Rochester, NY).

**Immunocytochemistry**

To determine the cellular localization of p53 protein, NT2-N neurons were plated on sterile 22 mm² glass coverslips (n=6 in 2 separate experiments) (No. 1, Corning Glassworks, Corning, NY). Neurons were treated with 100 µM Cu for 18 h. To test the interaction between Cu and Zn, cells were also treated simultaneously with 100 µM Cu and Zn at

25
concentrations of 100, 500 or 700 µM. Untreated cells served as controls. Cells were washed 3 times with PBS and then fixed by incubation with 3.7% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After 3 PBS washes, coverslips were incubated for 2 h at 37 °C with a commercially prepared goat polyclonal IgG antibody specific for the human tumor suppressor protein p53 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution in 10 mg/ml bovine serum albumin (BSA). Cells were then incubated (2 h, 37 °C) with a donkey anti-goat IgG secondary antibody conjugated to the fluorescent dye cyanine 3 (Cy3) (Jackson ImmunoResearch Laboratories, Westgrove, PA). Coverslips were mounted with a commercially prepared mounting medium (previously described) and examined using a Nikon Microphot-FX microscope equipped with epifluorescence. Photographs of all cells were taken and exposure times held constant to allow treatment group comparisons.

**DN-p53 transfection**

The role of p53 in the induction of Cu-mediated neuronal apoptosis was tested by transfection of a dominant-negative p53 construct (DN-p53) that has previously been shown to prevent the synthesis of p53 in NT2-N neurons (Curtin et al., 2001). DN-p53 and control plasmids were kindly provided by Dr. Michael Spinella, Dartmouth Medical School. Neurons were plated in 24-well plates on glass coverslips and transiently transfected with 1 µg of DN-p53 or control plasmid per well using the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 6 h cells were treated with 100 µM Cu for 18 h. Immunocytochemistry was performed as previously described to determine the abundance and localization of the tumor suppressor protein p53 and its effects on nuclear morphology.

**Results**

**Copper and Zinc Concentrations**

Increases in media Cu concentration resulted in significantly elevated intracellular levels of Cu following 6 h of treatment (Figure 2-1) such that treatment with 100 µM Cu resulted in a 23-fold increase in cellular Cu. The addition of Zn to Cu-treated neurons significantly increased Cu uptake (Figure 2-2A) to levels that were 5-fold higher than treatments with Cu alone. Following Zn treatment there was an increase in the intracellular concentration of Zn. Figure 2-2B shows that the addition of 100 µM Zn significantly
increased intracellular Zn (p<0.001). The addition of Cu did not alter Zn uptake in control or 100 µM Zn treated cells. However, addition of Cu to cells treated with 700 µM Zn appeared to facilitate Zn uptake as Zn concentrations in these cells were significantly higher than cells treated with 700 µM Zn alone (p<0.001) or with Cu and 100 µM Zn (p<0.001) (Figure 2-2B).

**Neuronal Viability**

Figure 2-3 shows the effect of Cu and Zn on NT2-N viability over a 24 h time course. Treatment with 100 µM Cu did not significantly alter viability through 6 h, but reduced viability to approximately 50% of control by 12 h (p<0.001). The addition of 100 µM Zn to Cu-treated cells resulted in a more rapid decline in cell viability. At the 6 h time point, Zn treatment (100 µM) of Cu-loaded cells resulted in a significant loss of viability compared to both control (p<0.001) and Cu-treated cells (p<0.001). In contrast, the addition of higher levels of Zn (700 µM) in conjunction with Cu prevented cell death such that viability was not significantly different from untreated control cells at any time point (Figure 2-3). We also tested the effects of increasing concentrations of Zn alone on cell viability. Treatment of cells with 100, 500 and 700 µM Zn led to significant cell death that was greater than that mediated by 100 µM Cu. The finding that Zn concentrations between 500 and 700 µM protected neurons from Cu-mediated cell death led us to perform the remaining experiments at these concentrations to determine the mechanisms responsible for this effect.

**Annexin V Staining and Nuclear Morphology**

As hypothesized Cu treatment led to evidence of apoptosis. By 6 h cells exhibited plasma membrane staining with annexin V that was not accompanied by nuclear staining (Figure 2-4). Untreated controls showed no signs of apoptosis (Figure 2-5A). However, the sign of early apoptosis seen with Annexin V staining was followed by later stages of apoptotic death at 18 h including chromatin aggregation, nuclear shrinkage (Figure 2-5B) and nuclear blebbing (Figure 2-5C). At this time point there were also cells that exhibited morphological signs of necrosis including nuclear swelling and rupture (Figure 2-5D). The addition of 100 µM Zn to Cu-treated cells was not effective in protecting cells from death (Figure 2-3 and Figure 2-5E). However, when 700 µM Zn was added to Cu-treated cells,
there was not only increased cell viability, but this was accompanied by normal nuclear morphology (Figure 2-5F).

**Regulation of p53**

Measurement of p53 mRNA abundance showed that the loss of neuronal viability after treatment with Cu or Cu and 100 \( \mu M \) Zn was accompanied by an increase in p53 mRNA (Figure 2-6). However, the addition of higher concentrations of Zn to Cu-treated cells not only prevented the increases in p53 mRNA, but reduced message levels. Specifically, 500 \( \mu M \) Zn reduced p53 mRNA to 50% of control, and 700 \( \mu M \) Zn reduced p53 mRNA to 10% of control.

Immunocytochemistry was used to further explore the location and abundance of p53 after treatment with Cu and Zn (Figure 2-7). While untreated control cells expressed minimal levels of intracellular p53 (Figure 2-7A), treatment with 100 \( \mu M \) Cu produced a significant increase in p53 (Figure 2-7B). Examination of these cells showed that the p53 in Cu-treated cells was not only localized to the nucleus, but also appeared in the nuclear blebs that are characteristic of apoptotic body formation (Figure 2-7B). There was a further increase in nuclear and cytoplasmic p53 in Cu-treated cells after the addition 100 \( \mu M \) Zn (Figure 2-7C). However, the addition of 500 \( \mu M \) or 700 \( \mu M \) Zn to Cu-treated neurons resulted in a distinctively different p53 distribution. These high concentrations of zinc prevented translocation of the p53 protein into the nucleus (Figure 2-7D) which was confirmed by Western analysis of nuclear proteins (Figure 2-8).

**p53 function**

Examination of nuclear morphology and p53 immunocytochemistry showed that Cu treatment of neurons transfected with the p53 control plasmid resulted in an elevation of nuclear p53 (Figure 2-9B) and nuclear morphology consistent with apoptosis (Figure 2-9A). In contrast, transfection with the DN-p53 construct not only eliminated the Cu-mediated expression and nuclear translocation of p53 (Figure 2-9D), but also prevented the development of apoptosis over an 18 h time period (Figure 2-9C).
Discussion

While Wilson’s Disease (WD) is most often associated with hepatic Cu toxicity, fibrosis, hepatitis and liver cancer, serious neurological consequences also frequently occur. In fact as many as 30% of WD patients present first with neuropsychiatric symptoms resulting from central nervous system (CNS) Cu accumulation. An examination of 9 studies of brain Cu in WD showed that Cu can accumulate in most regions of the CNS of WD patients, but that the amount of this accumulation varies by region (Scheinberg and Sternleib, 1984). For example, it was shown that WD can result in a 10-fold increase in cortical neuronal Cu compared to age-matched controls. Basal ganglia Cu increased over 7-fold in WD, while cerebellar Cu increased almost 20-fold. The highest region of accumulation was the thalamus that had increases of over 27-fold in WD (Scheinberg and Sternleib, 1984). Given the possibility for such high levels of redox-active Cu, it is not surprising that the clinical symptoms of WD include neurodegeneration. In an attempt to understand the mechanisms responsible for this neuronal damage and death, we developed an in vitro model of Cu toxicity that would permit the accumulation of Cu in human cultured neurons. Addition of 50 or 100 µM Cu to NT2-N neurons resulted in Cu accumulation consistent with that seen in WD (12-23 fold over untreated control). These neurons have previously been used for studies of apoptosis where we have shown that morphological analysis, combined with an examination of the tumor suppressor protein p53, allows the detection of apoptotic and necrotic death (Libonati et al., 2000). As expected, increases in neuronal Cu resulted in neuronal death. While we had hypothesized, based on our previous work with hepatocytes, that the mechanism of death would be apoptotic, in fact we found that these neurons were undergoing cell death that had characteristics of both apoptosis and necrosis. Annexin V staining showed that early cell death is apoptotic, while death at later time points may be mediated by both apoptosis and necrosis. Many neurons clearly had nuclei that fragmented into discrete blebs associated with apoptotic bodies. This is suggestive of apoptosis because necrotic nuclei do not bleb (Kerr et al., 1995). However, there were also neurons that had distinct characteristics of necrosis with irregularly shaped, swollen nuclei and evidence of nuclear rupture. The observation that both apoptosis and necrosis may occur in the same population of neurons is consistent with
previous observations suggesting that in neurons there may be a hybrid form of cell death that cannot be strictly characterized as apoptosis or necrosis (Martin et al., 1998).

