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Manganese (Mn) is an essential dietary element required for several important physiological processes. However, accumulation of Mn due to excessive environmental exposure is known to pose neurological health concerns that manifest as movement abnormalities and cognitive impairment. Oxidative stress has been hypothesized to play a role in this dysfunction. In these studies we examined the effect Mn exposure had on oxidative stress in the brain with or without antioxidant therapy, and how brain regional Mn accumulation affected stereotypic behaviors in rats. Sprague-Dawley rats raised on AIN-93G diet were randomized into a Mn free group (deionized water) and Mn exposed group (deionized water with 1 g Mn/L). Each group was then subdivided into three additional groups receiving injections of either saline (vehicle) or antioxidant therapy: 200 mg/kg N-acetyl-cysteine (NAC) or 5 mg/kg (-)-epigallocatechin-3-gallate (EGCG) to yield six groups total, each with an n=6. During the sixth week of treatment, stereotypic rat behaviors (total distance traveled, sleep, sniff, and groom) were monitored using Clever Systems Home Cage Scan. Upon completion of the sixth week the rat brains were removed with the caudate putamen (CP) and hippocampus (HC) sectioned out and analyzed for Mn and iron (Fe) concentrations, total glutathione (GSH) levels, lipid peroxidation in the form of F_2-Isoprostanes (F_2-IsoPs), along with glutathione peroxidase (GPx) and catalase mRNA levels. Results were considered significant when $p \leq 0.05$. Mn exposure significantly increased Mn concentrations in both the CP and HC, accompanied by significantly decreased Fe:Mn ratios in Mn-exposed groups. Mn significantly

increased total distance traveled in each Mn-exposed group and decreased sniff behavior in the Mn only group. Only modest alterations in GSH and catalase levels were present in each region, although, a significant positive correlation between Mn concentration and GPx mRNA expression was observed. Additionally, F_2-IsoP results showed no evidence of increased oxidative damage compared to CN regardless of EGCG or NAC therapy. These data, compared to the body of Mn toxicity research, suggest that oral Mn exposure may not generate deleterious levels of oxidative stress leading to overt neurological dysfunction. This exposure paradigm, instead, may result in subtle neurochemical alterations associated with behavior change and potential cognitive impairment.

EFFECTS OF MANGANESE EXPOSURE AND ANTIOXIDANT THERAPY ON

OXIDATIVE STRESS AND STEREOTYPIC BEHAVIORS IN RATS.

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Manganese (Mn) is a dietary metal essential for numerous cellular processes including macronutrient metabolism, bone growth, cellular free radical defense, and ammonia clearance from the brain. However, excessive exposure to Mn has been shown to result in brain Mn accumulation and subsequent neurotoxicity. Mn is known to accumulate in iron (Fe) rich regions of the brain resulting in neurological symptoms associated with movement and behavior abnormalities. Mn neurotoxicity or "Manganism" shares movement disorder symptoms with Parkinson's disease (PD) and initial psychiatric alterations analogous to schizophrenia. Mn accumulates in the basal ganglia, where it has been shown to disrupt neurotransmitter function and damage neurons and glial cells, potentially facilitating a neurodegenerative state.

Damage caused by Mn neurotoxicity is hypothesized to result from multiple factors. Mn is known to alter dopamine, γ-aminobutyric acid (GABA) and glutamate homeostasis. A disturbance in these neurotransmitters can propagate movement and behavior abnormalities due to modified signal transduction. Additionally, this may result in altered synaptic concentrations of these neurotransmitters disturbing the function of glial cells surrounding the synapse. Indeed, data suggest that Mn alters the ability of astrocytes to process synaptic glutamate (the primary excitatory neurotransmitter) resulting in over excitation. Furthermore, Mn indirectly stresses cellular redox status

albeit through excitotoxicity, free radical generation, and/or lowering glutathione (GSH) levels. Recent studies have identified a specific species of isoprostanes, F_2-Isoprostanes (F_2-Isops), as a direct biomarker of Mn induced oxidative damage, specifically lipid peroxidation.

Augmenting cellular redox potential is thought to potentially curb cellular damage caused by Mn-induced oxidative stress. *N*-Acetyl-Cysteine (NAC) and (-)-Epigallocatechin-3-gallate (EGCG) are two compounds known to have a sparing affect on endogenous antioxidant systems. NAC is an able cysteine donor to complete the GSH tripeptide with glutamate and glycine, ensuring substrate availability for GSH synthesis. EGCG has the ability to serve as an antioxidant with eight hydroxyl groups available to donate electrons, and *in vitro* studies demonstrate EGCG increased gene expression of glutamate-cysteine ligase, which creates the catalytic precursor for de novo GSH synthesis.

The primary goal of this study is twofold; 1) examine the effect brain Mn accumulation has on stereotypic behaviors of rats, 2) examine the effect of Mn on brain GSH levels and the overall antioxidant enzyme response, and to explore the effectiveness of antioxidant therapy in mitigating cellular damage and modulating GSH levels (damaged measured as F_2-Isoprostane production). The specific aims for this study are to:

1. Examine the effect of Mn exposure on behavior by monitoring alterations in stereotypic behaviors and relating them to regional brain Mn accumulation. Twenty-one day old Sprague-Dawley rats were exposed to Mn

and monitored for changes in behaviors associated with locomotor activity and anxiety (total distance traveled, sleep patterns, grooming, and sniffing). *The working hypothesis for this aim is that increased Mn levels will lead to changes in these behaviors indicative of increased anxiety and disturbed circadian rhythm. Furthermore, antioxidant therapy will help normalize disturbances in behavior initiated by Mn exposure.*

2. Demonstrate an effect of Mn exposure on glutathione levels and associate corresponding changes to endogenous markers of oxidative stress in the rat brain. Twenty-one day old Sprague-Dawley rats were used to measure *in vivo* GSH levels and F_2-Isoprostane formation with or without Mn exposure. *The working hypothesis for this aim is that Mn accumulation in the brain will lead to altered GSH levels increasing F_2-Isoprostane formation, and the addition of antioxidant therapy will limit F_2-Isoprostane formation by attenuating the damage caused by reactive oxygen species.*

The overall hypothesis of these studies is that Mn exposure indirectly leads to neurochemical alterations by disrupting GSH functionality, leaving the brain vulnerable to oxidative insult. The resulting damage may decrease normal neuron and glial cell function resulting in altered neurotransmission and manifesting as altered locomotion and behavior. Antioxidant treatment may help mitigate damage caused by oxidative stress and help restore normal behavior in Mn exposed rats. Results from these studies help to characterize the role of Mn in the sequelae of neurodegeneration, and may lead to the development of possible therapies.

CHAPTER II
REVIEW OF LITERATURE

Introduction

Manganese (Mn) is an essential dietary element that plays a role in the proper function of many biological processes including energy metabolism, bone growth, blood clotting, ammonia clearance and neurotransmitter synthesis in the brain, and cellular free radical defense. Though Mn plays an important biological role, the body necessitates minimal amounts to satisfy requirements, with an adequate intake (AI) established at 1.8 or 2.3 mg/day for women and men, respectively (National Academy of Science, 2002). Historically, Mn deficiency has not been recognized as a risk to the general public. Mn toxicity, however, has garnished the attention of clinicians and researchers by manifesting symptoms similar to Parkinson's disease (PD). The onset of Mn toxicity, coined "Manganism", is characterized with anxiety and schizophrenic behavior, followed by movement disorders, and impaired memory (Cersosimo and Koller, 2006). Initial cases of Mn toxicity were relegated to occupational exposure, such as mining or battery manufacturing. Though, emerging cases have identified drinking water as a potential source of Mn toxicity (Wasserman et al., 2006). Regardless of the source, occupational exposure to Mn remains the primary culprit of toxicity. Recent attention has focused on the anti-knock gas additive methylcyclopentadienyl manganese tricarbonyl (MMT). MMT has been used in Canada since 1976, and has recently been approved for use in the

United States (Barceloux 1999). Unlike acute occupational exposure, widespread use of MMT may expose sizeable populations to chronic low doses of aerosol Mn salts (e.g. Mn sulfate and Mn phosphate). These readily inhaled Mn compounds may enter the body via respiration into the lungs or to the brain along the olfactory nerve. It is unclear how chronic low dose exposure will affect exposed populations. Clinical studies have also uncovered formula fed infants and patients receiving total parnenteral nutrition (TPN) as populations at risk for Mn toxicity (Hsieh et al., 2007). Studies have shown infant formula to have Mn concentrations 100 times higher than breast milk (Lonnerdal, 1994). Lastly, iron deficiency, the most common micro-nutrient deficiency in the world, can promote Mn accumulation within the brain (Erikson et al., 2005a).

Manganese Exposure

Dietary

Mn is abundant in the food supply with nuts, legumes, vegetables, fruit, and brewed tea representing the primary sources followed by red meats and poultry to a lesser extent (Aschner and Aschner, 2005). Currently not enough data have been collected to establish an RDA for Mn intake; however, an AI has been set at 1.8 and 2.3 mg/day for adult women and men respectively. Mn requirements do change throughout the life cycle according to growth and developmental needs (Tasker et al., 2004). The AI for different developmental stages is as follows: 3µg/day for <6mo, 600µg for 7-12mo, and 1.2 to 1.5 mg/day for children 1-3 and 4-8 years, respectively (National Academy of Sciences, 2002). The average dietary intake of Mn has been estimated at 2-6 mg/day (Freeland-

Graves and Turnlund, 1996) with vegetarian diets increasing the amount to approximately 11 mg/day (Gibson, 1994).

Excess dietary Mn rarely results in toxicity due to tight regulation in the gut. It is estimated that 1-5% of Mn ingested is absorbed (Finley, 2004; Davis et al., 1993). Dietary and endogenous factors play a role in this regulation. One study by Finley (2004) using radio-labeled ^{54}Mn found that men absorb less dietary Mn than women, though men retained Mn longer systemically, likely due to plasma ferritin levels. This finding corresponds with duodenal biopsy data that showed divalent metal transporter (DMT-1) expression was inversely associated with serum ferritin levels (Zoller et al., 1999). DMT-1 is expressed in endothelial cells and serves a transporter for divalent metal ions such as iron (Fe), copper (Cu), Mn, and zinc (Garrick et al., 2003). Other factors altering absorption of Mn, include competition with Fe and other divalent metals for absorption or excessive amounts of phytic acid (Aschner and Aschner, 2005; Hurrell, 2004). Dietary Fe deficiency has also been shown to increase intestinal Mn absorption (Finley 1999), as well as transport into the brain (Erikson et al., 2005a). Once in the system Mn transport in the blood depends on its oxidation state. Mn^{2+} travels bound to albumin and gamma-globulin, where as trivalent Mn^{3+} readily attaches to unoccupied transferrin (Aschner et al., 2007). Mn^{2+} is the most prevalent species in biological media; however, the more cytotoxic free Mn^{3+} can exist in small quantities.

Occupational and Environmental Exposure

Occupational exposure to Mn is the most common cause of Mn neurotoxicity. Several cases of neurotoxicity have been linked to Mn exposure within mining,

manufacturing, and welding industries (Hochberg et al., 1996; Crossgrove and Zheng, 2004). Inhalation of dense Mn particulate, up to 100-fold higher than established safe limits, is not uncommon in workers who display neurological symptoms (Crossgrove and Zheng, 2004). Alternatively, chronic exposure to low dose airborne Mn in the form of methylcylopentadienyl manganese tricarbonyl (MMT) may present future health concerns, particularity in vulnerable infant and iron deficient populations. Furthermore, Mn toxicity has been linked with contaminated drinking water (Wasserman, 2006; Sahni et al., 2007). In Bangladesh, children drinking from wells with a high Mn content (793 μg/L, compared to the Environmental Protection Agencies (EPA) established safe limit of 300 μg/L) had significantly reduced verbal scores and overall intellectual performance compared to children consuming water Mn concentrations under the EPA's established safe limit (Wasserman, 2006).

Transport to the Brain

It is well known that Mn accumulates in Fe rich areas of the brain (Erikson et al., 2002a). To date several transport mechanism have been discovered, each contributing to the transport of Mn into the brain. The transport mechanism engaged depends largely on the species of Mn, divalent (Mn^{2+}) or trivalent (Mn^{3+}), and plasma concentrations. Entrance of Mn via the cerebral spinal fluid at the choroid plexus tends to favor high plasma concentrations, where as cerebral capillary transport at the blood brain barrier (BBB) predominate under lower physiologic concentrations (Aschner et al., 2007). It is thought that the majority of Mn is chaperoned into the brain by Mn^{3+} bound to transferrin or uptake of Mn^{2+} by divalent metal transporter DMT-1 (Aschner et al., 2007). However,

ancillary Mn transport also occurs through active transport, leak pathways, in the form of Mn^{2+} citrate, and through store-operated calcium channels (Aschner and Gannon, 1994; Crossgrove et al., 2003; Crossgrove and Yokel, 2005).

DMT-1 is a known transporter of Mn and Fe that is expressed on the apical wall of endothelial cells, cerebral capillaries, on foot processes of astrocytes (integral to the BBB), and choroid epithelia of the blood-CSF barrier (Garrick et al., 2003). A study by Chua and Morgan, (1997) helped characterize the role of DMT-1 in Mn transport using homozygous Belgrade (b/b) rats with a defective DMT-1 allele. Mn and Fe transport into the brain was impaired in b/b rats compared to heterozygous (+/b) and Wistar rats indicating the importance of DMT-1.