The strongest evidence for apoptosis was the induction of the tumor suppressor protein p53. Both Zn toxicity (Libonati et al., 2000) and deficiency (Reaves et al., 2000) have been shown to increase expression of this protein. The induction of p53 is one of the hallmarks of apoptosis (Oda et al., 2000) and may even be required for some types of neuronal death (Xiang et al., 1996). The increases in this protein following Cu treatment appear to be largely post-transcriptional, as significant increases in p53 were accompanied by only small increases in p53 mRNA. What is most significant is that upon Cu treatment, p53 is translocated to the nucleus. This is consistent with its role as a DNA-binding transcription factor active in the transcriptional regulation of a variety of down-stream pro-apoptotic genes (Oda et al., 2000). Furthermore, elimination of Cu-induced p53 expression by the DN-p53 construct prevented neuronal death and other apoptotic indices. This suggests that the induction of p53 is responsible for Cu-mediated neuronal death.

The role of zinc in the processes governing neuronal death, particularly in neurons that have been damaged by Cu, has not been fully explored. It is clear that in combination with Cu, concentrations of Zn that would normally be found in the synaptic cleft of “zinc-ergic” neurons increase p53 and induce neuronal death. However, higher concentrations of Zn inhibited neuronal death. Previous work has shown that Cu and Zn can act antagonistically (Powell, 2000). Thus, it would be reasonable to hypothesize that the addition of high concentrations of Zn would prevent neuronal Cu uptake. Indeed, this would explain why high concentrations of Zn prevented Cu-induced apoptosis. However, in the present study Zn consistently facilitated the uptake of Cu in NT2-N neurons. The transporters responsible for this uptake are not known but could include the recently identified Cu transporter Ctr-1 that has been localized to neurons (Lee et al., 2001). Thus, the question still remains why high concentrations of Zn prevented neuronal death while lower concentrations did not. The answer is suggested by examination of Zn levels in these cells. Zn uptake was facilitated by the addition of Cu only when Zn was added at 700 µM. Thus cellular Zn concentrations were significantly higher in Cu-loaded cells treated with 700 µM.

These data suggest that the protective effects of Zn are not due to antagonism with Cu but are rather the result of elevations in cellular Zn. There may be a number of mechanisms
responsible for this action. First, Zn has been shown to have antioxidant properties (Powell, 2000). Thus, it is possible that Zn is acting to counteract the prooxidant nature of Cu. Recent findings have also shown that Zn displaces redox-active Cu and Fe on the plasma membrane preventing lipid oxidation and cell death (Zago and Oteiza, 2001). However, it has previously been shown that not all of the effects of Cu are the result of oxidation as both Cu$^{+1}$ and Cu$^{+2}$ cause DNA damage equally, and free radical scavengers are unable to completely eliminate Cu-induced damage (Tkeshelashvili et al., 1991). This suggests that other mechanisms are involved. Zn has been shown to stimulate the anti-apoptotic proteins Hsp70 (Hatayama et al., 1993; Libonati et al., 2000) and Bcl-2 (Ishido et al., 1999) and inhibit the apoptotic protease caspase-3 (Perry et al., 1997).

It should be noted here that the anti-apoptotic effects of Zn do not appear to last indefinitely (Fraker et al., 1997). For example, while 700 µM Zn clearly protected thymocytes from glucocorticoid-induced apoptosis at 6 h, by 16 h the protective effects were diminished significantly (Fraker et al., 1997). In the current work high Zn protected NT2-N neurons from Cu-mediated death for 24 h. Given that neurons can sequester high concentrations of Zn and can be exposed to high concentrations of Zn in vivo, these data may represent cell specific differences.

This work suggests that p53-mediated apoptosis may play a role in the neuronal death associated with Cu toxicity. The regulation of p53 may also contribute to the transient protective effect of Zn in neurons. Furthermore, this work shows that there are two mechanisms responsible for the Zn regulation of p53 action. First, the addition of high concentrations of Zn to Cu-treated cells results in a reduction in p53 mRNA abundance. It is not known if this reduction is the result of decreased transcription or decreased p53 mRNA stability, but either way would likely result in the eventual reduction of p53 protein levels. More strikingly however, is the fact that the addition of high Zn resulted in the inability to translocate p53 into the nucleus of Cu-treated cells. Thus, p53 is not able to function as a transcription factor and regulate the transcription of downstream genes to induce apoptosis. The mechanisms responsible for the inhibition of translocation are not known. However, Zn is known to increase the activity of the Bcl-2 (Ishido et al., 1999). Transfection with the bcl-2 gene reduced nuclear translocation of p53 and prevented apoptosis (Beham et al., 1997). Thus, it is not unreasonable to hypothesize that the movement of p53 in Zn treated cells is
regulated by changes in Bcl-2 activity. This represents a potentially new avenue for research into the role of these trace metals in neuronal death and survival.
Figure 2-1. Effect of increases in media Cu concentrations on the intracellular levels of Cu. NT2-N neurons were treated with Cu (0, 50, 100, and 200 µM) for 18 h (n=3). Cu concentrations were measured in washed cell lysates by graphite furnace atomic absorption spectroscopy and normalized to total cellular protein (mean ±S.D.). *Different from control at p<0.001.
Figure 2-2. The effects of increases in media Cu and Zn concentrations on the intracellular levels of Cu and Zn.

A. NT2-N neurons were treated with Cu (100 µM) or Zn (100 and 700 µM) alone or in combination for 6 h. Cu concentrations were measured in washed cell lysates for each condition by graphite furnace atomic absorption spectroscopy and normalized to total cellular protein (mean±S.D.). Bars with different letters are significantly different from each other at p<0.001.

B. NT2-N neurons were treated as previously described. Zn concentrations were measured for each condition with graphite furnace atomic absorption spectroscopy and normalized to total cellular protein (mean ±S.D.). Bars with different letters are significantly different from each other at p<0.001.
Figure 2-3. The effect of Cu and Zn on neuronal viability. NT2 -N neurons were treated with Cu (100 µM) or with Cu and Zn (100 or 700 µM) over a 24 h time course. Viability was determined by the incorporation of the vital dye neutral red and expressed as percent of control, n=3 dishes for each time point and treatment in 2 separate experiments for a total of n=6. *Different from control at p<0.001.
**Figure 2-4.** Evidence for apoptosis by Annexin V staining. Following 6 h of Cu treatment cells were incubated with Annexin-V-FITC conjugate and counterstained with propidium iodide. This early apoptotic cell shows an Annexin V stained plasma membrane with no nuclear staining by propidium iodide. Photomicrograph is representative of 3 dishes in 2 experiments (n=6). Scale bar = 25 µm.
Figure 2-5. Effect of Cu and Zn on NT2-N nuclear morphology. Fluorescent microscopy of (A) normal nuclear morphology in untreated control cells, (B) nuclear condensation after treatment with 100 µM Cu for 18 h, (C) nuclear blebbing after treatment with 100 µM Cu, (D) nuclear swelling and rupture after treatment with 100 µM Cu, (E) nuclear condensation and blebbing after treatment with 100 µM Cu and 100 µM Zn, and (F) normal nuclear morphology in cells treated with 100 µM Cu and 700 µM Zn. Photomicrographs are representations of 3 dishes for each treatment in 2 separate experiments (n=6). Scale bar = 25 µm.
Figure 2-6. Effect of Cu and Zn on NT2-N p53 mRNA abundance. Total cellular RNA from 3 separate dishes was pooled for each treatment for Northern analysis. Bars represent relative p53 mRNA expressed as a function of 28S rRNA.
Figure 2-7. Immunolocalization of p53 after Cu and Zn treatment for 18 h. Fluorescent microscopy of (A) untreated control cells, (B) nuclear localization of p53 in Cu treated cells (100 µM), (C) nuclear and cytosolic localization of p53 after Cu and 100 µM Zn, and (D) cytosolic p53 after treatment with Cu and 700 µM Zn. Photomicrographs are representative of 3 dishes for each treatment in 2 separate experiments (n=6). Scale bar = 25 µm.
Figure 2-8. Effect of Cu and Zn on nuclear p53 protein abundance. Photo shows representative Western analysis after isolation of nuclear proteins cells treated with Cu (100 µM) or Cu and Zn (700 µM). Untreated cells served as controls.
Figure 29. Effects of Cu following transient transfection with a DN-p53 or control construct. After transfection with the control plasmid, Cu (100 µM, 18 h) induced apoptosis (A) determined by nuclear morphology and (B) nuclear p53 localization. Transfection with the DN-p53 construct resulted in (C) normal nuclear morphology and (D) the elimination of p53 expression following Cu exposure. Photomicrographs are representative of 6 dishes for each construct. Scale bar = 25 µm.
CHAPTER 3

COPPER REGULATION OF P53-RESPONSIVE GENES
IN CULTURED HUMAN NT2-N NEURONS

Introduction

The trace element copper (Cu) is required for the function of a variety of enzymes including the free radical scavenger superoxide dismutase (Ceballos et al., 1991), cytochrome c oxidase, that functions in the terminal oxidative step of the mitochondrial electron transport chain (Lappalainen et al., 1995), and dopamine β-monooxygenase, the enzyme responsible for synthesis of the catecholamine norepinephrine (Prohaska and Brokate, 2001). While it is clear that Cu is required for normal central nervous system (CNS) function, there is also evidence that toxic accumulation of Cu in the CNS is associated with neuronal damage and death (Johnson, 2001). Elevated levels of Cu in the CNS are most frequently seen with the autosomal recessive disorder, Wilson’s disease (WD) (Wender et al., 1993). This disease results from a mutation in the gene that codes for the P-type ATPase, ATP7B (Seidel, 2001). Malfunction of this Cu transporter results in the inability to excrete Cu from the liver and eventual accumulation of toxic levels of hepatic Cu (Harada, 2000). A recent report suggests that the WD mutation leads to abnormal intracellular localization of ATP7B. While wild-type ATP7B has been localized to the trans-golgi network, mutant forms have been localized to vesicular compartments of the cytoplasm (Huster et al., 2003).

In many cases of WD Cu accumulation appears to be the result of Cu-induced hepatocyte damage and lysis with subsequent release of toxic levels of Cu that are mobilized to the CNS (Waggoner et al., 1999). However, there is now evidence that the buildup of Cu in the CNS of WD patients may involve brain specific mechanisms, as recent reports have shown that the WD gene is expressed in the CNS (Cox, 1995; Saito et al., 1999). This may explain why many WD patients present first with neurological symptoms including cognitive deficits, (Bornstein et al., 1995; Medalia, 1991) bradykinesia, muscle rigidity, and tremor (Oder et al., 1993). Treatments include the Cu chelator, penicillamine and zinc (Zn), both of
which lead to improvements in motor (Brewer et al., 2001) and cognitive function (Akil and Brewer, 1995).

Increased neuronal Cu has also been implicated in other neurodegenerative disorders. High concentrations of Cu in vitro are associated with the Abeta protein and neurotoxicity suggesting a role for Cu-mediated neuronal death in patients with Alzheimer’s disease (Cuajungco et al., 2000). Studies have also shown that increased neuronal Cu selectively causes death in dopaminergic neurons cultured from rat substantia nigra implying that Cu overload may be involved in the etiology of some forms of Parkinson’s disease (Paris et al., 2001).

Given the association between Cu overload and these neurodegenerative disorders, the importance of the molecular mechanisms responsible for these observations is clear. Cu has been shown to increase hydroxal free radicals in both murine neocortical cell cultures (Sheline et al., 2002) and rat pheochromocytoma (PC12) cells (Kim et al., 2001). In the presence of hydrogen peroxide Cu generates hydroxal free radicals that cause DNA strand breaks and mutagenesis (Liang and Dedon, 2001). Furthermore, we now know that there are specific Cu binding sites on DNA that interact with nuclear Cu leading to genotoxic stress (Kim et al., 1997; Ueda et al., 1998).

In response to DNA damage the tumor suppressor protein p53 functions as a sequence-specific DNA transcription factor that regulates the expression of many downstream genes involved in cell cycle arrest and the initiation of apoptotic cell death (O’Rourke et al., 1990; Yonish-Rouach et al., 1993). While we have previously used a dominant-negative p53 construct to show that Cu-mediated neuronal death occurs through an apoptotic pathway that requires both the expression and nuclear translocation of p53 (VanLandingham et al., 2002), the transcriptional mechanisms downstream to p53 have not been investigated. Thus, this work was designed to identify the p53-dependent mechanisms of Cu-mediated neuronal cell death. Following expression profiling of p53-regulated genes by oligonucleotide array, the abundance and subcellular localization of several key gene products involved in both neuronal apoptosis and survival were examined.
Materials and Methods

Neuronal Culture

The human neuroblastoma cell line, NTERA-2 (NT2) (Stratagene, La Jolla, CA) was cultured in Dulbecco’s Modified Essential Medium supplemented with F-12 nutrient mixture (DMEM/F12; Life Technologies, Gaithersburg, MD), 10% cosmic calf serum, (Hyclone Laboratories, Logan, UT) and 0.1% antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cell cultures were induced to differentiate into post-mitotic neurons (NT2-N) with 10 μM retinoic acid (RA) 3 times a week for 4 weeks. Cultures were protected from light exposure to avoid photodestruction of RA while incubated in a 37 °C humidified chamber containing 5% CO2. Following a course of RA treatment, neurons were mechanically dislodged, transferred from T-75 flasks to 6-well plates, and further treated for 2 weeks with RA in combination with the mitotic inhibitors 5-fluoro-2’-deoxyuridine (10 μM), cytosine β-D-arabinofurano-side (1 μM) and uridine (10 μM). Cells were then treated with 100 μM Cu (as cupric sulfate) for 12 and 18 h.

p53 Target Gene Expression Profiling

Total cellular RNA was isolated from control and Cu treated neurons by the Trizol extraction method (Invitrogen, Life Technologies, Carlsbad, CA) for expression profiling of p53-responsive genes using the TransSignal p53 Target Gene Array kit (Panomics, Inc., Redwood City, CA). The integrity of the RNA was confirmed following spectrophotometry and etidium bromide visualization after electrophoresis on a denaturing formaldehyde-agarose gel using 10 μg of total RNA. The RNA was then reverse transcribed with AMV reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN) and labeled with biotin-dUTP using sequence specific primers supplied with the arrays. Duplicate arrays, each containing 145 spots of sense strand oligonucleotides specific for known human p53-regulated genes and controls (3), were hybridized to the labeled cDNA probes at 42 °C. Chemiluminescent detection with array exposure to Hyperfilm (Amersham Pharmacia Biotech, UK) followed by photodensitometry (BioRad, Hercules, CA) was used to identify differentially regulated genes using a 1.5 fold change in the expression ratio to determine Cu regulation. Spot intensities of regulated p53 target genes were normalized to the expression of glyceraldehyde 3’ phosphate dehydrogenase (GAPDH) mRNA abundance.
Immunocytochemistry

Immunocytochemistry was used to determine the abundance and subcellular localization of the p53 protein. NT2 -N neurons were cultured in 6-well plates on sterile 22 mm² coverslips (n=6 for each condition, 2 separate experiments) (No.1, Corning Glassworks, Corning, NY). Neurons were treated for 12 or 18 h with 100 µM Cu. Untreated neurons served as controls. Cells were washed with phosphate buffered saline (PBS) 3 times and then fixed with 3.7% paraformaldehyde (PFA) for 10 min. Cells membranes were then permeabilized using 0.2% Triton X-100 in PBS for 5 min. Following 3 washes with PBS, coverslips were incubated for 2 h at 37°C with a rabbit anti-human polyclonal antibody specific for the tumor suppressor protein p53 (Santa Cruz Biotechnology, Santa Cruz, CA), insulin-like growth factor binding protein -6 (IGFBP-6, Santa Cruz Biotechnology), heat shock protein 70 (Hsp 70, Santa Cruz Biotechnology), or c-jun (Cell Signaling Technology, Beverly, MA). Cells were incubated (90 min, 37°C) with a secondary antibody conjugated to the fluorescent dye cyanine -3 (Cy3) (Jackson ImmunoResearch Laboratories, Westgrove, PA). Coverslips were then washed and mounted with a commercially prepared medium (FluorSave Reagent, Calbiochem-Novabiochem, La Jolla, CA) and examined by fluorescent microscopy (Nikon Microphot-FX).

Western Analysis

Cu treated and control neurons were collected with NP-40 protein extraction buffer while on ice and were centrifuged at 10,000 rpm for 20 min. Supernatant was collected for protein analysis by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Protein (40 µg) was loaded for each condition and separated on a 12% polyacrylamide gel by SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk and incubated overnight at 4°C with a commercially prepared mouse monoclonal IgG primary antibody specific for Hsp 70 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-mouse secondary antibody (1:4000; Amersham-Pharmacia, Piscataway, NJ) for 90 min at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY) was used to visualize protein abundance. Films were analyzed by quantitative photodensitometry using the Bio-Rad Gel Doc system (Bio-Rad Laboratories,
Hercules, CA) in conjunction with QuantOne software (Bio-Rad Laboratories, Hercules, CA). The membrane was then stripped and hybridized as previously described with a mouse IgG anti-neuro-specific enolase primary Ab (Chemicon International Laboratories, Temecula, CA). The abundance of hsp 70 was normalized to that of neuro-specific enolase.

**Results**

*p53 regulation*

Untreated control neurons expressed minimal amounts of intracellular p53. Following 12 h of Cu treatment there was a significant increase in p53 that was distributed throughout the neuron. Continued exposure to Cu (18 h) resulted not only in an increase in p53, but the translocation of this transcription factor almost exclusively to the nuclei of NT2-N cells (Figure 3-1).

**Expression profiling**

Oligonucleotide arrays hybridized with mRNA from NT2-N cells revealed the expression of 42 p53-responsive genes. Of these genes, 18 mRNAs were differentially regulated upon the addition of Cu to the culture media. The remaining 24 mRNAs, and the control messages GAPDH, β-actin and ubiquitin were not differentially expressed. Using a 1.5 fold expression ratio as the cutoff for transcriptional regulation, our examination of p53-target genes showed Cu treatment resulted in the up-regulation (Table 1) as well as down-regulation (Table 2) of transcripts that function in p53-mediated apoptosis. Representative signals from the oligonucleotide array are shown in duplicate (Figure 3-2). The c-jun transcript was increased 17.5-fold in Cu-treated neurons compared to control. IGFBP-6 showed a 3.9-fold increase in mRNA abundance after Cu treatment. Hsp 70, increased 3.7-fold following Cu treatment compared to control. Similarly, Hsp 27 mRNA was increased 2.5-fold in the Cu-treated neurons compared to control.