Aside from DMT-1, Mn can cross the BBB by a few different mechanisms. Mn complexed with transferrin can bind to transferrin receptors at the cerebral capillaries for endocytosis into the capillary endothelium (Fishman et al., 1987). Within the endothelial cell Mn is liberated from systemic transferrin to complex with brain synthesized transferrin for transport into the brain. A small amount of Mn in the blood is bound to citrate, this Mn-citrate complex is thought to cross the BBB through monocarboxylate transporter (MCT) (Aschner et al., 2007). Other small scale transport mechanisms include the zinc family transporter (ZIP) and leak pathways (Aschner et al., 2007). The mechanisms by which Mn travels within the brain are still largely undefined; however, transferrin is thought to play a role.

Lastly, Mn transport to the brain has also been demonstrated to occur along olfactory neurons (Dorman et al., 2002). Solubility of the Mn species appears to play a

role in transport, with more soluble $MnCl_2$ and $MnSO_4$ concentrations peaking rapidly compared to less soluble $MnHPO_4$ and insoluble Mn_3O_4 (Dorman et al., 2001; Dorman et al., 2002; Brenneman et al., 2000). Inhaled Mn concentrates primarily in the olfactory bulb, but the mechanism governing Mn relocalization once it has entered the brain has yet to be fully elucidated (Leavens et al. 2007).
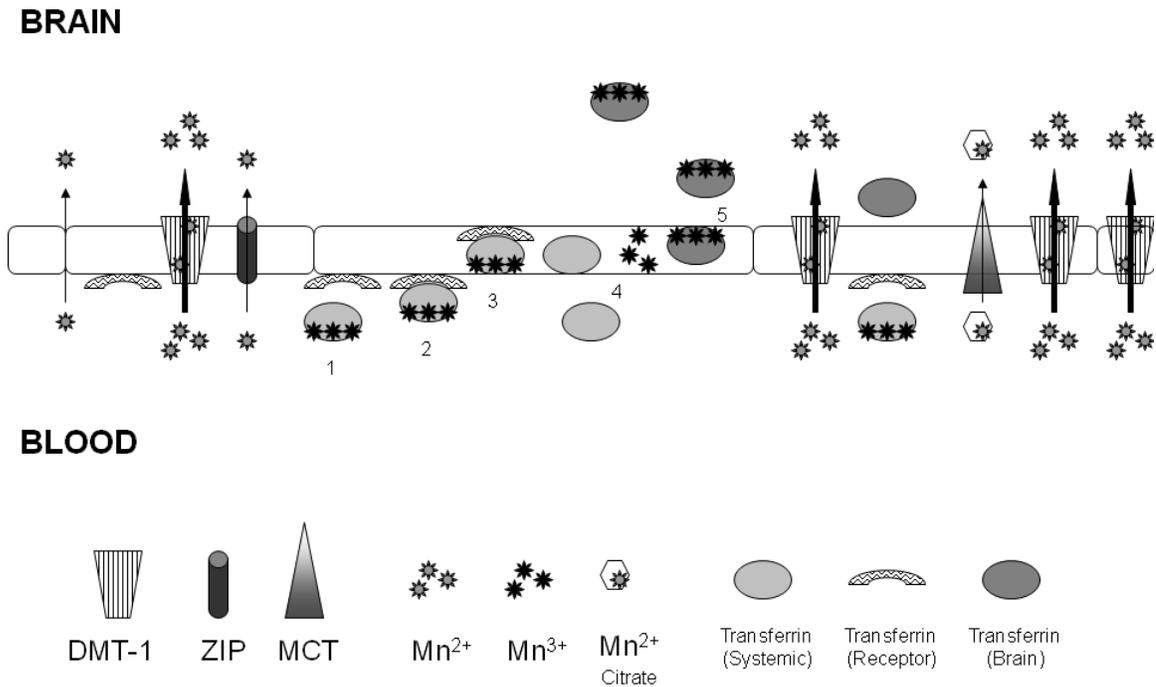
Figure 2-1. **Manganese Transport at the Blood Brain Barrier –** The above figure is a schematic of the Mn transport mechanism across the BBB. Primary transport routes (DMT-1 and transferrin endocytosis) are depicted with more abundant BBB density and bold arrows compared to the other transport mechanism (ZIP, Mn^{2+}-Citrate, and leak pathways). Endocytosis of Mn-bound transferrin 1) Mn bound to systemic transferrin reaches the BBB. 2) Mn-bound transferrin binds to transferrin receptor. 3) Endocytosis of the receptor bound complex 4) Mn is liberated from systemic transferrin and binds to brain-produced transferrin where it is transferred across the basolateral surface of the capillary endothelium into the brain.

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Manganese Toxicity

Systemic

Mn toxicity is known to affect multiple organ systems within the body. The liver plays a critical role in Mn homeostasis, accounting for approximately 95% of Mn clearance via biliary excretion (Crossgrove and Zheng, 2004). Hepatic failure poses a serious threat to brain and systemic Mn levels. In fact, decreased liver function due to alcoholism, cirrhosis, and hepatitis have been linked to hyper-intensity of T-1 weighted MRI's in human subjects (Malecki et al, 1999). Liver damage can also result from acute Mn exposure, as Mn has been shown to concentrate in the mitochondria of hepatic cells altering Ca^{2+} levels and electron transport chain (ETC) function (Zhang et al., 2003). Altered ATP production may play a critical role in biliary excretion according to a study conducted in marine vertebrates that suggests Mn efflux from the liver is influenced by an ATP sensitive mechanism (Madejczyk et al., 2009). Additionally, Mn exposure can also affect the kidneys and lungs. Both ingested (Huang and Lin, 2004) and inhaled (Tapin et al., 2006) Mn has been shown to accumulate in the kidneys leading to renal failure. Occupational asthma has also been documented in welders attributed to breathing Mn particulate (Beach et al., 1996). Further investigation in lung epithelial cells exposed to Mn revealed increased cytokine production suggesting an inflammatory response in the lungs due to Mn toxicity (Pascal and Tessier, 2004). Pulmonary absorption of Mn does occur across the alveolar epithelium through non-selective Ca^{2+} channels (Heilig et al., 2006).

Neurotoxicity

Mn accumulation in the brain, due to overexposure, is centralized in the basal ganglia, an area critical to modulating fine-motor skills and a target for the pathology of many neurodegenerative diseases. Similar to Parkinson's disease, Mn neurotoxicity inflicts damage to the globus pallidus and substantia nigra leading to altered dopamine levels in the striatum (Cersosimo and Koller, 2006). The loss of striatal dopamine contributes to the symptomatic dysfunction shared by the two disorders. The majority of Mn research has focused on perturbations in the nigrostriatal dopaminergic pathway, however, it has recently come to light that preceding alterations in γ-aminobutyric acid (GABA) and norepinephrine may contribute to dysfunction (Anderson et al., 2008, 2009, respectively).

Within the brain, excess Mn is primarily sequestered in astrocytes (Aschner et al. 1999). Dysfunction is thought to occur when excess Mn burden on astrocytes and other glial cells disrupts their ability to modulate the neuronal environment. This leaves neurons vulnerable to excitotoxicity, reactive oxygen species (ROS), and other toxic byproducts generally processed by astrocytes. Astrocytes express a glutamate/aspartate transporter (GLAST), clearing extracellular glutamate after excitatory neurotransmission (Erikson et al., 2002b). An important function of astrocytes is to recycle glutamate to glutamine by a Mn-dependant astrocyte-exclusive enzyme glutamine synthetase (GS). Under normal conditions GS accounts for the majority of Mn in the brain Glutamate is converted to glutamine via GS in the presence of ammonia, released from the astrocyte, and taken up by neurons for synthesis of GABA or new glutamate by way of glutamic

acid dehydrogenase or glutaminase, respectively (Fitsanakis et al., 2006). This process not only promotes neurotransmitter recycling, but also clears ammonia from the brain by using it to form glutamine when condensed with glutamate. Mn can alter this process as evidenced by the use of nuclear magnetic resonance (NMR) spectroscopy in rats after 50 mg/kg $MnCl_2$ injections, which identified decreased glutamine in the globus pallidus and altered glutamate-glutamine-GABA metabolism (Zwingmann et al., 2007). Additionally, in primary astrocyte cell culture 300 μM $MnCl_2$ significantly decreased GLAST mRNA expression (Erikson et al., 2002b). The role that astrocytes play in supporting neurons is not exclusive to neurotransmitter metabolism. Astrocytes also provide neurons with cysteine and glycine precursors necessary for neuronal GSH synthesis (Dringen, 2000). These data show that astrocyte dysfunction impedes normal neuronal function; therefore, it is reasonable to consider astrocytes a target of Mn neurotoxicity.

Dysfunction resulting from a Mn toxic environment is ultimately due to Mn induced neuronal death. Whether or not neuronal death is instigated by Mn directly, or through Mn-induced neuronal/astrocyte dysfunction has yet to be determined. Additionally, the exact mechanisms responsible are by in large undefined. However, multiple hypotheses exist as to how Mn toxicity leads to neurodegeneration. Implications of Mn-induced glutamate excitotoxicity, the generation of ROS, and altered energy metabolism are commonly thought to be putative mechanisms.

Mechanisms of Toxicity

Excitotoxicity

Glutamate, the most abundant excitatory neurotransmitter in the brain can activate both ionotropic and metabotropic receptors (Meyer and Quenzer, 2005). Ionotropic receptors such as N-methyl-D-aspartic acid (NMDA) are fast acting ion channels allowing Na^+ and Ca^{2+} to enter a cell upon glutamate binding. Slower acting metabotropic receptors function through G-protein linked cell signalling cascades, with longer lasting effects. Excitotoxicity is the result of prolonged activation of both receptor species propagating cell death. Increased intracellular Ca^{2+}, due to excitotoxicity, can alter the permeability of the inner mitochondrial membrane diminishing respiration and leading to cell death (Hunter and Haworth, 1979; Zoratti and Szabo, 1995). Excess intracellular Ca^{2+} can also disrupt mitochondrial membrane potential initiating apoptosis (Lemasters et al., 1987). Furthermore, a study by Brecht et al. 2001 showed that excitotoxicity led to caspase-3 activation and DNA fragmentation in primary neuron-glial co-cultures isolated from the hippocampus of mice demonstrating the vulnerability of both neurons and glial cells to an excitotoxic assault.

Excess synaptic glutamate can result from disturbances in neurotransmitter levels causing disregulation of glutamatergic neurons, neuronal death releasing large concentrations of neurotransmitters into surrounding synapses, or an inability of astrocytes to clear synaptic glutamate. Mn neurotoxicity may play a role in each of these scenarios.

As previously mentioned, astrocytes remove glutamate from the synaptic cleft, partially due to GLAST function (Aschner 1999; Erikson et al., 2002b). However, a study by Hazell and Norenberg (1997) demonstrated a decrease in glutamate uptake by astrocytes exposed to 100 μM $MnCl_2$. It was unclear if this decrease was due to direct inhibition of GLAST or altered Na/K-ATPase function, responsible for establishing ion gradients necessary for proper GLAST and glutamate transporter (GLT-1) function (Rose et al., 2009). Concurrent experiments in primary astrocytes exposed to $MnCl_2$ and $MnSO_4$ revealed significant decreases in GLAST mRNA levels, corroborating previous findings (Erikson et al., 2002b). In GABAergic AF5 neuronal cells, low level Mn treatment increased intracellular and extracellular glutamate by 170% and 198%, respectively (Crooks et al., 2007). Microdialysis experiments show that Mn spiked artificial cerebral spinal fluid decreased synaptic GABA, glutamate, and aspartate levels (Takeda et al., 2002). Alternatively, Mn may lead to increased extracellular GABA in the striatum due to decreased GABA transporter (GAT-1) protein expression (Anderson et al., 2008). Likewise, rats injected with 200 μM Mn/kg had significantly increased striatal glutamate with amplified cell death (Xu et al., 2009). This finding supports evidence of Mn increased spontaneous glutamate-mediated excitatory postsynaptic potentials (EPSPs) in the cortical-striatal glutamate pathway (Centonze, 2001), and increased glutamate in the caudate putamen of iron deficient-manganese exposed rats (Erikson et al., 2002a). It has also been suggested that Mn may activate NMDA receptors leading to direct over excitation (Spadoni et al., 2000). Together, these studies establish that altered glutamate levels due to Mn toxicity are a putative cause of excitotoxicity and

neurodegeneration. Cell death associated with excitotoxicity may be due to caspase-3 activation and DNA damage (Brecht et al., 2001), or altered cellular respiration (Zoratti et al., 1995). Additionally, excitotoxicity via kainate administration increases reactive oxygen species (ROS) leading to increase hippocampal cell death (Liang et al., 2000), suggesting ROS play a role in excitotoxicity.

Reactive Oxygen Species

The brain is an organ highly susceptible to oxidative stress because of its high energy requirements. Since glucose is the primary fuel of the brain, the energy demand is met through glycolysis and oxidative phosphorylation. This results in the production of superoxide radicals, which are a natural byproduct of oxidative respiration. Under normal conditions superoxide radicals are converted to hydrogen peroxide (H_2O_2) and O_2 by the superoxide dismutase enzyme family (Cu/Zn-containing SOD in the cytosol, or Mn-containing superoxide dismutase (MnSOD) in the mitochondia), catalase further processes H_2O_2 into H_2O and O_2. However, in the presence of iron, H_2O_2 is converted to the highly reactive hydroxyl radical OH^-, by way of the Fenton reaction. This is important with respect to neurodegeneration, as Mn accumulates in Fe rich regions of the brain. To date it is unclear if Mn can initiate a Fenton like reaction directly; rather, evidence suggests that Mn may indirectly cause production of reactive oxygen species. This may be achieved by Mn altering enzymes involved in cellular oxidative defense (GSH, catalase, glutathione peroxidase), by decreasing the efficiency of the electron transport chain (ETC) leading to increased superoxide production, or by reacting with dopamine to produce cytotoxic dopamine quinones. Dopaminergic cell death is a

hallmark of Mn toxicity, and evidence suggests that Mn and dopamine have a synergistic effect on ROS generation leading to DNA damage and cell death (Oikawa et al., 2006; Prabhakaran et al., 2008).