**IGFBP-6**

IGFBP-6 protein expression levels were minimal in control neurons. Following 12 h of treatment with 100 µM Cu there was a significant increase in both nuclear and cytoplasmic IGFBP-6 protein levels. Analysis of IGFBP-6 after 18 h of Cu-overload showed further increases in protein expression which coincided with increasing levels of neuronal apoptosis. Furthermore, use of the nuclear stain DAPI showed that increased levels of
IGFBP-6 were correlated with nuclear shrinkage, a morphological sign of neuronal apoptosis (Figure 3-3).

**Hsp 70**

Hsp 70 protein abundance increased with increasing concentrations of Cu (Figure 3-4). Prior to treatment Hsp 70 protein levels were nearly undetectable. However, following the addition of Cu (100 µM) there was an increasing amount of Hsp 70 expression throughout the neuron at both 12 and 18 h (Figure 3-5).

Western blot analysis of the Hsp 70 protein showed an increase in Hsp 70 expression following 12 h of Cu treatment compared to untreated control neurons (Figure 3-6). Graph insert shows quantitative differences in abundance between treatment groups normalized to neuro-specific enolase abundance.

**c-jun**

As expected, immunocytochemistry confirmed nuclear localization of the transcription factor c-jun in control neurons. Consistent with increases in c-jun mRNA (Table 1), Cu-treatment resulted in an overall increase in c-jun protein. However, in treated cells, the protein was localized both to the nucleus, and to extra-nuclear structures (Figure 3-7).

**Discussion**

DNA damage and neuronal degeneration have been linked to cellular Cu accumulation (Levay et al., 1997). In an attempt to understand the molecular mechanisms responsible for Cu-induced cell death, we developed an in vitro model of Cu toxicity using human cultured NT2-N neurons that would represent neuronal Cu overload similar to that seen in the degenerating neurons of Wilson’s disease patients (VanLandingham et al., 2002). We showed that Cu-mediated neuronal death employs apoptotic mechanisms that are dependent on the expression and nuclear translocation of p53 (VanLandingham et al., 2002). Other models of neuronal injury, such as cerebral ischemia (Li et al., 1997), epilepsy (Tan et al., 2002) and traumatic brain injury (Raghupathi et al., 2000; Napieralski et al., 1999) have also shown that p53 modulates neuronal viability.

While the molecular mechanisms of p53 action are not fully understood, it appears that DNA damage induces the tetramerization of p53 and binding to two copies of the consensus sequence 5’ -PuPuPuC(A/T)(T/A)GPyPyPy-3’ separated by 0-13 base pairs (El -
Deiry et al., 1992). p53 binding to these sites on the 5’-flanking regions of p53-responsive genes, results in regulation of gene transcription. Clearly, these half-sites provide a great deal of heterogeneity to the sequence that can bind p53 oligomers. Furthermore, it appears that p53 binding can occur even when several bases of the sequence do not conform to the recognized p53 response element (Bargonetti et al., 1993; Foord et al., 1993). Thus, it is not surprising that a large number of genes, both pro- and anti-apoptotic are transcriptionally regulated by p53 (Qian et al., 2002).

**Regulation of Pro-Apoptotic Genes**

Use of an oligonucleotide array system designed to evaluate the differential regulation of p53-responsive genes showed a significant increase in the mRNA for both pro-apoptotic and anti-apoptotic genes. An example of a pro-apoptotic gene is the inducible transcription factor c-jun, a member of the AP-1 complex that has been implicated in the early molecular mechanisms leading to neuronal apoptosis. Jun acts to initiate the series of events leading to cytochrome c release from the mitochondria, a well-known trigger of apoptosis (Whitfield et al., 2001).

Both microinjection of dominant-negative c-jun gene constructs (Ham et al., 1995) and the use of immunoneutralization of c-jun (Estus et al., 1994) eliminated neuronal apoptosis, suggesting that in at least some neurons c-jun is required for apoptosis. Additionally, there is evidence in these studies that Cu-mediated apoptosis was accompanied by significant increases in c-jun mRNA. Increases such as this are most frequently associated with the formation of jun/fos heterodimers that bind to AP-1 regulatory sites on gene promoter regions (Hughes et al., 1999). However, there is evidence that jun/jun, but not fos/fos, homodimers can also act to regulate transcription from AP-1 sites (Gass et al., 1995). This pattern of jun/jun homodimerization is a likely one in Cu-mediated apoptosis because we show here that increases in c-jun mRNA and jun protein were accompanied by a decrease in fos expression. Interestingly, we also found that RB-1 is up-regulated in Cu-treated cells. RB-1 has previously been shown to repress c-fos expression (Robbins et al., 1990). Given that jun is a transcription factor, its immunocytolocalization to the nucleus in both control and Cu-treated cells is not surprising. However, in treated cells c-jun was also localized to cellular structures that closely resemble the localization of proteins that are targeted to lysosomes (Goldshmidt et al., 2002). Furthermore, it appears that both fos (Aniento et al.,
ubiquitin-dependent pathways. Ubiquinated fos is translocated to the lysosomes for degradation (Aniento et al., 1996). Given that c-jun has an estimated half-life of approximately 1 min (Treier et al., 1994), this work suggests that excess c-jun, synthesized following Cu-overload, may be ubiquinated and targeted to the lysosome for degradation.

Insulin-like growth factors (IGF-I and IGF-II) have been shown not only to play a role in neuroblastoma proliferation and differentiation (El-Badry et al., 1991), but also appear to protect cells from apoptosis (Butt et al., 1999). There are six known IGF binding proteins (IGFBP 1-6) that modulate the bioavailability and function of IGF. Of particular interest is IGFBP-6, which can successfully compete with the type -1 IGF receptor for IGF-II binding, thus inhibiting the anti-apoptotic function of IGF-II (Grellier et al., 2002). Copper treatment increased mRNA and protein levels of IGFBP-6 by approximately 4-fold. Overexpression of IGFBP-6 in other cell types results in reduced proliferation (Sueoka et al., 2000a) and apoptosis (Sueoka et al., 2000b). The NT2-N neurons studied in the current work are postmitotic. Thus, it appears likely that the p53-regulated increases in IGFBP-6 in these cells contributed to neuronal apoptosis following Cu overload.

Other p53-associated, pro-apoptotic genes that were regulated in Cu-treated neurons included p14/ARF a regulator of p53 stability. Overexpression of p14/ARF increases cellular sensitivity to p53-mediated apoptosis (Tango et al., 2002). p53 has also been shown to activate the transcription of a group of the redox regulator genes known as PIGs (Polyak et al., 1997). These genes are responsible for the generation of oxygen radicals that appear to play role in apoptosis. To our knowledge, this is the first report suggesting that the p53-regulated gene PIG-8 participates in neuronal death.

Regulation of Anti-Apoptotic Genes

Copper-treatment resulted in increased expression of the heat shock proteins Hsp 70 and Hsp 27, both of which have been implicated in the protection of neurons from apoptosis (Yenari, 2002). These chaperone proteins prevent cell death by regulating protein folding during periods of cellular stress, and are part of the machinery that is responsible for protein unfolding and translocation to the mitochondrial matrix (Strub et al., 2003). Furthermore, Hsp 70 has been shown to act as an antioxidant (Chong et al., 1998) and prevent the release of cytochrome c from the mitochondria (Tsuchiya et al., 2003). An analysis of the 5'-flanking
region of the hsp 70 gene recently published by Fiszer-Kierzkowska et al., 2003, revealed the presence of two putative p53 binding sites starting at position –1883 (GAGCAAGAGTN_3 AGGCTAGCCT) and position –1468 (AAACAGGCTCN_11 GGGCTAGCCT). Each of these sites is composed of one half-site that conforms to the consensus sequence, with the other deviating by only one base pair. While future work will be needed to confirm the direct transcriptional regulation of hsp 70 by p53, it is clear that regulation of these chaperone proteins play a role in the response to Cu overload in NT2-N neurons, potentially serving to provide protection from apoptotic signals.