Production of free radicals from mitochondrial energy metabolism is a normal function of living cells. Under physiological conditions, these ROS are quenched by antioxidant enzymes before deleterious processes can occur. Normal production of ROS has also been recognized to play a role in cell signaling cascades (Kamata and Hirata, 1999). However, the presence of excessive Mn augments ROS production (Ali et al., 1995), and has been shown to alter mitochondrial membrane potential (Zhang et al., 2004; Rama Roo and Norenberg, 2004). Using isolated mitochondria, Zhang et al. (2004) demonstrated that 50μM $MnCl_2$ was enough to significantly decrease the function of ETC complexes I, II, and III, while 500 μM and 1000 μM $MnCl_2$ significantly decreased all four ETC complexes. These effects were significantly attenuated by pretreatment with the antioxidant *N*-Acetylcysteine (NAC), suggesting ROS generation contributed to the damage. This confirms a previous finding by Bautista et al. (2000) sighting dysfunction in complex I of the ETC, beyond threshold level, of H_2O_2 induced oxidative stress, and suggests that Mn-induced ROS may contribute to mitochondrial dysfunction. Additionally, Mn^{3+} has been shown to be a more potent inhibitor of complex I, compared to the less reactive Mn^{2+}, in cultured PC12 cells (Chen et al., 2001). Interference with ETC enzymes leads to increased ROS, specifically superoxide, by blocking the normal passage of electrons down the chain to reduce molecular oxygen to water (Cassarino and Bennett, 1999). These studies demonstrate a mechanism by which

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Mn may lead to altered energy metabolism and mitochondrial dysfunction creating increased oxidative stress in the cell.

Excessive Mn-induced reactive oxygen species are known to alter several cellular processes. If oxidative damage is apparent in the mitochondria, Ca^{2+} homeostasis may be altered leading to apoptosis (Orrenius, 2007). A study using primary rat astrocytes by Zhaoobao et al. (2008) demonstrated Mn-induced apoptosis via a decrease in inner-mitochondrial membrane potential, phosphorylation of the ERK pathway, and activation of caspase-3, of which the authors attributed partial cause to ROS. ROS were also implied in ERK and c-Jun N-terminal kinase (JNK) activation of Bax and caspase-3 proteins, leading to apoptosis in ceramide treated primary mouse astrocytes (Oh et al., 2006). Again, these apoptotic pathways are triggered by excessive production of ROS, and may be blunted by proper function of antioxidant enzymes, such as GSH, catalase, and SOD.

Manganese role in glutathione biology

The tripeptide glutathione (GSH) is one of the most abundant endogenous antioxidants, comprising approximately 90% of non-protein thiols in the brain (Anderson and Meister, 1983). Glutathione has the ability to reduce free radicals, in the presence of glutathione peroxidase, forming oxidized glutathione (GSSG), which is subsequently reduced back to GSH via glutathione reductase. A strong link has been established between GSH and the pathology of neurodegenerative disorders (Schulz et al., 2000 for review). In PD patients, specifically, total GSH levels in the substantia nigra where 40% lower than controls, with a modest increase in oxidized GSSG (Sian et al., 1994).

Animal and cell studies have further characterized the role Mn plays in altering GSH levels and other markers of oxidative stress. Inhalation studies utilizing $MnSO_4$ and $MnPO_4$ unveiled age, sex, and species related differences on metallothionein (MT) and glutamine synthetase (GS), protein and mRNA levels (indirect markers of oxidative stress), as well as total GSH (Erikson et al., 2007; Taylor et al., 2006; Weber et al., 2002). Monkeys exposed to airborne Mn concentrations ranging from 0.06-1.5 mg Mn/m^3 exhibited a significant decrease in GS protein within the globus pallidus, showed decreased MT mRNA expression in the cortex of animals exposed to >0.3 mg Mn/m^3, and presented increased GSH in the cortex while decreases were seen in the caudate nucleus at 1.5 mg Mn/m^3 (Erikson et al., 2007). Results were similarly heterogeneous in another study by Erikson et al. (2004) using airborne Mn on 16 month old male rats and juvenile male and female rats. An increase in GS protein was observed in both young and old male rats within the hippocampus and thalamus, while the olfactory bulb was the lone region in female rats with increased GS protein. Decreases in GS and MT mRNA appeared to be age and sex dependent affecting the cerebellum, olfactory bulb, and hippocampus of young males, though decreased GS and MT mRNA were observed in the hypothalamus of young females and hippocampus of old male rats. Decreases in GSH were also seen in the striatum of the old male and young female rats, and in the olfactory bulb of young males. Interestingly young females exhibited an increase in GSH within the olfactory bulb. These results indicate that while there are alterations in the antioxidant defense system, inconsistencies suggest the role of oxidative stress in neurodegenerative pathologies may be lower that originally thought. A similar stance

was taken by Weber et al. (2002) when rat pups exposed to $MnCl_2$ showed significant increases in cortical GSH, but no changes were observed in GSSG, MT, or GS levels. Conversely, data produced by Ali et al. (1995) identified a dose-dependent increase of ROS in the caudate nucleus of Mn exposed rats, a region that significant decreases in GSH were reported by Desole et al. (1995), along with decreased GSH in isolated synaptosomes.

In vitro studies also tend to support the theory of Mn-induced oxidative stress. Significant decreases in GSH levels of RBE4 rat brain endothelial cells exposed to 200 μM Mn was associated with increased cell death (Marreilha dos Santos et al., 2008). Pretreatment with 500-1000 μM NAC spared RBE4 cell death, suggesting apoptosis was oxidative stress-induced. Furthermore, SK-N-SH neuroblastoma cells treated with L-buthionine sulfoximine (L-BSO), used to decrease cellular GSH, exhibited increased apoptosis when exposed to Mn or dopamine (DA) (Stokes et al., 2000). Stredrick et al. (2004) examined the effects of 60 μM Mn on dopamine producing CATH.a cells and non-producing SK-N-SH cells, associating increase toxicity with CATH.a cells even after decreasing dopamine production with 100 μM α-methyl-para-tyrosine (AMPT). Formation of ROS was thought to play a role in apoptosis as the addition of 5 mM GSH or 10 mM NAC significantly protected CATH.a cell death. Most recently, microglia are thought to contribute to Mn-induced free radical generation in dopaminergic neurons (Zhang et al., 2009). Neuron-glial co-culture (containing microglia) and neuron-enriched culture (no microglia) were treated with 10-300 μM $MnCl_2$. Neuronal death was significantly more apparent in the neuron-glial co-culture than the neuron-enriched.

Furthermore, when microglia were introduced to the neuron-enriched culture similar cell death was observed as the neuron-glial co-culture. Exposure to $MnCl_2$ caused a robust increase in ROS that preceded neuronal damage, and the addition of SOD, catalase, GSH, or NAC significantly improved viability of cells exposed to Mn. This finding provides evidence that intra- and extra-cellular Mn can induce ROS capable of damaging neurons. These data also suggest that astrocytes may play an important role in preventing neuronal damage by sequestering and concentrating extracellular Mn.

F_2-Isoprostanes

Overwhelming opinion suggests that oxidative stress plays a role in neurodegeneration. However, until recently the only method to quantify the amount of oxidative stress *in vivo* was to observe the response of oxidatively sensitive proteins such as GSH, GPx, catalase, glutamine synthetase (an astrocyte specific enzyme highly sensitive to oxidative stress), and metallothionein. No biological method had been identified to detect actual damage caused by oxidative injury until the development of F_2-Isoprostane (F_2-IsoPs) quantification. Isoprostanes (IsoPs) are a family of prostaglandin-like molecules distinct in the fact that they are formed by free radical peroxidation of arachidonic acid independent of cyclooxygenase (Milne et al. 2008). Formation of IsoPs occurs while esterfied to phospholipids, and are released by phospholipase activity (Roberts and Milne 2008). Because they are membrane bound when formed, it is thought that membrane fluidity and overall integrity may be altered as a result. The F_2 member of the IsoP family has become a reliable measure of oxidative injury, and F_2-IsoP production has been implied in neurodegenerative disorders (Montine et al. 2004). F_2-

Isops are stable molecules easily quantified; however, F_2-IsoPs readily convert to E_2 and D_2 IsoPs in the presence of depleted cellular reducing agents such as GSH (Montine et al. 2003). E_2/D_2 IsoPs can later dehydrate to form A_2 and J_2 IsoPs, which due to their electrophilic nature, conjugate with GSH via GSH s-transferase for removal from the body (Milne et al. 2008; Chen et al. 1999). Other than a marker for oxidative stess, F_2-IsoPs also have biological functions related to vasoconstriction. F_2 and E_2 IsoPs have been shown to act on thromboxane receptors and to induce endothelin release, leading to vasoconstriction in the brain (Morrow et al. 1992). It is likely that the biological effect of IsoP formation encompasses more that what is known to this point. However, given what has yet to be elucidated, IsoP quantification is still regarded as the gold standard of measuring *in vivo* oxidative damage.

Treatments for Manganese Neurotoxicity

To date very few treatment options have been proposed for Mn neurotoxicity. Other than immediate removal from the Mn toxic environment two clinical treatments have been documented to improve neurotoxic symptoms, $CaNa_2EDTA$ and para-Aminosalicylic Acid (PAS).

$CaNa_2EDTA$ is a synthetic compound used in detergents and food preservatives that is known to bind divalent and trivalent metal ions. A study by Hernandez et al. (2006) used $CaNa_2EDTA$ to treat seven welder/foundry workers presenting Mn induced Parkinson's symptoms. Five of the seven workers showed improvement in muscle rigidity and postural tremor. The use of PAS as treatment for Mn intoxication was investigated in a case study of a 50 year old woman who had been exposed to airborne

Mn for 21 years. All Mn-induced symptoms were significantly alleviated upon receiving PAS therapy, and the patient presented close to normal clinical, neurologic, MRI and handwriting scores in a follow up examination 17 yrs post treatment (Jiang et al. 2006).

Though CaNa$_2$EDTA and PAS treatment have shown positive results, preventing Mn accumulation through reduced exposure is the best option to decrease neurotoxic damage due to Mn. The use of antioxidant therapy has been suggested to do help protect against cellular damage associated with Mn neurotoxicity. Several compounds have been identified as potential neuroprotectants including; ascorbic acid, α-tocopherol, NAC, and a growing number of polyphenolic compounds (PC). These compounds help maintain cellular redox status protecting against Mn-induced free radical damage. One PC, in particular, that has shown promise as a neuroprotectant is (-)-epigallocatechin-3-Gallate (EGCG).

EGCG is thought to have strong antioxidant properties, stemming from multiple OH$^-$ groups projecting off of an electron stabilizing phenolic ring (Esposito et al., 2002). The antioxidant capability of catechins has been evidenced by EGCG attenuating the rise of SOD and catalase in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity (Levites et al., 2001). Not only did EGCG attenuate the rise of SOD and catalase seen in MPTP toxicity, but it also decreased dopaminergic neuron loss in the mouse substantia nigra suggesting that electron donating from EGCG helped mitigate damage normally managed by cellular enzymes (Levites et al., 2001). Additionally, EGCG has been shown to bind Fe^{2+}, significantly decreasing DNA damage caused by the Fenton reaction (Perron and Brumaghim, 2009). Microarray analysis of specific apoptotic genes

was conducted in SH-SY5Y neuronal cell exposed to 1μM EGCG. EGCG decreased expression of the pro-apoptotic genes bax, bad, caspase-1, caspase-6, p21, and gadd45 (Weinreb et al., 2003). Similarly, a study using mice fed 2 mg/kg EGCG showed significant decreases in Bax gene expression within the substantia nigra and hippocampus (Mandel et al., 2004). Together these data support the hypothesis of antioxidant therapy as protection against Mn neurotoxicity. However, questions remain regarding the bioavailability of EGCG in concentrations relevant to neuroprotection.

Conclusion

While there is a solid foundation of literature examining Mn neurotoxicity, little experimental data has been published on the use of *in vivo* antioxidant therapy to attenuate Mn-induced damage. The protective effect of antioxidant treatment *in vitro* has been well characterized in both neurons and glial cells exposed to Mn. However, it is still questionable whether the positive results seen *in vitro* can translate into a neuroprotective effect *in vivo*. Additionally, if antioxidant therapy is successful in attenuating Mn-induced damage, will this be reflected in the behavior of these rats (e.g., normalize locomotor and anxiety-like neurological behaviors associated with neurotoxicity). Mn has also been implied in the etiology of Parkinson's disease, and attaining this information may play a critical role in testing the efficacy of antioxidant therapy as a potential treatment for neurodegenerative diseases. To date it is unclear how much of a role Mn-induced oxidative stress plays in the neurochemical alterations present in Mn target regions. Furthermore, does this dysfunction manifest as stereotypic

behavior changes analogous to those observed in early stage manganism, or motor function abnormalities indicative of Parkinson's disease.

CHAPTER III

THE EFFECT OF MANGNESE EXPOSURE, WITH AND WITHOUT N-ACEYTL-CYSTEINE AND (-)-EPIGALLOCATECHIN-3-GALLATE THERAPIES, ON ANTIOXIDANT SYSTEMS IN THE RAT BRAIN

Abstract

Manganese (Mn) is an essential element implicated in the etiologies of metal induced neurological disorders. Over-exposure to Mn results in its accumulation in iron-rich brain regions leading to symptoms analogous to Parkinson's disease. Mechanisms governing this response are largely undefined though it is hypothesized that Mn-induced oxidative stress may play a role. Previous studies have examined indirect oxidative markers such as glutamine synthetase, metallothionein, and glutathione (GSH) with mixed results. In this study we examined weanling rats exposed to Mn (1 g/L d.i. water) for total GSH, glutathione peroxidase and catalase mRNA , and F_2-isoprostane (F_2-IsoP) production in the caudate putamen (CP) and hippocampus (HC), two regions previously identified to be affected by oxidative stress. Randomly selected rats also received i.p. N-acetyl-cysteine (NAC; 200 mg/kg) or (-)-epigallocatechin-3-gallate (EGCG; 5mg/kg) injections to observe any protective effect against Mn-induced oxidative stress. Mn exposure resulted in significant Mn accumulation versus control in each region (p <

0.05); however, this did not correspond to alterations in total GSH or production of F_2-IsoPs. EGCG was also associated with significant Mn accumulation in the CP ($p < 0.05$). Significant up-regulation of GPx gene expression ($p < 0.05$) observed in the HC correlated with increased Mn levels, and was augmented by EGCG. These data suggest a biological response in antioxidant gene expression to Mn exposure, even in the absence of identifiable oxidative stress. Additionally, antioxidant therapy may enhance this response.

Introduction

Manganese (Mn) is an essential dietary element involved in important biological processes related to energy metabolism, bone formation, blood clotting, cellular free radical defense, and neurotransmitter synthesis in the brain (Aschner et al., 2005). However, when exposed to excess amounts of environmental manganese neurological symptoms present in a condition coined "manganism." The symptomology of manganism is similar to that of Parkinson's disease in that each disrupts dopaminergic neurons in the basal ganglia leading to alterations in fine motor control, increased resting tremor, gait disturbances, and bradykinesia (Cersosimo and Koller, 2006). Clinically, manganism is distinct in the lack of response to L-dopa treatment and early onset of anxiety and schizophrenic-like behavior (Cersosimo and Koller, 2006). Cases of Mn toxicity have been reported in miners, welders, battery manufacturers, individuals exposed to contaminated drinking water, and in conditions altering hepatic function (Barceloux, 1999). Other populations at risk include neonates, patients receiving total parenteral nutrition, and those that are iron deficient (Aschner et al., 2005). Recently, approval of

the anti-knock gas additive methylcylopentadienyl manganese tricarbonyl (MMT) has raised concern of the possible effect chronic low dose Mn exposure will have on toxic accumulation.

Mn toxicity is characterized by the accumulation of Mn in iron (Fe) rich regions of the brain, including the striatum, globus pallidus (GP), and substantia nigra (SN) (Erikson et al., 2005a). Altered dopamine transporter density in the striatum has been shown to facilitate Mn accumulation (Erikson et al., 2005b). Additionally, damage to the GP and SN lead to perturbed dopaminergic communication between the SN and the striatum (Cersosimo and Koller, 2006). Recent studies have shown that disturbances in γ-aminobutyric acid (GABA) and norepinephrine may precede dopaminergic changes and contribute to this dysfunction (Anderson et al., 2008, 2009).

Oxidative stress associated with Mn accumulation potentially contributes to neurochemical alterations observed in manganism. Ali et al. (1995) reported a dose-dependent increase in reactive oxygen species (ROS) in the caudate putamen (CP) and hippocampus (HC) of Mn-exposed rats. Increased ROS generation has been linked to disturbances in mitochondrial membrane potential (Zhang et al., 2004) and inhibition of the electron transport chain (Bautista et al., 2000). This may further propagate ROS through generation of superoxide radicals (Cassarino and Bennett, 1999). Damage to mitochondria alters cellular Ca^{2+} homeostasis leading to apoptosis (Orrenius et al., 2007). Zhaoobao et al. (2008) demonstrated that ROS due to Mn compromised mitochondrial membrane potential leading to caspase-3 activation and subsequent apoptosis in primary

rat astrocytes. This evidence provides reason to suspect that oxidative damage may lead to dopaminergic neuron loss reported in this pathway (Jenner and Olanow, 1996).

Glutathione (GSH) is an endogenous tripeptide that, under normal conditions, functions to conjugate and reduce cellular radicals (Dringen et al., 2000). Aberrations in GSH due to Mn appear to be age and brain region specific. Desole et al. (1995) reported decreased GSH in the CP of Mn-exposed aged rats, while increases in cortical GSH where found in Mn-exposed rat pups (Weber et al., 2002). Inhalation studies in monkeys revealed increased GSH in the frontal cortex and decreased GSH in the caudate nucleus with 1.5 mg Mn/m^3 $MnSO_4$ exposure (Erikson et al., 2007). The reason behind region specific GSH alterations has yet to be determined, and the biological response of GSH to Mn needs to be further characterized to understand these inconsistencies. If indeed Mn-induced oxidative stress plays a role in this relationship, it would be prudent to examine additional endogenous antioxidants that may affect GSH levels and function such as glutathione peroxidase (GPx) and catalase.

GPx is an enzyme that aids GSH in the reduction of cellular peroxides by transferring an electron from GSH, leaving GSH in its oxidized form (GSSG). Catalase, a ubiquitous antioxidant enzyme, is responsible for converting H_2O_2 to H_2O and O_2. Altered function of GPx may decrease the ability of GSH to reduce free radicals, and decreases in catalase could increase the antioxidant burden of GSH. In theory, the use of neuroprotective antioxidant therapy could spare these systems in a pro-oxidant environment, and help illustrate if changes in GSH levels are due to Mn-induced oxidative stress.

N-acetyl-cysteine (NAC) and (-)-epigallocatechin-3-gallate (EGCG) are two antioxidants known to cross the blood brain barrier. NAC is a cysteine donor for GSH synthesis, and may directly scavenge free radicals with its thiol functional group (Zhang et al., 2008). Recently, EGCG has gained interest as a neuroprotective polyphenolic compound in the prevention of β-amyloid plaque formation in Alzheimer's disease (Rezai-Zadeh et al., 2008). EGCG may also act as an antioxidant using the electron donating potential of eight hydroxyl groups to reduce free radicals (Sajilata, et al., 2008). There is a growing body of research inspecting the effect of *in vitro* antioxidant treatments in the presence of Mn; however, little has been reported on the efficacy of *in vivo* antioxidant therapy in conjunction with Mn exposure.

The purposes of this study were to: 1) Examine the effect of Mn on GSH in two brain regions known to accumulate Mn (CP and HC); 2) Inspect the response of the antioxidant enzymes GPx and catalase to investigate their role in GSH functionality in an oxidative stress response; 3) Indirectly measure oxidative damage, in the form of the lipid peroxidation byproduct F_2-Isoprostanes (F2-IsoPs), to determine if Mn-induced oxidative stress plays a role in this process; 4) Establish whether *in vivo* antioxidant therapy can modulate these antioxidant systems in the presence of Mn-induced oxidative stress.

Materials and Methods

Animals

A total of 36 male 21-day-old Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly divided into two exposure groups: control (CN; AIN-93G diet, deionized water containing no Mn, n=18), or manganese exposed (Mn; AIN-

93G diet, deionized water containing 1g Mn/L in the form of $MnCl_2$, n=18). Rats had free access to AIN-93G diet (Dyets Inc, Bethlehem, PA), and water 24 h/day. Exposure groups where then assigned to receive one of three treatments via i.p. injection 3x per week: vehicle (Saline, n=6), N-acetyl-cysteine (NAC; 200mg/kg), or (-)-epigallocatechin-3-gallate (EGCG; 5mg/kg) (Sigma-Aldrich Inc., St. Louis, MO) to yield a total of six treatment groups (CN, Mn, NAC, NAC-Mn, EGCG, EGCG-Mn). Room temperature was maintained at 25 ± 1 °C, with dark cycle occurring between 1800 and 600 h. After six weeks of treatment, rats were sacrificed, brains removed with the caudate putamen (CP) and hippocampus (HC) sectioned out using mapped stereotaxic coordinates (Paxinos and Watson, 1998). Trunk blood and liver samples were also collected. Animal procedures were approved by The University of North Carolina at Greensboro Animal Care and Use Committee.

Metal Analysis

Mn and Fe levels were quantified in dissected brain regions using graphite furnace atomic absorption spectrophotometry (Varian AA240, Varian Inc., USA). Tissue was diluted 1:10 (w/v) in ultra pure nitric acid and placed in a 60°C sand bath overnight for digestion. 100 µl of diluted sample brought up to 1 ml with 2% nitric acid was used for analysis. A bovine liver internal standard (10 µg Mn/g; 184 µg Fe/g) (NBS Standard Reference Material, USDC, Washington DC, USA) was used for quality control. All metal results are expressed as µg/mg protein. Protein levels were quantified using Pierce BCA Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL).

Glutathione Analysis

Brain tissue was diluted 1:10 w/v in phosphate buffered saline (pH 7.4), homogenized via sonication, and centrifuged at 10,000 x g for 15 min. Supernatant collected was then deprotenated by adding an equal amount of metaphosphoric acid (5 g dissolved in 50 ml water) (Sigma-Aldrich Inc., St. Louis, MO), vortexed for 30 sec, and centrifuged at 3,000 x g for 2 min. 50 μl of 4 M triethalolamine (TEAM) (Sigma-Aldrich Inc., St. Louis, MO) was then added per ml of supernatant to increase sample pH. Total glutathione levels were measured immediately after the addition of TEAM reagent using Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI). Results are expressed as total glutathione equivalents per mg protein.

GPx and Catalase mRNA

At sacrifice tissue samples of each sectioned brain region were submerged in 1ml RNA*later*® solution (Ambion Inc., Austin, TX), and stored at -80°C. RNA was isolated using the ToTALLY RNA™ kit (Ambion Inc., Austin, TX) as per manufacturer's instructions. RNA yield and purity was assessed using the A260:A280 ratio produced by NanoDrop spectrophotometry (Thermo Scientific, Delaware, MD). To create cDNA a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA) was used following manufacturer's instructions. TaqMan® Gene Expression Assays were purchased to probe for GPx and catalase gene expression, in conjunction with Universal PCR Master Mix (Applied Biosystems, Foster City CA). GPx and CAT expression was normalized to β-actin expression for the same sample.

F$_2$-Isoprostane Quantification

Isoprostane analysis was conducted in the Dr. Michael Aschner lab at Vanderbilt University using a gas chromatography/mass spec. method designed at Vanderbilt to specifically detect F$_2$-Isoprostanes as outlined by Milatovic et al., 2007 and Morrow and Roberts, 1999.

Statistical Analysis

Data were analyzed with SPSS version 14 for windows (Chicago, IL). Outliers were removed using boxplot analysis, and resulting data were analyzed using two-way ANOVA with Mn levels and antioxidant treatment as between subjects' effects. Dunnett's procedure was applied when statistical between means was significant, $p<0.05$, to assess mean difference between treatment groups and control. Pearson's correlational analyses were used to examine the relationships between metal concentrations and GPx and catalase mRNA levels.

Results

Metal results

Mn concentrations in both the HC and CP were significantly higher ($p < 0.05$) in each Mn-exposed group compared to control (Fig. 3-1a, b). Additionally in the CP, EGCG treatment was independently associated with significant increases in both Mn ($p = 0.01$) (Fig. 3-1a) and Fe ($p = 0.006$) (Data not shown). In the HC, a similar trend was observed between EGCG and Mn though not significant ($p = 0.108$) (Fig. 3-1b).

Mn and Fe share a common transporter (DMT-1) at the blood brain barrier (Erikson et al., 2003). Due to this intricate relationship an Fe to Mn ratio (Fe:Mn) was

used to observe changes Mn-exposure had on Fe levels (Fig. 3-1c, d). All Mn-exposed groups in the CP accumulated significantly less Fe than Mn-unexposed groups ($p < 0.005$). The NAC treatment group exhibited the only significant ($p < 0.005$) Fe:Mn ratio in the HC with increased levels compared to control. This indicates an increase in Fe accumulation, in the HC, with NAC treatment under normal levels of Mn.
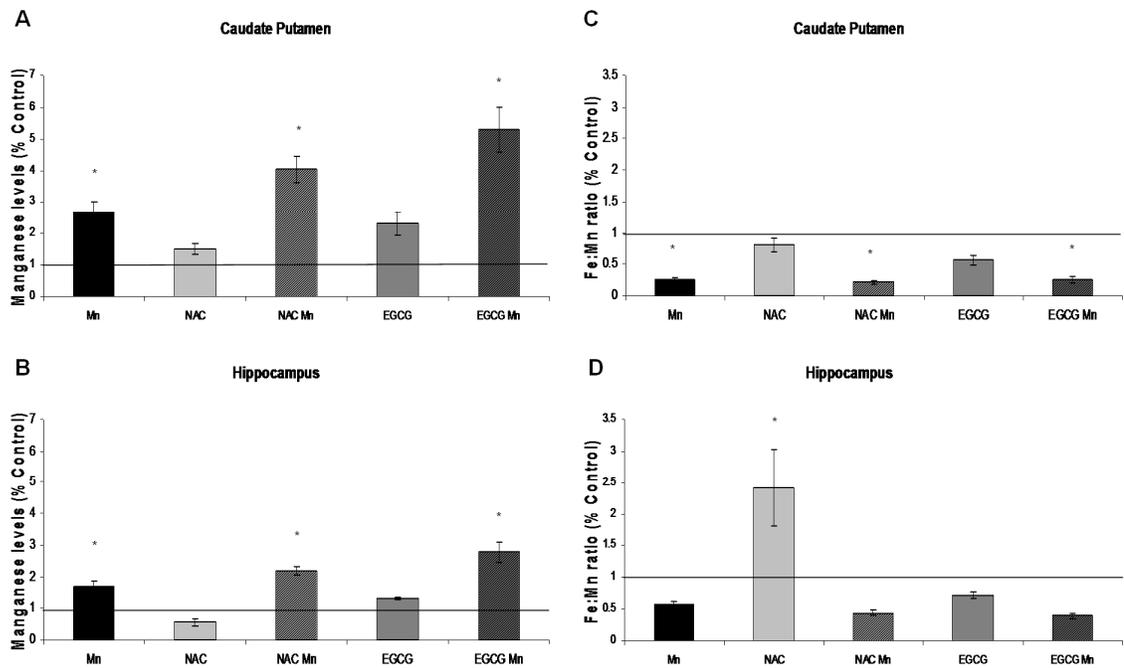
Figure 3-1. **Brain Metal Concentrations** – CP and HC metal values expressed as percent control ± SEM, with the straight line representing mean control values. Mn exposure significantly increased Mn accumulation in the caudate putamen (A) and the HC (B) versus control. C) Decreased Fe:Mn ratios in the caudate putamen indicate Mn-exposure significantly decreases Fe concentrations in these groups versus control. D) A significant increase in the Fe:Mn of the NAC treatment group was observed compared to control. *$p < 0.05$ according to Dunnett's post-hoc analysis.