It appears that the balance between anti-apoptotic and pro-apoptotic signals determines the response to p53 in Cu-treated cells. In contrast to the up-regulation of hsp 70 and 27, several other anti-apoptotic mRNAs associated with p53 expression were down-regulated in Cu-treated NT2-N cells. For example, mRNA for Bcl-2, which is known to block the release of cytochrome c from the mitochondria (Hou et al., 2003), was reduced in Cu-treated cells, as was glutathione peroxidase (GPx) mRNA, a neuroprotective enzyme that removes hydrogen peroxide (Ben-Yoseph et al., 1996). Creatine kinase (CK), which was down-regulated in the current work, is required for ATP production. While CK is most abundantly expressed in skeletal muscle, expression of the brain isoform has previously been shown to be down-regulated by p53 (Zhao et al., 1994). p73 expression was also down-regulated in these cells. p73 is a member of the p53 family of transcription factors that has been shown to be required for neuronal survival in vivo (Pozniak et al., 2002). Thus, the down-regulation of these anti-apoptotic genes likely plays a role in Cu-induced neuronal apoptosis.
TABLE 1
Up-regulation of mRNA abundance of p53-regulated genes in copper-treated human NT2-N neurons.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun</td>
<td>17.5</td>
<td>pro-apoptotic early immediate gene</td>
<td>Ham et al., 1995</td>
</tr>
<tr>
<td>PIG-8</td>
<td>6.5</td>
<td>pro-apoptotic redox regulator gene</td>
<td>Polyak et al., 1997</td>
</tr>
<tr>
<td>Reprimo</td>
<td>4.5</td>
<td>cell cycle arrest</td>
<td>Taylor &amp; Stark, 2001</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>3.9</td>
<td>pro-apoptotic, IGF-II binding</td>
<td>Grellier et al., 2002</td>
</tr>
<tr>
<td>Hsp 70</td>
<td>3.7</td>
<td>anti-apoptotic, chaperone protein</td>
<td>Yenari et al., 1999</td>
</tr>
<tr>
<td>RB-1</td>
<td>2.8</td>
<td>tumor suppressor, fos repression</td>
<td>Robbins et al., 1990</td>
</tr>
<tr>
<td>Hsp 27</td>
<td>2.5</td>
<td>Anti-apoptotic, chaperone protein</td>
<td>Yenari et al., 1998</td>
</tr>
<tr>
<td>p14/ARF</td>
<td>2.0</td>
<td>regulates p53 stability</td>
<td>Tango et al., 2002</td>
</tr>
</tbody>
</table>

Note. Expression levels indicated are -fold induction over expression in control neurons normalized to the abundance of glyceraldehyde 3-phosphate dehydrogenase mRNA.

TABLE 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-LO</td>
<td>0.4</td>
<td>anti-inflammatory enzyme</td>
<td>Kekavjar et al., 2000</td>
</tr>
<tr>
<td>a-actin</td>
<td>0.4</td>
<td>neurite outgrowth</td>
<td>Zhou &amp; Cohan, 2001</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.4</td>
<td>anti-apoptotic</td>
<td>Hou et al., 2003</td>
</tr>
<tr>
<td>GPx</td>
<td>0.4</td>
<td>Anti-oxidant, neuroprotective</td>
<td>Ben-Yoseph et al., 1996</td>
</tr>
<tr>
<td>CK</td>
<td>0.5</td>
<td>creatine kinase, ATP production</td>
<td>Zhou et al., 1994</td>
</tr>
<tr>
<td>P2RXL1</td>
<td>0.5</td>
<td>P2X nucleotide recept., neurite outgrowth, apoptosis</td>
<td>Neary et al., 1996</td>
</tr>
<tr>
<td>14-3-3 sigma</td>
<td>0.6</td>
<td>Anti-apoptotic, Bad repressor</td>
<td>Subramanian et al., 2001</td>
</tr>
<tr>
<td>fos</td>
<td>0.6</td>
<td>AP-1 binding</td>
<td>Hughes et al., 1999</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.6</td>
<td>Growth factor, neuroprotection</td>
<td>Svensson et al., 2002</td>
</tr>
<tr>
<td>p73</td>
<td>0.6</td>
<td>Neuronal survival</td>
<td>Poziniak et al., 2002</td>
</tr>
</tbody>
</table>

Note. Expression levels indicated are a function of expression in control neurons normalized to the abundance of glyceraldehyde 3-phosphate dehydrogenase mRNA.
Figure 3-1. Copper (Cu) treatment results in an increase in p53. Cultured human NT2 -N neurons were treated with 100 µM Cu for 12 and 18 h. Cells were then fixed and immunostained with a p53 primary Ab followed by a fluorescently tagged secondary Ab. Photos represent control neurons with minimal p53 expression followed by Cu -treated neurons for 12 h with increased cytoplasmic p53, and Cu -treated neurons for 18 h showing that p53 is translocated to the nucleus. Photomicrographs are representative of 6 dishes for each treatment. Scale bar = 10 µm.
Figure 3-2. Cu-overload alters the regulation of p53-responsive genes in NT2-N neurons. Cells were treated with Cu for 12 h. Total cellular RNA was collected for Cu-treated and control neurons. Following reverse transcriptase and biotin labeling of transcripts, oligonucleotide arrays revealed the differential regulation of a variety of pro- and anti-apoptotic mRNAs. The figure shows representation of mRNAs (c-jun, IGFBP-6, Hsp 70, and Hsp 27) in control and Cu-treated conditions. GAPDH served to normalize spot intensities between mRNA groups.
Figure 3-3. Copper induction of p53 results in an increase in immunodetectable IGFBP-6 in cultured human NT2-N neurons. Neurons treated with Cu (100 µM; 12 and 18 h) and immunolabeled with primary Ab for IGFBP-6 followed by a fluorescently tagged secondary Ab. Untreated cells served as controls. Neurons were stained with DAPI to label nuclei. Photos represent untreated control cells with minimal IGFBP-6 expression followed by 12 and 18 h of Cu where IGFBP-6 expression is increased both in the cytoplasm and nuclei of the neurons. Photomicrographs are representative of 6 dishes for each treatment. Scale bar =10 µm.
Figure 3-4. Immunolabeling of Hsp 70 is elevated with the addition of increasing concentrations of Cu. Cultured human NT2-N neurons were fixed and immunolabeled with a primary Ab for Hsp 70 followed by a secondary Ab with a fluorescent tag. Photos represent neurons treated with increasing concentrations of Cu at 50, 100, and 200 µM. Photomicrographs are representative of 6 dishes for each treatment. Scale bar= 10 µm.
**Figure 3-5.** Addition of toxic levels of Cu to cultured human NT2-N neurons results in increased immunodetection of Hsp 70. Neurons were treated with 100 μM Cu, fixed and stained with a primary Ab for Hsp 70 followed by a secondary Ab with a fluorescent tag. Photos represent untreated control neurons with low expression of Hsp 70 followed by Cu addition at 12 and 18 h both of which showed an increase in Hsp 70 expression throughout the cell. Photomicrographs are representative of 6 dishes for each treatment. Scale bar=10 μm.
Figure 3-6. Copper increases Hsp 70 in cultured human NT2-N neurons. Neurons were treated with Cu (100 µM) for 12 h. Untreated neurons served as controls. Cells were collected in NP-40 extraction buffer for protein isolation and separated on a 12% polyacrylamide gel. Following electro-transfer, a primary Ab for Hsp 70 was hybridized to the nitrocellulose membrane overnight. On day 2 the membrane was hybridized with a secondary Ab conjugated to HRP and chemiluminescence was used to determine the expression of Hsp 70. Photo represents 4 lanes each of control and Cu-treated protein labeled for Hsp 70 and neuro-specific enolase. Graph represents differences in Hsp 70 protein abundance between Cu and control neurons normalized for neuro-specific enolase. *Significantly different from controls at p<0.001.
Figure 3-7. Copper treatment results in an altered localization of immunolabeled c-jun. Cultured human NT2-N neurons were treated with Cu (100 µM) for 12 h. Cells were fixed and incubated with a primary Ab for c-jun followed by a fluorescently tagged secondary Ab. Photos show untreated control neurons with nuclear localization of c-jun followed by Cu-treated neurons expressing c-jun in both the nucleus and extra-nuclear structures. Scale bar=10 µm.
CHAPTER 4

MOLECULAR REGULATION OF ZINC IN THE Olfactory Bulb: Gene Discovery in a Model for Neuropsychiatric Disorders

Introduction

Zinc (Zn) is an essential trace metal which plays a role in multiple biochemical functions. It is required for the activity of >300 enzymes, and is a member of all 6 enzyme classifications. Furthermore, Zn has both catalytic and structural roles in its varying enzymatic functions (McCall et al., 2000). Zn is needed for nucleic acid and protein synthesis and is critical for the function of a group of transcription factors (TFs) known as Zn-finger proteins (Huang et al., 1997). The configuration of these finger motifs are determined by one Zn atom and it is this structural component that allows such TFs to bind to metal response elements on the promoters of multiple genes and regulate their expression (Hambridge, 2000; Litchlen et al., 1999). In addition, Zn modulates neurotransmission (Trombley and Shepard, 1996), prevention of oxidative stress (Powell, 2000) and feeding (Shay and Mangian, 2000). While many studies have shown the importance of dietary Zn in food intake, including findings that Zn deficiency is linked to anorexia nervosa, these studies have not revealed how Zn in the diet effects gene expression involved not only in food consumption, but also in clinical findings of depression, anxiety and cognitive impairments strongly linked to anorexia and Zn deficiency. Furthermore, caloric restriction has been correlated to increased neurogenesis and cell survival (Mattson, 2000). This is interesting in light of the findings that Zn deficiency, known to decrease food intake, is linked to changes in neuronal morphology indicative of pathological cell death (Ahn et al., 2000).