Glutathione Results

The effect of Mn on GSH levels varied by brain region resulting in a 20% increase in the CP as opposed to a 20% decreased in the HC; however, neither was significant compared to control (Fig. 3-2a, b). Treatment with NAC was independently associated with significantly decreased GSH levels compared to no antioxidant treatment ($p = 0.010$) and EGCG treatment ($p = 0.018$) in the CP (Fig. 3-2c). A similar result was found in the HC, though NAC significantly decreased GSH only compared to the antioxidant free treatments ($p = 0.031$) (Fig. 3-2b).
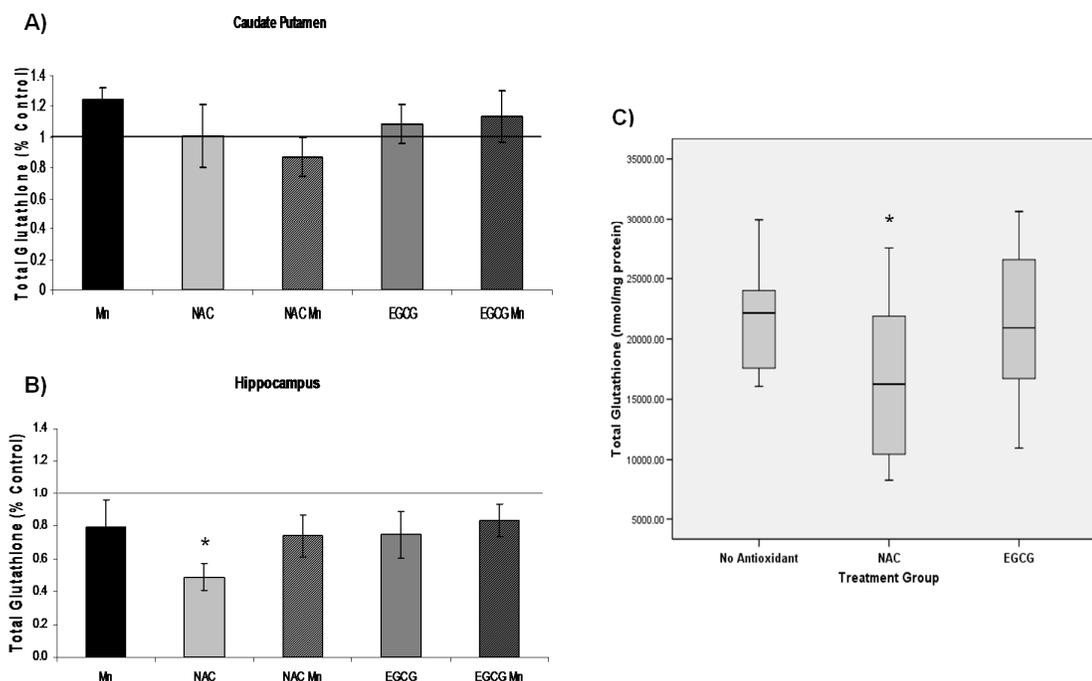
Figure 3-2. **Glutathione Levels** – Total glutathione levels in the caudate putamen and the hippocampus expressed as percent control ± SEM, with the straight line representing mean control values. A) No significant changes in glutathione levels were found in the caudate putamen. B) A significant decrease in glutathione was observed in the hippocampus with the NAC treatment group. C) NAC treatment independently decreased total glutathione levels compared to EGCG and no antioxidant treatment. *$p < 0.05$ according to Dunnett's post-hoc analysis.

GPx and Catalase mRNA Results

Gene expression of GPx and catalase were quantified in the CP and HC to assess the response of antioxidant enzymes in the presence of Mn. No significant changes were observed in catalase gene expression, in either the CP or HC, with most groups exhibiting slightly decreased values compared to control (Fig 3-3a, b). Mn exposure in conjunction with NAC treatment significantly increased GPx expression in the CP ($p = 0.001$). A

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similar increase was observed in the HC, though not significant. In the HC, EGCG treatment and Mn-exposure caused a near significant ($p = 0.059$) increase in GPx gene expression suggesting a biological response of GPx to Mn-exposure in the presence of antioxidants. A significant positive correlation ($p = 0.005$) was also found in the HC between Mn and GPx with increased Mn levels associated with increased GPx gene expression.
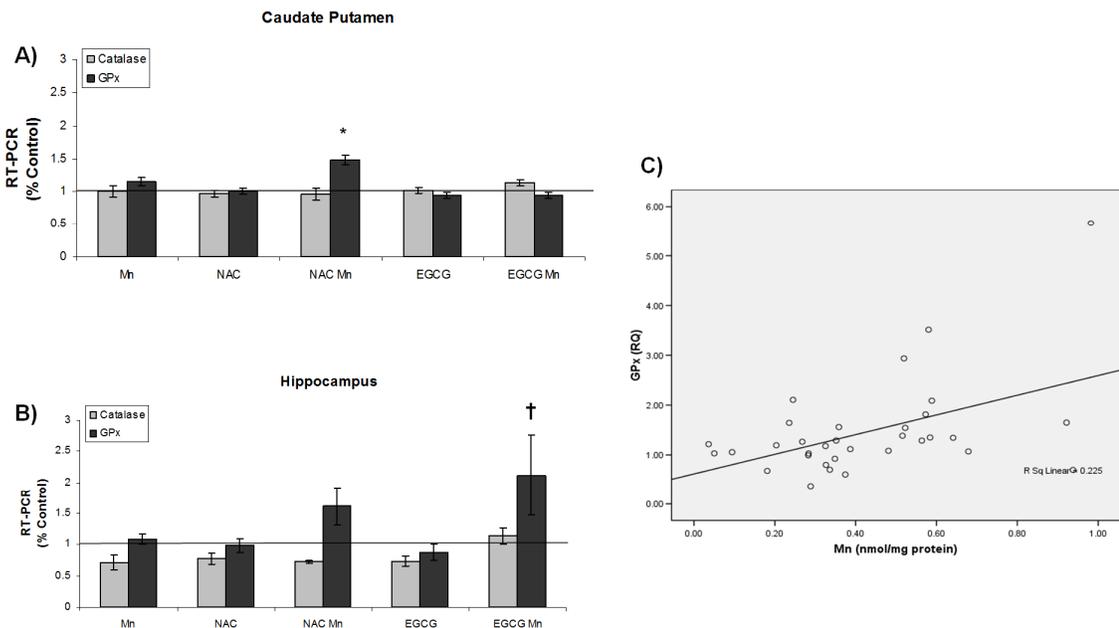
Figure 3-3. **GPx and Catalase Gene Expression** – Effect of Mn and antioxidant therapies on catalase and glutathione peroxidase (GPx) gene expression. A) In the caudate putamen up-regulated GPx gene expression was observed in the NAC-Mn treatment group. B) A near significant ($p = 0.059$) increase in GPx gene expression was observed in the hippocampus of Mn-exposed rats receiving EGCG therapy. C) A positive correlation between Mn accumulation and increased GPx gene expression was observed in the hippocampus of juvenile rats. *$p<0.05$; † - $p<0.1$ according to Dunnett's post-hoc analysis.

F₂-Isoprostane Results

No significant changes in F_2-IsoPs were apparent in the HC with or without Mn exposure and/or antioxidant treatment (data not shown). In the CP significant decreases in F_2-IsoPs were observed in each group compared to control (data not shown).

Discussion

These data suggest that Mn accumulation elicits a biological response in antioxidant enzymes, specifically GPx, which is amplified by antioxidant therapy. Mn accumulated significantly in the CP and HC of Mn-exposed rats (Fig. 3-1a, b). This was accompanied by a significant positive correlation between Mn and GPx gene expression (Fig. 3-3c). NAC and EGCG therapies amplified this relationship (Fig. 3-3) which appeared to be independent of changes in total GSH levels (Fig. 3-2). Slight alterations were observed in GSH levels, though none were significant in the presence of Mn (Fig 3-2a, b). However, NAC therapy significantly decreased GSH levels compared to EGCG and antioxidant free treatments (Fig. 3-2c). Interestingly, no evidence of increased oxidative damage was observed via F_2-IsoP analysis (data not shown). However, this finding may not be representative of the biological environment due to limited availability of tissue for F_2-IsoP analysis and possible auto-oxidation of the control samples which measured abnormally high (discussed below). Taking this into account, increased GPx gene expression suggests that Mn toxicity elicits an oxidative stress response, even in the absence of apparent oxidative stress. Furthermore, antioxidant therapy seemed to reinforce the antioxidant gene expression response.

Mn exposure via drinking water significantly increased Mn levels in both the CP and the HC and decreased the Fe:Mn ratio (a marker of divalent metal homeostasis) (Fig. 3-1c, d), consistent with findings previously reported in our lab (Anderson et al., 2008). However, the introduction of NAC and EGCG therapies produced a few novel findings with respect to brain metal homeostasis. EGCG therapy significantly increased Mn accumulation in the CP in both EGCG and EGCG-Mn treatment groups (Fig 3-1a). EGCG was also independently associated with increased Fe levels in the CP (data not shown). A 2007 study by Perron et al. identified the ability of EGCG to mitigate DNA damage by chelating Fe, thereby decreasing Fe-induced ROS. Perron demonstrated that EGCG forms a complex between its three polyphenolic gallate substituents and multiple Fe ions. Because Fe, Mn, and other divalent metals have comparable reactive properties, the possibility of Mn forming a similar complex with EGCG is likely. Additionally, the chelating properties of EGCG may decrease the oxidative threat of divalent metals in the brain. This raises an interesting question as to whether or not EGCG may chaperone Fe and/or Mn to, from, or within the brain. Evidence of EGCG in the brain after oral administration confirms permeability at the blood brain barrier (BBB), and it is suggested that methylation of EGCG in the liver may aid in this process (Youdim et al., 2004; Mohsen et al., 2002). Methylation may create a more hydrophobic compound allowing for passive transcellular diffusion at the BBB and choroid plexus (Youdim et al., 2004). To date no specific transporters have been identified, though there seems to be interaction between EGCG and P-glycoprotein efflux pumps. Jodoin et al. (2002) demonstrated that EGCG and other green tea polyphenols reversibly inhibit P-glycoprotein pumps allowing

for delivery of cancer drugs to the multi-drug resistant cell line CH^{R}C5. This introduces the possibility that EGCG may alter other transport proteins allowing for its passage after binding Fe, or potentially Mn.

Interestingly, the increased metal concentration in the CP did not lead to significant alterations in GSH, GPx, or catalase. Discrete changes in GSH were observed in the CP (Fig. 3-2a), with the Mn group exhibiting the largest change (a 20% increase in GSH levels compared to control). This finding, albeit not significant, is contradictory to reports of decreased GSH in the CP of aged rats (Desole et al., 1995), but complementary to previous findings by Erikson et al. (2004) and Dobson et al. (2003) in which no significant changes in striatal GSH levels in rats exposed to airborne Mn were shown. Alternatively, neonatal rats exposed orally to MnCl$_2$ exhibited increased cerebrocortical GSH levels while no changes in metallothionein (MT) or glutamine synthetase (GS) were observed (Weber et al., 2002). Brain regional GSH in the basal ganglia were not reported by Weber et al., and the authors felt it was unlikely oxidative stress played a role in GSH changes. MT and GS proteins, like GSH, are sensitive indirect markers of oxidative stress. MT is a metal binding protein with cysteine residues capable of neutralizing free radicals (Erikson et al., 2008), and GS is an astrocyte specific enzyme highly sensitive to oxidation (Stadtman and Berlett, 1997). Mn inhalation studies in primates use all three of these indexes in accessing oxidative stress. Two studies by Erikson et al. (2007; 2008) exposed monkeys to airborne Mn-sulfate, each reporting decreased GSH in the caudate nucleus along with decreased GS in the globus pallidus after 45 and 65 day exposure, respectively. Interestingly, GSH decreases in the caudate nucleus were accompanied by

increased GSH in the putamen (the caudate and putamen collectively form the striatum) after 65 day exposure suggesting that Mn exposure affects individual brain regions differently. These alterations persisted even after 90 day removal from airborne exposure. Inhalation studies in rats using Mn-sulfate revealed similar decreases in GSH within the CP (Taylor et al., 2006). Differences between exposure protocols, inhaled v. ingested, may influence the impact of Mn accumulation on GSH, and overall oxidative stress. Studies, such as ours, using oral Mn exposure via drinking water show little evidence of Mn-induced oxidative stress compared to inhalation studies. Furthermore, brain Mn levels in our study were 10-100 times lower than brain regional Mn reported by Dorman et al., 2006 in rhesus monkeys. However, we did observe similar percent control increases in Mn-exposed groups. Decreased Mn levels (µg Mn/g tissue) in oral exposure could stem from intestinal regulation of metal absorption by DMT-1. Airborne Mn is not subject to this homeostatic regulation in either the lungs or by transport down the olfactory nerve, resulting in greater brain Mn accumulation and potentially increased oxidative damage. This suggests that oral Mn exposure, while still accumulating significantly compared to control, may not reach levels high enough to induce notable oxidative stress.

Antioxidant therapy had little impact on GSH levels. In the HC, decreased GSH found in the NAC group was the only significant change we observed (Fig. 3-2b), and this effect was abolished by Mn. One would expect an increase in GSH levels due to NAC, an active cysteine donor and GSH precursor, though this was not the case. In fact NAC treatment significantly reduced GSH levels compared to EGCG and antioxidant

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free groups (Fig. 3-2c). The reduction in GSH that we observed could be due to NAC directly scavenging free radicals, in effect sparing GSH thereby decreasing cellular production. This finding opposes the growing body of *in vitro* research illustrating increased GSH levels in response to NAC treatment under Mn exposure: NAC (500-1000 µM) increased GSH and spared Mn-exposed RBE4 cell death (Marreilha do Santos et al., 2008); CATH.a cells treated with 10 mM NAC and 5 mM GSH protected against Mn-induced cell death (Stredrick et al., 2004); NAC treatment improved viability of Mn-exposed neuron-glial co-cultures (Zhang et al., 2009). A critical difference between these *in vitro* studies and our study is that we were not able to demonstrate a depletion of GSH definitively due to oxidative stress. The biological response of NAC *in vivo* may differ from that of cell studies, and the protective effect of NAC could be a graded response to declining GSH levels. It is also important to note that necropsy analysis of animals receiving i.p. NAC injections revealed significant inflammation of the liver and intestinal tract accompanied by a substantial decrease in gut motility. To our knowledge this is the first time NAC has been administered via i.p. injection for a chronic treatment protocol (3x per week for 6 weeks). Oral, topical, and intravenous administration are commonly used to deliver NAC in colon cancer, HIV, and cardio pulmonary studies (Atkuri et al., 2007).

We observed that Mn had a significant effect on GPx gene expression (Fig. 3-3c), and that EGCG and NAC amplified this response (Fig. 3-3a, b). These results did not appear to be driven by oxidative stress, as there was no significant increase in F_2-IsoP levels observed (data not shown). Isoprostane analysis has emerged as a reliable

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indicator of lipid peroxidation (Montine et al. 2004). However, proper tissue preservation is critical to accurately quantify F_2-IsoPs. F_2-IsoPs readily convert to E_2 and D_2 IsoPs in the presence of depleted cellular reducing agents such as GSH, and propagate in a pro-oxidant environment (Montine et al. 2003). The control tissue we analyzed for F_2-IsoPs measured abnormally high skewing comparisons between groups. It is possible that the brain regions in these animals were not dissected or flash frozen quickly enough to prevent auto-oxidation of the tissue. Because we were unable to quantify F2-IsoPs in our control samples we can not definitively say that no oxidative damage was present due to Mn exposure compared to control. Additionally, increased GPx gene expression did not correspond with alterations in GSH. Together these findings suggest that Mn-induced oxidative stress appears to play a minimal role in up-regulated antioxidant enzymes, but the presence of Mn did lead to increased GPx, perhaps by an alternate mechanism. Catalase levels, alternatively, remained stable across all treatment groups in the CP, decreasing slightly in the HC of all treatment groups compared to controls (Fig 3b). Neither antioxidant treatment nor Mn-exposure altered mRNA levels. This finding supports our thought that the free radicals generated, if any, were not significant enough to tax cellular antioxidant defense.

Data obtained in this experiment align with previous findings that Mn exposure leads to significant Mn accumulation in the CP and HC of rats. Additionally, our results reveal a positive correlation between Mn accumulation and increased GPx gene expression. Antioxidant therapy with NAC and EGCG amplified this response implying a potential neuroprotective effect. Oxidative stress was not evident in these brain regions, and only

modest alterations were observed in total GSH levels. This could suggest that oral Mn exposure, though still neurotoxic, may not be as deleterious as inhalation exposure. Increased Mn due to oral exposure may elicit subtle neurochemical alterations leading to altered behavior and cognitive function, whereas airborne exposure leads to more robust Mn accumulation, oxidative stress, and potentially a neurodegenerative state. However, further work needs to examine the effect of oral Mn exposure in other regions of the basal ganglia, such as the globus pallidus and the substantia nigra. These regions, along with the cortex, are integral to the neural circuitry associated with processing and executing movement patterns and behaviors commonly altered by Mn neurotoxicity.

CHAPTER IV

THE EFFECT OF WATER-BORNE MN EXPOSURE AND SUBSEQUENT ANTIOXIDANT TREATMENT ON STEREOTYPIC BEHAVIORS IN RATS

Abstract

Manganese (Mn) is an essential element critical to normal neurological function. It has been well documented that excessive exposure to Mn can result in neurotoxic brain accumulation leading to symptoms resembling neurodegenerative disorders, such as Parkinson's disease. Behavioral alterations are known to occur in these disorders, but few studies have examined behavior abnormalities associated with neurotoxic Mn exposure. In this study we examined the effect of water-borne Mn exposure on stereotypic behaviors in rats. Additionally, oxidative stress has been implied in the etiology of Mn neurotoxicity, because of this we applied *N*-acetyl-cysteine (NAC) and (-)-epigallocatechin-3-gallate (EGCG) antioxidant therapies to observe if protection from oxidative stress would correct any behavior abnormalities. Twenty-one day old Sprague-Dawley rats were exposed to Mn via drinking water (1 g Mn/L) and either 200 mg/kg NAC or 5 mg/kg EGCG thee times a week for six weeks. Rat behaviors were monitored for a 24 hour period during the sixth week with Clever Systems Home Cage Scan video surveillance. Locomotor behavior was assessed by calculating total distance traveled,

and time spent sleeping, sniffing, and grooming were monitored to reflect anxiety-like changes in behavior. Mn significantly accumulated in the caudate putamen and hippocampus of exposed rats, and was positively correlated with total distance traveled ($p < 0.05$). Mn also significantly decreased sniff behavior, but little effect was observed in sleeping and grooming behaviors. Antioxidant therapy had no effect on anxiety behaviors in Mn-exposed groups, but was associated with increase locomotor activity. These data indicate that oral Mn exposure is associated with locomotor abnormalities, but minimally alter anxiety-like behavior. Additionally, NAC and EGCG antioxidant therapy plays a minimal role in preserving behavior changes in the presence of Mn.

Introduction

Dietary metals play an essential role in many biological processes ranging from energy metabolism and bone health to neurological function (Aschner et al., 2005). Manganese (Mn), specifically, functions as a cofactor for glycolytic enzymes, aids in blood clotting, and is required for ammonia clearance from the brain (Aschner et al., 2005; Takeda, 2003). Perturbed metal homeostasis can have detrimental effects on these systems, and has been reported in occupational settings related to mining, welding, and battery manufacturing (Dobson et al., 2004). In these settings over exposure to Mn usually occurs due to increased airborne Mn particulate, giving rise to a neurotoxic condition known as "manganism."

A hallmark of manganism is increased accumulation of Mn in iron (Fe) rich areas of the brain. Studies in these populations using magnetic resonance imaging (MRI) repeatedly show increased Mn accumulation in the globus pallidus compared to normal

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individuals (Kim et al., 2007; Park et al., 2007; Kim, 2004). Manganism is also accompanied by neurological symptoms related to locomotion and alter behavior (Dobson et al., 2004). General symptoms include fatigue, loss of appetite, insomnia, and headache (Pal et al., 1999), with progression to violent behavior, emotional distress, and hallucinations (Aschner et al., 2005). Early onset of the disorder is associated with anxiety and schizophrenic like behavior (Pal et al., 1999). It is believed that alterations in neurotransmitter systems, in particular, dopamine (DA) drive these symptoms (McDougall et al., 2008).

Mn neurotoxicity is also similar to Parkinson's disease with respect to damage within the globus pallidus and substantia nigra leading to altered dopamine levels in the striatum (Cersosimo and Koller, 2006). The loss of striatal dopamine contributes to the symptomatic dysfunction shared by the two disorders. The majority of Mn research has focused on perturbations in the nigrostriatal dopaminergic pathway, however, it has recently come to light that preceding alterations in γ-aminobutyric acid (GABA) and norepinephrine (NE) may contribute to dysfunction (Anderson et al., 2008, 2009).

Relationships between behavior, locomotion, and Mn exposure have been further studied in rodent models with varying results. A study conducted by Calabresi et al. (2001) reported that rats exposed to Mn exhibited significantly increased activity in open field tests with little habituation. This contradicts a previous finding by Talavera et al. (1999) demonstrating decreased activity in Mn-exposed mice. Additionally, no significant activity changes were observed in neonatal rats receiving oral Mn, though increased startle response was noted (Dorman et al., 2000). Evidence from other rodent

studies supports the involvement of DA in locomotor behavior. When the DA transporter (DAT) gene expression was disrupted in mice resulted in hyper-locomotion (Giros et al., 1996). This was supported in DAT knockout mice that had high extracellular dopamine and increased locomotion (Spieleway et al., 2000). In rat models of hyperactivity, intracisternal injections of 6-hydroxydopamine increased gene expression of striatal N-methyl-D-aspartic acid (NMDA) receptors and decreased GABA transporter expression suggesting a role of GABA and glutamate in increased locomotor activity (Masuo et al., 2004).

Though Mn is the principle metal of interest in locomotor abnormalities associated with Manganism, the relationship between Mn and Fe homeostasis should be considered as well. An intricate relationship exists between Mn and Fe as the two share common absorption and transport mechanisms within the body, primarily via the divalent metal transporter DMT-1 (Aschner, 2006; Erikson and Aschner, 2006). In fact, as Fe levels decline, increased Mn is absorbed in the intestines followed by augmented transport of Mn into the brain (Erikson et al., 2002a). Decreased Fe levels have also been associated with decreased activity and increased anxiety-like behaviors in rats (Beard et al., 2002). This suggests that neither should be exclusively examined in behavioral studies.

Previous data collected in our lab uncovered some interesting associations between dietary Fe deficiency, Mn exposure, and behavior alterations in the home cage environments of rats. The purpose of this study was to further characterize the relationship between Mn and Fe homeostasis with behavior by using 24-hour video

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surveillance, and to assess any effect of antioxidant treatment on the behavior of Mn-exposed rats.

Materials and Methods

Animals

A total of 36 male 21-day-old Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly divided into two exposure groups: control (CN; AIN-93G diet, deionized water containing no Mn), or manganese exposed (Mn; AIN-93G diet, deionized water containing 1g Mn/L in the form of $MnCl_2$). Rats had free access to AIN-93G diet (Dyets Inc, Bethlehem, PA) and water 24 h/day. Exposure groups where then randomly assigned to receive one of three treatments via i.p. injection: vehicle (Saline, n=6), *N*-acetyl-cysteine (NAC; 200mg/kg), or (-)-Epigallocatechin-3-gallate (EGCG; 5mg/kg) (Sigma-Aldrich Inc., St. Louis, MO) to yield six groups CN, Mn, NAC, NAC-Mn, EGCG, and EGCG-Mn. Room temperature was maintained at $25 \pm 1°C$, with dark cycle occurring between 1800 and 600 h. Animal procedures were approved by The University of North Carolina at Greensboro Animal Care and Use Committee.

Metal Analysis

After week six rats were sacrificed, brains removed, and brain regions of interest sectioned out (caudate putamen (CP) and hippocampus (HC)) using mapped stereotaxic coordinates (Paxinos and Watson, 1998). Mn and Fe levels were quantified in dissected brain regions using graphite furnace atomic absorption spectrophotometry (Varian AA240, Varian Inc., USA). Tissue was diluted 1:10 weight to volume (w/v) in ultra pure nitric acid and placed in a 60°C sand bath overnight for digestion. 100 μl diluted sample

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brought up to 1 ml with 2% nitric acid was used for analysis. A bovine liver internal standard (10μg Mn/g; 184μg Fe/g) (NBS Standard Reference Material, USDC, Washington DC, USA) was used for quality control. All metal results are expressed as μg/mg protein. Protein levels were quantified using Pierce BCA Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL).

Behavior Analysis

Behavior analysis was collected during weeks four, five, and six of dietary and antioxidant protocol using Clever Systems Home Cage Scan (HCS) video equipment and software (Reston, VA). This software was designed to automatically score behavior by analyzing sequential frame by frame animal postures in space. Animal postures are determined 30 frames per second by referencing anatomical features in relationship to one another against other customized cage landmarks. Accuracy of HCS scoring has been verified by manual assessment to be ≥ 90% (Steele et al., 2007). Rats are placed in shoebox cages including food, water, and minimal bedding and individually recorded for a continuous 24hr period. During the dark cycle cages were backlit with red light to allow for video capture. Behaviors monitored include: drinking, eating, sleeping, grooming, and sniffing. Total distance traveled was also calculated to assess activity. Only week six data is reported as no significant differences emerged between groups in weeks four and five.