Olfactory bulbectomy is a well-documented model for the study of depression and anxiety in experimental animals (Kelly et al., 1997). Removal of the olfactory bulbs is associated with a variety of behavioral abnormalities that can be used to evaluate the function of antidepressant therapy (Mar et al., 2000). Interestingly, some of the highest concentrations of Zn in the brain are found in the olfactory bulb (OB) (Gulya et al., 1991; Ono and Cvherian, 1999). Electrophysiological studies have been used extensively to study the role of Zn in olfactory
neuron activity and synaptic transmission. Zn is co-localized with a subset of glutamate releasing neurons and is associated with GABA and glycine neurotransmission. Zn is therefore critical for the regulation of both excitatory and inhibitory communication in the OB (Trombley and Shepard, 1996). Zn modulates the activity of these neurotransmitters and is hypothesized to regulate other neurotransmitters and neuromodulators including NPY which is known to be involved in food consumption (Selvais et al., 1997). Interestingly, NPY may also act as an anxiolytic through neuromodulation in the limbic structures of the brain (Kask et al., 2002). A complete understanding of the role of Zn in the OB is important as Zn deficiency has been linked to a number of neuropsychiatric disorders including major depression (Nowak, 1998), anxiety (Chu et al., 2003), schizophrenia (Andrews, 1992), Parkinson’s (Forsleff et al., 1999) and Alzheimer’s disease (Constantinidis, 1991). Many of these disorders are also associated with OB dysfunction. The OB not only participates in the processing of olfactory information but has been shown to regulate emotion and memory (Holmes et al., 2002). While many functions for Zn in OB have been characterized little is known about the molecular roles of Zn in the OB. This work is critical as 1% of the entire human genome codes for Zn-finger proteins and over 220 proteins have Zn-finger motifs (Wu et al., 1995). Two of these proteins have been identified that are specifically expressed in the OB, these include Olf-1 and Roaz, which is involved in olfactory neuron differentiation (Tsai and Reed, 1998). The high concentrations of Zn in the OB, and the known role of Zn in transcriptional regulation make it reasonable to hypothesize that there is a significant molecular role for Zn in the OB. Furthermore, it could be suggested that alterations in OB neuronal Zn and subsequent changes in molecular expression may be involved in multiple neuropsychiatric disorders. Taking into account the effects of altered Zn on food intake, the connectivity of the OB to feeding, and the effects that Zn may have on molecular expression in the OB, findings in this area may aide in elucidating mechanisms involved in certain disease states such as depression linked anorexia.

In order to determine the molecular roles of Zn in the OB this work employed multiple techniques for high throughput analysis of gene expression using a model of dietary Zn deficiency. A subtracted library was created to enhance the finding of genes down-regulated under conditions of Zn deficiency. The decision to evaluate down-regulated genes by Zn deficiency was based on the requirement of Zn in a multitude of transcription factors potentially involved in OB gene regulation. A microarray chip was then designed with 1,000 clones chosen
from our subtracted library. The use of microarray allowed us to screen for a higher magnitude of transcripts and diminish the amount of false positive genes seen as differentially expressed by subtraction. Furthermore, this work used the well-documented technique of differential hybridization to screen for genes differentially expressed by dietary Zn deficiency in the OB. By using this technique we were allowed not only to screen for the expression of thousands of genes but also determine which genes may be differentially up-regulated as opposed to down-regulated under conditions of Zn deficiency. By identifying changes in the abundance of OB transcripts in response to dietary Zn deficiency, our aim was to correlate changes in gene expression to physiological abnormalities that are related to neuropsychiatric disorders.

**Materials and Methods**

**Animals**

To induce Zn deficiency, weanling male Sprague-Dawley rats were fed a commercially prepared (Research Diets Inc., New Brunswick, NJ) Zn adequate (+Zn, 30 ppm) or Zn deficient (-Zn, 1 ppm) diet for 15 days (n=15/group). Because Zn deficiency is known to cause anorexia, an additional group of rats (n=15) was pair-fed to the –Zn rats. Pair-fed rats were provided with the weighed amount of +Zn food eaten by the –Zn rats on the previous day. Deionozed water was provided ad libitum to all rats.

**OB Zn Concentrations**

To determine the effect of dietary Zn restriction on OB Zn content, bulbs were removed from 5 rats/group after anesthetization with methoxyflur-ane (Schlering-Plough, Union, NJ) and decapitation. Samples were disrupted by sonication in 1 ml of phosphate buffered saline and protease cocktail inhibitors (1:20 dilution) (Sigma-Aldrich, St. Louis, MO). Total protein concentrations were measured by the BCA assay (Pierce, Rockford, IL). Zn content between treatment groups in the OB was measured by graphite furnace atomic absorption spectroscopy and expressed as ng Zn/mg protein.
Creation of cDNA libraries

Total RNA was isolated from remaining 10 rats/group (+Zn/-Zn) using the Trizol extraction method. RNA was pooled between groups and two rounds of oligo (dT) cellulose mRNA (Invitrogen Life Technologies, Carlsbad, CA) isolation was used to acquire transcripts for cDNA synthesis. A cDNA library representing genes from both +Zn and -Zn rats was created using the Uni-ZAP XR vector (Stratagene, La Jolla, CA) following the protocol of the manufacturer. Briefly, 5 µg of mRNA from each group was reverse transcribed with StrataScript reverse transcriptase for first strand cDNA synthesis followed by addition of DNA polymerase I for the synthesis of second strand cDNA. Size selection was performed (>400 bp) and cDNA was unidirectionally inserted into the Uni-ZAP XR vector using EcoRI (5′) and XhoI (3′) restriction sites. The cDNA was packaged using the Gigapack III Gold Cloning Kit according to manufacturer’s protocol. The packaged library was amplified by transfecting XL-1-Blue MRF′ cells. Library tittering showed that both +Zn and -Zn deficient cDNA libraries contained 1x10^6 clones per µg of DNA. Libraries were subsequently stored at 4°C for later use.

PCR amplification of lambda phage DNA inserts

The cDNA inserts were PCR amplified by using 100 pfu (plaque-forming units) as template along with T3/T7 primers and HiFi SuperScript DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The PCR reactions were performed in multiple 50 µL reactions to acquire adequate amounts of PCR product for subtraction application. The following PCR parameters were used: 2 min 94°C (ensures phage lysis and double strand cDNA separation) followed by 30 cycles at 94°C for 30 sec; 40°C for 1 min; 68°C for 3 min. Following 30 cycles of PCR a 7 min 68°C cDNA elongation step was performed to ensure full length polymerization of cDNA inserts. All PCR products were purified using a Qiagen PCR Purification kit (Qiagen, Valencia, CA) and checked on 1% agarose gels for abundance and size followed by spectroscopy (260/280) to determine cDNA concentrations.

Preparation of a Subtracted cDNA library

Photoprobe Biotin (Vector Laboratories, Burlingame, CA) was thermo-coupled at 95°C for 30 minutes for direct nucleic acid labeling at multiple sites over the length of the cDNA acquired from PCR products of -Zn cDNA. The -Zn biotinylated cDNA (50 µg) was used as the
driver to subtract from unlabeled +Zn cDNA (10 µg) (5:1). Following hybridization of mixed cDNAs at 68 °C for 24 h a Vectrex Avidin D slurry (Vector Laboratories, Burlingame, CA) was used per protocol to remove cDNA hybrids between +Zn and -Zn groups and unhybridized cDNA from the -Zn population. This method of subtraction therefore left behind unhybridized cDNA from the +Zn population for collection. 1% agarose gels were run to determine cDNA size and abundance both pre- and post-cDNA hybridization and after Avidin D separation. A second round of subtraction was performed to further enhance for transcripts down-regulated in the -Zn group, this subtraction also used a 5:1 ratio of -Zn biotinylated cDNA compared to +Zn controls.

**Preparation of Zn-regulated Subtracted Library for Microarray analysis**

Following the second subtraction, un-amplified (to prevent selective amplification of small cDNA fragments) and un-hybridized cDNA selected for from the +Zn population of cDNAs was run on a 1% agarose gel and selectively cut for two different size populations at >1.5 Kb and 600 bp to 1.5 Kb. Following gel purification an “A” overhang was added to the 3′ ends of the cDNA products, then cDNAs were ligated into the pCR 2.1 -TOPO TA cloning vector (Invitrogen Life Technologies, Carlsbad, CA) with subsequent transformation into competent Ecoli cells. An aliquot (50 µl) of each transformation reaction was plated on IPTG/Xgal, LB/Ampicillin agar plates and incubated overnight at 37 °C. Following confirmation of successful transformation and calculations of colony number based on blue/white selection both size groups of cDNA clones were separated in aliquots of 250 µl and diluted 1:1 in glycerol. Additionally, 30 white colonies from both groups were picked for PCR amplification of the inserts using T3 and T7 primers to confirm insert size distribution. Concomitantly these PCR products were chosen for confirmation of differential expression by Reverse Northern analysis. Briefly, PCR product concentration was quantified and 25 ng of each selected product was covalently bound to a nylon membrane using the standard protocol for slot blotting (Current Protocols in Molecular Biology, Vol. 2, 2000). DNA samples were transferred to membranes through the use of a blotting manifold, DNA denaturation and binding was promoted through the addition of samples to an alkaline buffer prior to spotting. Duplicate blots were created and probed respectively with +Zn and -Zn radiolabeled probes that were generated as previously described. Autoradiography was used to identify differential regulation followed by photodensitometry to quantify expression levels. GAPDH served as a control to normalize spot
intensities between labeled blots. The cDNAs with greater than 2-fold changes in expression ratios were then chosen for DNA sequencing and sent to the Florida State DNA Sequencing Core Facility. Database searches of cDNA sequences obtained from screening were conducted using the BLAST database search program from the National Center for Biotechnology.