Statistical Analysis

Data were analyzed using SPSS version 14 for windows. Outliers were removed using boxplot analysis, and resulting data were analyzed using analysis of variance.

When statistical analyses yield significance, p<0.05, Dunnett's procedure was used to analysis mean difference between treatment groups and control. Pearson's correlational analysis was used to report associations between metal levels and reported behaviors. Results were considered significant with $p < 0.05$.

Results

Metal Results

Mn concentrations in both the HC and CP were significantly higher ($p < 0.05$) in each Mn-exposed group compared to control (Fig. 4-1a, b). Additionally in the CP, EGCG treatment was independently associated with significant increases in both Mn ($p = 0.01$) and Fe ($p = 0.006$) (Data not shown). In the HC, a similar trend was observed between EGCG and Mn though not significant ($p = 0.108$) (Fig. 4-1b).

Mn and Fe share a common transporter (DMT-1) at the blood brain barrier (Erikson et al., 2003). Due to this intricate relationship an Fe to Mn ratio (Fe:Mn) was used to observe changes Mn-exposure had on Fe levels (Fig. 4-1c, d). All Mn-exposed groups in the CP accumulated significantly less Fe than Mn-free groups ($p < 0.005$). The NAC treatment group exhibited the only significant ($p < 0.005$) Fe:Mn ratio in the HC, with increased levels compared to control. This indicates an increase in Fe accumulation, in the HC, with NAC treatment under normal levels of Mn.
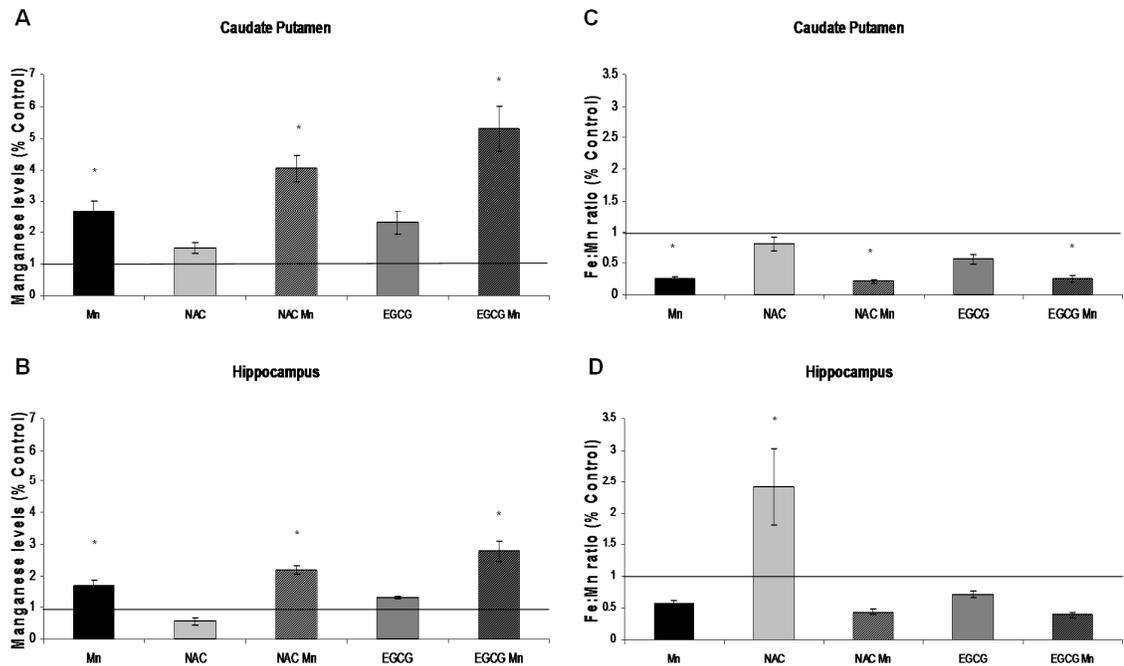
Figure 4-1. **Brain Metal Concentrations** – CP and HC metal values expressed as percent control ± SEM, with the straight line representing mean control values. Mn exposure significantly increased Mn accumulation in the caudate putamen (A) and the HC (B) versus control. C) Decreased Fe:Mn ratios in the caudate putamen indicate Mn-exposure significantly decreases Fe concentrations in these groups versus control. D) A significant increase in the Fe:Mn of the NAC treatment group was observed compared to control. *$p < 0.05$ according to Dunnett's post-hoc analysis.

Distance Traveled

Distance traveled was significantly increased in the Mn ($p = 0.02$) and EGCG-Mn ($p = 0.029$) groups compared to control (Fig. 4-2a), with a trend toward significance observed in the NAC-Mn group ($p = 0.071$). Increased CP and HC Mn levels were significantly correlated with this result ($p = 0.017$ and 0.001, respectively) (Fig. 4-2c, d), as well as decreased Fe:Mn ratios in the CP ($p = 0.008$) and HC ($p = 0.001$) (Fig. 4-2e, f).
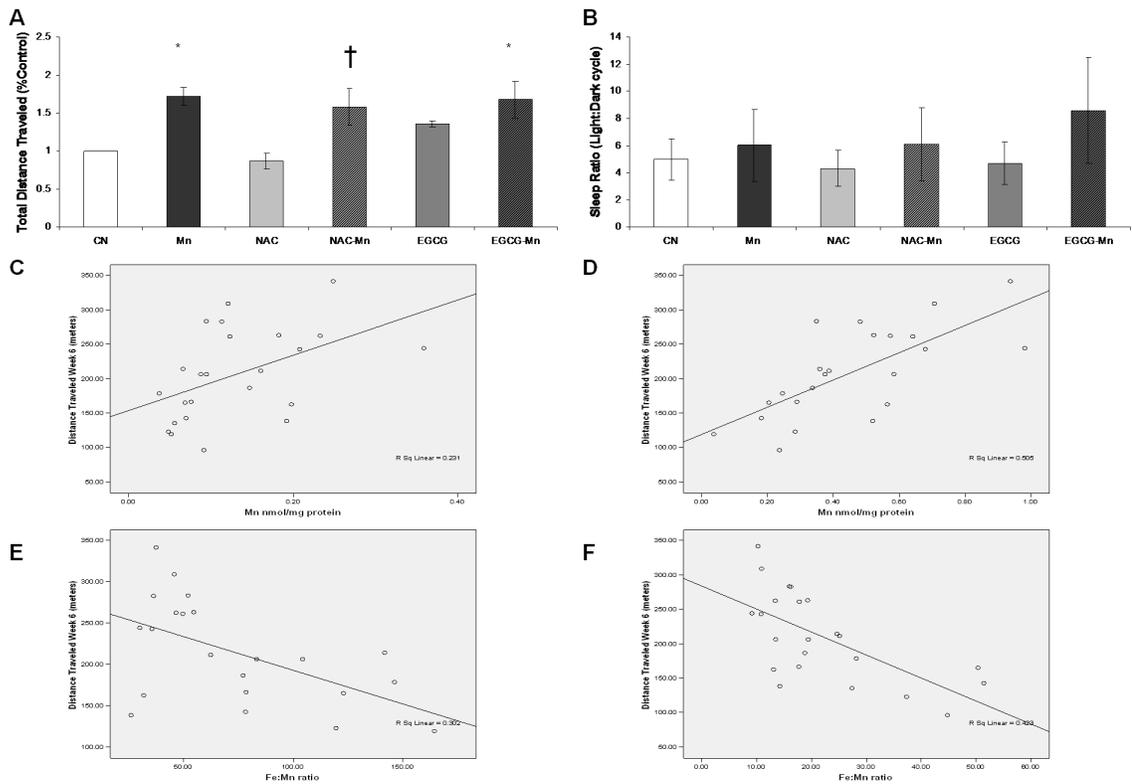
Figure 4-2. **Distance Traveled and Sleep Patterns** A) Total distance traveled during week six, expressed as % Control. Mn exposure significantly increased total distance traveled during week six. B) Sleeping patterns expressed as a Light:Dark ratio (time spent sleeping during the light cycle divided by time sleeping during the dark cycle). Mn-exposed rats slept less during the dark cycle, which corresponds with increased total distance traveled. A positive correlation was found between Mn levels in the CP (C) and the HC (D) and total distance traveled ($p = 0.017$ and 0.001, respectively). A negative correlation was observed between Fe:Mn ratio in the CP (E) and the HC (F) and total distance traveled ($p = 0.008$ and 0.001, respectively). *$p < 0.05$ †$p < 0.1$ according to Dunnett's post-hoc analysis. Pearson's analysis used to establish correlational significance.

Sleeping

No significant differences were observed between groups after analyzing sleep behaviors (Fig. 4-2b). Mn exposure did not appear to alter circadian rhythm, evidenced

by slight increases in Light:Dark ratio (Fig. 4-2b). This increase indicates the majority of sleep occurred in the light cycle, consistent with normal behavior.

Grooming

Mn exposure caused an increase in grooming activity during the dark cycle (Fig. 4-3a). A trend toward significance was observed in the dark to light cycle groom ratio with CP Fe:Mn ratio (p = 0.106) (Fig. 4-3a inset). However, the EGCG-Mn group did not display as marked an increase over EGCG as Mn v. CN or NAC-Mn v. NAC.

Sniffing

A significant decrease in sniffing behavior between the Mn and CN group was observed (p = 0.05) (Fig. 4-3b). A similar, near significant (p = 0.068), decrease was seen in the EGCG-Mn group versus CN (Fig. 4-3b). Overall, decreased sniffing behavior correlated with increased HC Mn levels (p = 0.044) (Fig. 4-3b inset).
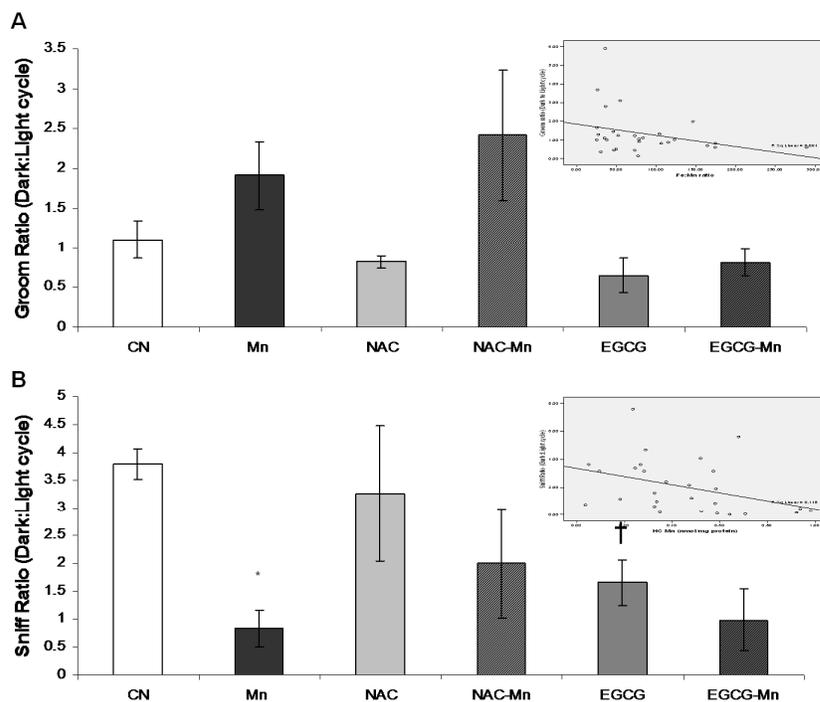
Figure 4-3. **Groom and Sniff Behaviors** A) Time spent grooming expressed in a Dark:Light ratio (time spent grooming during the dark cycle divided by time in the light). Increased grooming behavior was observed in Mn and NAC-Mn groups, which correlated with decreased Fe:Mn ratios in the CP (figure inset) ($p = 0.106$). B) Sniff behavior expressed in a Dark:Light ratio. The Mn groups engaged in significantly less sniffing behavior in the dark, than light, compared to CN ($p = 0.05$). Similarly, a trend toward significance was observed in decreased sniffing behavior of EGCG-Mn rats compared to CN ($p = 0.065$). Overall, decreased Dark:Light sniff ratios were correlated with increased Mn levels in the HC ($p = 0.044$). *$p < 0.05$ [†]$p < 0.1$ according to Dunnett's post-hoc analysis. Pearson's analysis used to establish correlational significance.

Discussion

Results obtained from this study indicate that Mn exposure does correlate strongly with altered locomotor activity. We did not measure neurotransmitter levels directly in this experiment, but neurochemical alterations in rodents are generally reflected by changes in locomotor activity (Flagel and Robinson, 2007). Additionally, Mn exposure

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alone was sufficient to produce Mn accumulation in the CP and HC, as well as perturb Fe levels to an iron deficient (ID) like state in the brain. Both ID and Mn toxicity are known to affect neurochemistry (Beard et al., 2002; Anderson et al., 2008; 2009), and may drive changes observed in stereotypic behaviors. Our results also indicate that NAC and EGCG antioxidant therapy plays a minimal role in preserving behavior changes in the presence of Mn.

Delivery of Mn via water consumption was successful in producing Mn accumulation in the CP and the HC of rats exposed. This result corroborated previous findings in our lab (Anderson et al., 2008; 2009). However, the preceding behavior trial utilized manipulations in Mn as well as dietary ID. This treatment protocol only yielded significant ID in the CP and globus pallidus (GP), with significant Mn accumulation limited to the CP and HC of rats exposed to both Mn and dietary ID. The inability to produce a robust brain metal response with the treatment protocol may have muted the behavioral response to altered metal homeostasis. Taking this into account, our current study eliminated the ID aspect of the treatment protocol. Mn exposure alone was sufficient to induce brain Mn accumulation and decrease brain Fe levels by presumably overwhelming the shared transport mechanism, DMT-1. Indeed, by solely manipulating Mn we observed a significant increase in Mn accumulation along with significantly decreased Fe:Mn ratios. Due to this protocol adjustment we were unable to examine the behavioral effects of frank ID, which is associated with neurochemical changes of its own, as well as propagating Mn accumulation. However, this allows us to observe the

behavioral changes associated with Mn-driven alteration in metal homeostasis, perhaps more analogous to those experienced in manganism.

Locomotor abnormalities have been associated with manganism (Dobson et al., 2004). When assessing locomotor activities in rats, total distance traveled is widely accepted to reflect neurochemical alterations (Flagel and Bobinson, 2007). We observed an increase in total distance traveled due to Mn exposure and a decreased Fe:Mn ratio. Both Mn and Fe are known to alter locomotor activity, and it is thought that changes in DA levels drive these alterations (Beard et al., 2002; Normandin et al., 2004; Le Moal, 1995). There are, however, different reports on locomotor behaviors associated with ID versus Mn toxicity. Beard et al. (2002) reported decreased overall locomotor activity in ID rats associated with decreased DA receptor and transporter densities. While Mn studies have revealed both increased and decreased activity levels depending on route and duration of Mn exposure. Specifically, thirteen week exposure to 30, 300, or 3000 μg/m^3 airborne Mn phosphate resulted in no changes in locomotor activity in rats (Normandin et al., 2002). A later study by the same group revealed that utilizing the same dosing protocol with a Mn sulfate/Mn phosphate mix cause a significant decrease in spontaneous motor activity (Normandin et al., 2004), suggesting that the species of Mn delivery may play a role in its neurotoxic effects. This differential effect corroborated an earlier toxicokinetic study examining the effect of Mn particle size and relative solubility on toxic outcomes (Barceloux, 1999 for review). Using the identical Mn sulfate/Mn phosphate inhalation model, Tapin et al. (2006) reported a significant increase in total distance traveled, contradictory to previous findings. Neuropathological analysis of the

GP and CP, conducted by Tapin, revealed a significant decrease of pallidial neurons in Mn exposed groups compared to control. This could be a potential upstream trigger to increased DA release in the nigrostriatal pathway (which has been associated with increased locomotion; Le Moal, 1995), by decreasing GABAergic firing from the GP onto the subthalamic nuclei (STN), which in turn would cause dysinhibition of glutamate firing from the STN into the substantia nigra (SN) leading to increased DAergic neurotransmission into the CP. This would be consistent with findings in $GABA_B^{-/-}$ receptor null mice that reported hyperlocomotion upon introduction to a novel environment (Vacher et al., 2006), and reported decreased $GABA_B$ mRNA levels in the SN due to Fe:Mn alterations (Anderson et al., 2008). This hypothesis hinges on the loss of neurons due to Mn exposure, and suggests coordination of multiple neurotransmitter systems demonstrating the complexity of a neurobehavioral response.

Similar variations in locomotor behaviors are found in studies using oral Mn exposure. We observed an increase in total distance traveled with 1g Mn/L via $MnCl_2$ in the drinking water after 6 weeks of exposure. Calabresi et al. (2001) reported a significant increase in activity during open field tests in rats ingesting 20 mg Mn/ml water for 10 weeks. Dorman et al. (2000) observed no change in motor activity neonatal rats exposed to 0, 25, or 50 mg/kg/day via $MnCl_2$ drinking water, with increase striatal DA levels. And significant decreases in total distance traveled were reported after 19 week oral exposure to 550 mg Mn/kg/day (Torrente et al., 2005). It is clear that variations in dose and duration of oral Mn exposure have an effect on observed locomotion, with longer durations at higher doses resulting in decrease motor activity.

Even thought brain accumulation of Mn is similar in these cases, chronic exposure may lead to increase neuronal damage eventually decreasing neurotransmitter output, specifically DA. Acute Mn exposure is known to alter neurotransmitter transporter and receptor gene expression resulting in increased synaptic neurotransmitter concentrations (Anderson et al., 2008; 2009). These alterations may induce locomotor abnormalities (increased activity) over short durations (6-10 weeks). Decreased activity observed with chronic exposure (11+ weeks) may be indicative of further neuronal dysfunction in an advanced diseased state. Similarly, age may play a role in the behavioral response to Mn accumulation. Increases in striatal DA is normally associated with increased locomotion (Le Moal, 1995); however, this finding in neonatal rats versus weanling rats may be the result of more severe developmental problems resulting in a general malaise.

Other behaviors examined in the current study (sleeping, sniffing and grooming) seemed to be less sensitive to acute Mn exposure. Mn-exposed rats spent comparatively more time sleeping during the light cycle than dark. This is contradictory to data previously collected in our lab suggesting that decreased Fe:Mn ratios lead to a partial reversal in circadian rhythm. ID has been shown to alter circadian rhythm (Youdim et al., 1980), as have disturbances in GABA biology (Crosio et al., 2000; Vacher et al., 2006). Decreases in Fe:Mn ratio in the current study may not elicit the same neurochemical response as frank ID which may account for differences in this observed behavior.

Sniffing and grooming behaviors were used as exploratory behaviors in the current study to assess anxiety-like behavior alterations. While we did see a significant

decrease in sniffing behavior in the Mn treated group, grooming increased during the dark cycle of Mn and NAC-Mn groups, though not significant. This differs from a study by Vezer er al. (2005) where increased HC Mn concentrations were correlated with decreased grooming at exposure week 10. Once again exposure duration may play a role in this difference.

For the most part, antioxidant therapy had little effect on any of the observed behaviors, which seemed to be driven largely by metal homeostasis. Oxidative stress has been theorized to play a role in the sequelae of Mn neurotoxicity and neurodegenerative disorders (Sian et al., 1994; Schulz et al., 2000; Erikson et al., 2004; Weber et al., 2002). The lack of effect antioxidant therapy had on behavior suggests that oxidative stress plays a minimal role in Mn-induced behavioral alterations. A more putative cause of Mn-induced behavior changes (e.g. increased locomotor activity) may be neurochemical alterations in GABA or DA biology.

Overall, our results show that oral Mn exposure does indeed alter locomotor activity, as seen with increases in total distance traveled, and altered groom and sniff behaviors. Though we did not examine neurotransmitter functionality directly, our results suggest some degree of underlying neurochemical alterations. It is also apparent that antioxidant therapy with NAC and EGCG does not play a significant role in correcting alterations in stereotypic behaviors as a result of Mn accumulation.

CHAPTER V

EPILOGUE

It is well established that manganese (Mn) and other divalent metals play a role in neurological dysfunction. The effect Mn has on neurobiology is multifaceted. Mn accumulation in the brain can disturb neurochemical homeostasis, as Mn is known to alter dopamine (DA), γ-aminobutyric acid (GABA), glutamate, and norepinephrine (NE) functionality (Fitsanakis et al., 2006; Anderson et al., 2009). In the basal ganglia these neurotransmitters work in concert to modulate motor control. A disturbance in one can disregulate the entire system resulting in loss of fine motor control, gait disturbances, and resting tremor, among other symptoms related to neurologic disorders. Mn can disrupt neurotransmission by altering neurotransmitter receptor and transport proteins (Anderson et al., 2008; 2009), or by inhibiting the proper function of surrounding glial cells (Erikson et al., 2002b). Glial cells, primarily astrocytes, help modulate the synaptic environment by clearing excess neurotransmitters along with extraneous metals (e.g. Mn accumulation in Mn neurotoxicity). Astrocytes also support neuronal function by recycling amino acids (glutamate to glutamine) for ammonia clearance and neurotransmitter recycling, as well as providing substrate precursors for neuronal glutathione (GSH) synthesis (Dringen et al., 2000;). Mn, however, can disturb these astrocytic functions leaving neurons vulnerable to excitotoxicity and oxidative stress.

The purpose of this project was to further characterize the relationship between Mn accumulation in the brain and oxidative stress. We examined the effect of oral Mn exposure on total GSH levels, mRNA levels of glutathione peroxidase (GPx) and catalase, the lipid peroxidation byproduct F_2-Isoprostanes (F_2-IsoPs), as well as altered stereotypic behaviors associated with Mn accumulation. Additionally, we coupled *N*-acetyl-cysteine (NAC) and (-)-epigallocatechin-3-gallate (EGCG) with Mn exposure to observe any protective benefits with antioxidant therapy. We hypothesized: 1) If Mn exposure created oxidative stress (indirectly measured by altered GSH, GPx, catalase, and increased F_2-IsoPs) NAC and EGCG therapy would attenuate those effects offering protection against Mn neurotoxicity. 2) If Mn-induced oxidative stress played a role in neurochemical changes leading to altered behavior, NAC and EGCG would help normalize movement and behavior abnormalities.

We found that while Mn did accumulate significantly in the caudate putamen (CP) and hippocampus (HC) of exposed rats, no significant signs of oxidative stress were present. Only modest changes were observed in GSH levels suggesting minimal free radical generation. No appreciable increase in F2-IsoP production and undisturbed catalase mRNA levels corroborate this finding. Mn did, however, significantly increase GPx mRNA. With respect to behavioral alterations, Mn significantly increased total distance traveled suggesting underlying neurochemical alterations. Decreased sniffing behavior in the Mn treatment group was also significant, and a trend toward significantly increased grooming was related to Mn exposure. Again, NAC and EGCG had little effect on behavior alterations. These data suggest that oral Mn exposure may not incur

significant oxidative stress or drastically alter neurotransmitter functionality; however, subtle neurochemical changes may result from this exposure paradigm.

The result of minimal Mn-induced oxidative stress was not a novel finding. Previous studies have shown increased, decreased, and no change of indirect markers of oxidative stress due to Mn exposure (Weber et al., 2002; Erikson et al., 2008; Taylor et al., 2006). What is interesting is that we observed minimal changes in GSH, but increased GPx mRNA. Increased GPx would, in theory, allow GSH to more efficiently quench peroxides. This would suggest that an increase in reactive oxygen species (ROS) were present, but we had little evidence to substantiate that claim. It would have been interesting to measure oxidized GSH (GSSG) along with total GSH levels to obtain a better picture of GSH redox status. Measuring GSSG may have provided insight as to why GPx levels were increased in the presence of no apparent oxidative stress. Alternatively, we could have directly measured ROS in tissue samples in addition to indirectly measuring F_2-IsoP production. Even though we did not see an effect of Mn on ROS generation, ROS can not be ruled out entirely in the etiology of Mn neurotoxicity. It just appears as though oral exposure to Mn may not cause severe enough damage to create substantial ROS. Conversely, individuals exposed to airborne Mn may be at greater risk of oxidative injury. In rats and monkeys, airborne exposure to Mn did result in altered biomarkers of oxidative stress (Taylor et al., 2006; Erikson et al., 2007, 2008). Perhaps the use of NAC and EGCG in airborne studies may be more indicative of their efficacy as antioxidant therapies.

Oral Mn exposure did have an effect on behavioral patterns in this study. Rats exposed to Mn experienced an increase in total distance traveled, which is indicative of neurochemical alterations (Flagel and Robinson, 2007). We did not measure neurotransmitter levels directly in this study, but Mn exposure has been linked to altered DA, GABA, and NE biology (Fitsanakis et al., 2006; Anderson et al., 2009). Furthermore, these alterations have been associated with changes in locomotor and anxiety like behavior (Anderson et al., 2008, 2009). We used sniffing and grooming behaviors, detected by Home Cage Scan (HCS) software, to represent changes in anxiety level. A limitation to this is that there is no behavior universally regarded as an index for anxiety. Solely monitoring changes in these behaviors may not be evidence enough to categorize the rats as more or less anxious. While using the HCS system may be convenient and is reportedly accurate (Steele et al., 2007), performing traditional behavior tests (e.g. open field test, Morris water maze, or radial arm maze) in conjunction with HCS may be useful to cross reference behavioral findings.

It is clear that Mn toxicity has an effect on neurochemical homeostasis; however it is difficult to say whether or not these alterations are driven by oxidative stress. Additionally, route of Mn exposure may dictate the type and severity of damage. Oral exposure may lead to subtle changes in neurobiology that manifest as cognitive or behavioral changes. While airborne Mn may lead to increased oxidative stress potentially causing neuronal damage and a subsequent neurodegenerative state. Future research should investigate the efficacy of antioxidant therapy in airborne Mn exposure. While oral Mn exposure studies may need to address the role of Mn in cellular signaling.

Mn exposure has been shown to alter protein kinase C (PKC) and intracellular calcium levels (Latchoumycandane et al., 2005), both of which are involved in cell signal cascades. This could help elucidate mechanisms related to altered neurotransmitter homeostasis, neurotransmitter receptor and transporter changes, and modified gene expression profiles in the presence of Mn exposure. Additionally, the relationship between Mn neurotoxicity and other divalent metals should be explored (e.g. copper) as a putative contributor to this neurological dysfunction. Ultimately, data presented in the current study along with future research will help to develop treatment modalities for Mn neurotoxicity and other neurodegenerative diseases like Parkinson's disease.

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