**Spotting of Microarray chips**

Glycerol stocks from both size groups of TA clones were sent to the Vanderbilt Microarray Shared Facility (VMSR) for the development of a 1,000 clone cDNA microarray chip. Transformed clones were plated on LB/Ampicillin plates and 800 colonies were picked from the 600 bp-1.5Kb range and 200 colonies were picked for cDNAs ranging in size from 1.5 Kb and higher using a Robotic Colony Picker. Overnight bacterial lyses was followed by PCR amplification using M13 forward and reverse primers. PCR product size and abundance was confirmed by gel electrophoresis. 1, 000 clones plus controls (GAPDH, Actin, NPY, ZnT-1, and MCCY (fluorescently-labeled oligonucleotide)) were spotted in triplicate from 3 separate 384-well plates onto poly-lysine coated slides (CEL Associates, Santa Clara, CA). DNA was printed from a proprietary buffer at a concentration of 150 ng/µl and an average spot size of 130 µm. Spoting was performed at room temperature at a relative humidity of 45%. 60 identically spotted microarray chips were returned along with 96-well plates containing bacterial plasmids specific for each colony picked for spotting.

**Labeling of mRNA targets**

Rats were placed on +Zn, -Zn and PF diets as previously described (10/group). Isolation of mRNA from OB total RNA for each group was performed as previously described. The CyScribe cDNA Post Labeling Kit (Amersham Biosciences, Piscataway, NJ) was used to produce Cy3 and Cy5 labeled cDNA targets for the use of dual fluorescent color microarray hybridizations. Briefly, random nonamers and oligo (dT) primers were annealed to isolated mRNA (500 ng), followed by the addition of a nucleotide mix and an amino -allyl coupled dUTP for generation of first strand cDNA. Next, mRNA was removed from the first strand cDNA to allow for amino allyl labeled cDNA to bind to its complement on the microarray chip. This was accomplished with alkaline treatment (2.5 M NaOH and 2 M HEPES) which causes fragmentation of mRNA. GFX PCR DNA and Gel Band Purification Kit columns (Amersham Biosciences, Piscataway, NJ) were then used to remove short mRNA oligomers. Modified cDNA was then coupled to respected CyDyes (Cy3 and Cy5 for separate cDNA groups) by the reaction
of CyDye NHS-esters with incorporated amine allyl groups on the cDNA. 4M hydroxylamine was added after CyDye labeling to inactivate any unreacted CyDye NHS ester molecules and cDNA was purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA). All CyDye labeling was done in the dark to prevent direct exposure to fluorescent light and subsequent photodestruction of CyDye fluorophores.

**Microarray Hybridization**

Prior to hybridization microarray chips were pre-washed following protocol from VMSR to specifically enhance fluorescent binding of labeled cDNA targets in accordance with manufacturer's cDNA spotting. Briefly, slides were vigorously washed with 0.2% SDS at room temperature, immediately transferred to a prehybridization solution (5X SSC; 1% Bovine Serum Albumin; 1% SDS) at 55°C for 45 min. followed by 7 washes in dH2O and 1 wash in isopropanol. CyDye labeled cDNA was then placed over the array, covered with a 22x25 mm coverslip (Corning, Corning, NY) and placed in the hybridization chamber (ArrayIt Brand products, TeleChem International Inc., Sunnyvale, CA). Microarray chips were hybridized for 18 h at 42°C then removed and washed per protocol (CyScribe Post-Labeling kit). The first group of microarray chips (n=3) was labeled with +Zn (Cy5 fluorophore) and -Zn (Cy3 fluorophore) labeled-cDNA to evaluate differential gene expression between these groups. The second group of microarray chips (n=3) were hybridized with +Zn (Cy3 fluorophore) and PF (Cy5 fluorophore) labeled-cDNA to compare differences in gene expression to group one chips. This comparison was used to determine transcripts specifically regulated by diminished food intake. All microarrays were air dried, placed in 50 ml conical tubes, wrapped in foil to prevent light exposure and stored at -80°C.

**Microarray data analysis**

Several microarray chips were non-discriminantly labeled with SyberGreen (Molecular probes, Eugene, OR) per protocol to determine location and measure abundance of bound DNA. After hybridization and air drying SyberGreen labeled microarrays were scanned on a ScanArray 5000XL scanner (GSI Lumonics, Moorpark, CA) in the FITC channel. Scanned microarrays were then analyzed by QuantArray imaging software (GSI Lumonics, Moorpark, CA) and a grid was created to account for the locality of all spots and used for locating and quantifying DNA expression on subsequent hybridized microarrays. Fluorescent cDNA target microarrays were scanned at 535 nm for Cy3 and 625 nm for Cy5. Background signal to noise ratio was quantified
for each probed element. The ratio was corrected for the two signal intensities and calculated as the differential expression ratio between the two target cDNA populations. Data was then analyzed in Microsoft Excel following global normalization to account for overall intensity differences between the two fluorescent dyes. A 4-fold increase or decrease in expression ratios was used as the cut-off for differential expression. The cDNAs differentially expressed in the in both pair-fed and Zn deficiency compared to Zn adequate controls were chosen for DNA sequencing.

**Differential Hybridization**

Radiolabeled cDNA probes were generated from PCR amplified cDNA inserts from both +Zn and –Zn lambda phage libraries of the rat olfactory bulb using a Random Prime Labeling kit (Invitrogen Life Technologies, Carlsbad, CA) with α-[\(^{32}\)P] dATP (Du Pont NEN Products, Boston, MA). Parameters for the generation of PCR products has been previously described. 50 ng of cDNA was radiolabeled for each condition. XL-1-Blue MRF′ bacterial culture was transfected with lambda phage from the +Zn cDNA library and was plated on LB/Ampicillin plates (25,000 plaques per plate/5 plates). The plates were incubated overnight in a 37 °C incubator. Nylon filters (MAGNAPROBE) (Osmonics Inc, Minnetonka, MN) were used for replica plaque lifts following the standard protocol of lysis - denaturing-neutralization (Current Protocols in Molecular Biology, Vol.1, 2000). Extracted DNA was then crosslinked into membranes by heating in a vacuum oven for 1 h at 85 °C. Membranes were prewashed at 45°C for 4 h then hybridized to individual probes (+/−Zn radiolabeled cDNAs) at 45 °C in 50% formamide, 5X SSPE, 1X Denhardt’s, 0.1% SDS, 100 µg/ml salmon sperm DNA. Just prior to hybridization probes were heat denatured to optimize for single strand binding to membrane complements. All membranes were hybridized for 16 h in a total volume of 25 ml and shaken. Following hybridization radiolabeled membranes were washed 4 x 15 min. with 5X SSC and 1% SDS at 45°C. Autoradiography was used to visualize binding of probes to plaque DNA.

Differential signals identified from autoradiographs allowed for selection of corresponding plaques bearing phage DNA for a second round of hybridization where plaques were purified to further confirm differential expression of the hybridization signal seen in the first hybridization. The second hybridization was performed and plaques selected were collected in SM buffer. The PCR protocol as previously described was used to amplify phage DNA inserts. Confirmation of differentially expressed cDNAs was confirmed by Reverse Northern analysis.
and autoradiography as previously described. The cDNAs with greater than 2-fold changes in expression ratios were then chosen and sequencing as previously described.

Results

Dietary Zn deficiency and/or restriction resulted in differential expression of multiple transcripts. Differentially expressed genes identified by cDNA library subtraction or differential hybridization were confirmed by reverse Northern analysis as shown in Figure 4-1. The combined results acquired from cDNA library subtraction, differential hybridization, and microarray are listed in Table 3.

Discussion

This work was designed to determine the effects of Zn deficiency on gene expression in the OB. Because dietary Zn deficiency results in decreased food consumption (Reeves et al., 2003, Evans et al., 2004), it was necessary to design this experiment to enable us to distinguish between the molecular effects of caloric restriction and the effects of Zn deficiency. This was accomplished by the inclusion of a pair-fed group that was subjected to caloric restriction matching the intakes of Zn deficient rats. An additional benefit of this design was that it enabled us to identify genes in the OB that were regulated by caloric restriction. While the effects of caloric restriction are currently receiving a great deal of attention, to our knowledge the molecular effects of caloric restriction on the OB have not been previously explored. Thus, the first part of this discussion will address the changes found in the OB following caloric restriction, while the second part will discuss the significance of Zn deficiency.

Caloric Restriction

Caloric restriction (CR) has been shown to decrease neuronal death (Kanda, 2002) and increase neurogenesis (Mattson, 2000). For example, in the hippocampus CR increased neurogenesis and decreased cell death following excitotoxic injury induced by kainate acid (Lee et al., 2003). Furthermore, dietary restriction has been shown to increase brain derived neurotrophic factor (BDNF) in the hippocampus, cerebral cortex and striatum (Duan et al., 2001). As suggested previously, the effects of CR or dietary restriction in the OB have not been studied. However, like the hippocampus, the OB has neuroregenerative ability (Arlotta et al.,
Furthermore, high concentrations of free Zn in many OB neurons increase the susceptibility to excitotoxic damage. Thus, the finding that CR altered the expression of several genes known to be involved in both apoptosis and neuroplasticity is significant.

**Apoptosis-Related Genes**

**ARC (Apoptosis Repressor with CARD).** This gene product is an apoptotic regulator with a CARD domain linked to caspase inhibition. Lifelong CR models have shown an increase in ARC, correlated with improved cognition in aging rats (Shelke and Leeuwenburgh, 2003). There are two known mechanisms of ARC action. First, when neurons are faced with challenging levels of oxidative stress, ARC prevents receptor-mediated neuronal apoptosis by interacting with caspase 2 and 8 and preventing calcium release from the mitochondria. Second, ARC may directly inhibit the release of mitochondrial cytochrome c, a known activator of caspase 3 and subsequent apoptosis (Shelke and Leeuwenburgh, 2003). Furthermore, studies using a rat model of transient global brain ischemia have shown that there is a decrease in ARC protein levels in the CA1 region of the hippocampus where selective death is occurring. These findings were further validated by an increase in cell death of cultured hippocampal neurons subjected to hypoxia that was reversed by overexpression of the full length ARC message (Hong et al., 2003).

Our work shows a 4-fold increase in OB ARC mRNA following less than two weeks of CR. This appears to be not only the first report of changes in ARC in the OB, but also the first report of changes in ARC mRNA abundance following CR. Future work will be needed to determine the mechanism responsible for this regulation and the role of ARC in the OB.

**Mdm2.** Mdm2 functions as a regulator of the tumor suppressor protein p53. p53 induces the transcription of mdm2. In turn, Mdm2 both trans-represses p53 expression and promotes the ubiquitin-mediated proteasome degradation of p53 (Paitel et al., 2003; Tan et al., 2000). Thus, increases in mdm2 expression decrease p53 abundance and activity. Here we report a 4-fold increase in mdm2 mRNA abundance under conditions of CR, suggesting a reduction in p53-mediated apoptosis. Not only is this consistent with the increased ARC expression discussed previously, it appears to be the first report of dietary regulation of mdm2.

**Neurogenesis and Neuroplasticity-Related Gene**

**Neuroserpin.** Neuroserpin is a serine protease inhibitor including tissue type plasminogen activator (Hastings et al., 1997). Because serine proteases have been associated with synaptic
plasticity, neuronal migration, and axogenesis, neuroserpin has been linked to repression of neurite outgrowth and neurogenesis. For example, neuroserpin transfection of PC12 cells led to a decrease in the number of cells extending neurites and reduced total neurite length. Furthermore, the cells did not respond to nerve growth factor-induced neurogenesis in the presence of elevated neuroserpin (Parmar et al., 2002). It has been proposed that neuroserpin is axonally secreted and regulates extracellular proteolysis (Osterwalder et al., 1996). During neurogenesis it may attenuate the proteolytic processes required for synaptic plasticity related to neuronal migration (Krueger et al., 1997). Interestingly, studies have shown high levels of neuroserpin expression in the OB, the subventricular zone, and the hippocampus, all known as regions with neurogenesis throughout life (Krueger et al., 1997). In these studies we have shown that CR decreases neuroserpin gene expression by 4-fold. This is consistent with previous work showing that CR increases neurogenesis (Mattson, 2000) and suggests that CR may enhance neurogenesis in the OB. Future work will be needed to test this hypothesis.

**Zinc Deficiency**

In vitro, Zn deficiency has been shown to induce neuronal apoptosis (Ahn et al., 2000; Ho et al., 2000). And, although the mechanisms have not been identified, it has been hypothesized that Zn is required for neurogenesis (Bhatnagar and Taneja, 2001). In this report, we have shown that dietary Zn deficiency regulates a number of OB genes that have been linked to apoptosis and neurogenesis. This work not only provides new information on the molecular role of Zn in the OB, but may also provide insight into the roles of Zn in apoptosis and neuroplasticity.

**Apoptosis-Related Genes**

Consistent with reports that Zn deficiency induces neuronal apoptosis, expression of the apoptosis repressors ARC and mdm2 was decreased in the OB of rats fed a Zn deficient diet. It should be noted that these decreases were not the result of a reduction in food intake, because both ARC and mdm2 mRNA increased 4-fold in pair-fed rats. These findings suggest that Zn deficiency may mediate apoptosis in the OB. Future studies will be needed to examine the cell type(s) that express these genes, the mechanisms of Zn that are responsible for this regulation, as well as the implications for OB function.
Neurogenesis and Neuroplasticity-Related Genes

**β**-tubulin. The cytoskeleton-associated protein **β**-tubulin is a specific marker for identifying neuronal precursor cells (Braun et al., 2002; Howell et al., 2003). It has been localized to proliferating cells that migrate from the adult subventricular zone (Katsetos et al., 2003) and to developing olfactory receptor neurons (Ronnett et al., 2003). Exercise-induced hippocampal neurogenesis revealed an increase in **β**-tubulin associated with neurogenic cells (Fabel et al., 2003). The work here shows a 2-fold decrease in **β**-tubulin expression suggesting a reduction in the neurogenic potential of the OB during states of Zn deficiency. Future work will be needed to explore the mechanisms responsible for this regulation and determine the effects of CR on expression of this marker of early neuronal growth.

**Neuroserpin.** While this regulator of neurite outgrowth was decreased in the OB of pair-fed (CR) rats, Zn deficiency did not alter neuroserpin mRNA abundance. Given that pair-fed and Zn-deficient animals were consuming the same daily amount of food, this suggests that the increases in neuroserpin associated with CR are dependent in the trace metal zinc. This suggests that neuroserpin may be an important target for the role of Zn in neurogenesis.

**Conclusions**

The work reported here supports the hypothesis that CR increases neurogenesis and decreases apoptosis in the CNS. This work has not only identified potential new regulators of neuronal survival and growth during CR (mdm2 and neuroserpin), but extends our current knowledge of the known neuronal regulator of apoptosis, ARC, to the OB. It is particularly interesting to note that many of the molecular changes seen in the OB following CR are reversed by Zn deficiency. It was fully expected that some genes, such as the putative zinc-finger protein BN/SsNH, would be decreased in both Zn-deficient and pair-fed rats. This means that BN/SsNH, whose function is not yet known, is regulated by CR, but it is unlikely that Zn deficiency regulates this gene. However, the genes associated with apoptosis and neurogenesis followed a different pattern. Neuroserpin was regulated only in pair-fed rats, while ARC, mdm2 and ATP synthase were all up-regulated by CR and down-regulated in Zn-deficient animals consuming the same amount of food. Thus, Zn deficiency appears to be able to reverse the effects of CR. This finding has implications for future studies on the effects of CR on learning, memory, neuronal...
survival, aging, and neurodegenerative diseases. Simply put, we now know that interpretation of any work employing CR is dependent on adequate Zn status. This seems to be particularly pertinent for studies involving long-term CR.

This work also has implications in the understanding and treatment of clinical disorders characterized by neuronal damage and death in the OB. There are a wide variety of disorders including Major Depression, Alzheimer’s disease, Parkinson’s disease, and schizophrenia that are characterized by neuronal death in the OB and diminished OB volume (ter Laak, 1994). In fact, in the case of Parkinson’s disease, loss of olfactory senses has been proposed as an early diagnostic tool (Becker et al., 2002). Furthermore, depression has repeatedly been linked to Zn deficiency in humans (Little et al., 1989; McLoughlin and Hodge, 1990; Maes et al., 1994; Maes et al., 1997a; Maes et al., 1997b; Maes et al., 1999). The current work makes this particularly significant because not only do antidepressant drugs increase neurogenesis, but it now appears that neurogenesis is required for the antidepressent effect (Santarelli et al., 2003). Thus, the role of the trace element zinc in OB apoptosis, neurogenesis, and a variety of neurodegenerative and neuropsychiatric disorders should be investigated in future work.
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<tr>
<th>Gene</th>
<th>Expression</th>
<th>Methods</th>
<th>Function</th>
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<td>putative zinc finger protein</td>
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<td>↑ PF 4-fold ↓ -Zn 2-fold</td>
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<td>Apoptosis repression protein with CARD domain represses p53, increases p53 degradation</td>
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<tr>
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PF, pair-fed animals representing the caloric restricted rats; -Zn, zinc restricted rats
Figure 4-1. Differentially expressed genes from cDNA library subtraction and differential hybridization were confirmed by Reverse Northern. Photo is representative of transcripts down-regulated 2-4 fold in the olfactory bulb following dietary Zn deficiency. GAPDH was used to normalize expression ratios between Zn adequate and deficient groups.
REFERENCES


BIOGRAPHICAL SKETCH

Jacob Wade VanLandingham (Jake) was born in Camp Leijune, North Carolina in 1973. He grew up in the small town of Juniper in Northwest Florida. In his youth he worked on the family farm helping to grow tomatoes and watermelons. Jake graduated from Florida High School here in Tallahassee on the campus of Florida State University. Following a year of playing college football at Valdosta State University in Georgia he returned home and received his Associate of Arts degree from Tallahassee Community College. In 1995 Jake entered the Physical Therapy Program at Florida A and M University. After two years he received his Bachelor of Science Degree and went on to work as a Physical Therapist in Neurological Rehabilitation. His career in Physical Therapy took him from Fort Myers to Pensacola and back to Tallahassee where he was the coordinator for services at the Dick Howser Center for kids. In 1999 Jake entered the Neuroscience Program at Florida State University and has now completed his PhD. in Neuroscience. With much family and friend support Jake will be moving to Atlanta, GA where he has accepted a post-doctoral fellowship in the Department of Emergency Medicine at Emory University. Furthermore, Jake will be working in the newly developed NeuroInjury Program which is designed to bring benchwork to the bedside.