DISSERTATION

GLIAL INFLAMMATORY SIGNALING IN MANGANESE NEUROTOXICITY

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Julie Ann Moreno

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WE HEREBY RECOMMEND THAT THE DISSERTATION
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Committee on Graduate Work

Colin Clay, Ph.D

William H. Hanneman, Ph.D.

Marie E. Legare, D.V.M, Ph.D

Ronald B. Tjalkens, Ph.D. Adviser

Paul Layborn, Ph.D., Program Director

ABSTRACT OF DISSERTATION

GLIAL INFLAMMATORY SIGNALING IN MANGANESE NEUROTOXICITY

Degenerative movement disorders affecting the basal ganglia, including, Parkinson's (PD) and Huntington's diseases, are debilitating and currently incurable. Increased inflammatory gene expression in astrocytes promotes neuronal loss in these disorders, but the signaling mechanisms underlying this phenotype are not fully understood. In order to enhance understanding of this phenotype, the degenerative movement disorder, manganism, is a useful model, because patients suffering from excessive exposure to manganese (Mn) develop a neurodegenerative condition affecting the same brain region and with clinical features resembling PD. Recently, the potential effects of Mn on the developing brain have gained attention due to an increase in cognitive deficits with overexposure to Mn. Moreover, astrocytes are a known target of Mn, and reactive gliosis seems to precede neuronal injury. Mn toxicity enhances production of the inflammatory mediator nitric oxide (NO) in astrocytes by a mechanism involving NF-κB, the principal transcription factor responsible for expression of inducible nitric oxide synthase (NOS2). However, the role Mn toxicity plays in the developing brain along with the signaling mechanism(s) by which Mn enhances activation of NF-κB remains poorly understood in astrocytes. Therefore, in order to address the gap in knowledge I have characterized the role of glial cells in the promotion of neuronal damage in the developing and adult brain in a

mouse model of Mn neurotoxicity, as well as the mechanism by which Mn enhances inflammatory activation of NF-κB dependent genes in astrocytes. First, it was identified that sGC relays signals to ERK and NF-κB, initiating NO signaling in astrocytes. Also it was determined that the glial inflammatory response leads to an age- and sex-dependent vulnerability of the basal ganglia which can be modulated by E_2. This indicates that Mn toxicity in the developing brain results in locomotor deficits, reduction in normal dopaminergic neurotransmitter release, increased NOS2 expression in glial cells and neuronal injury. These findings are significant because once the mechanism of Mn-induced inflammatory activation of glial cells is understood, it will promote a better understanding of manganism and potentially other disorders of the basal ganglia.

Julie Ann Moreno
Graduate Degree Program in Cell and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Spring 2009

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LITERATURE REVIEW

PHYSIOLOGIC ROLE OF MANGANESE

Manganese (Mn) is a paramagnetic heavy metal present in 0.1% of the earth's crust and located in soil, water, air and food (ATDSR, 2008). Mn is the 4th most utilized metal across the world following iron, aluminum and copper, respectively. There are 11 oxidation states of Mn with 2+, 3+ and 7+ found in the environment with 2+ and 3+ located in living tissues (Archibald and Tyree, 1987). Mn is an essential dietary nutrient with adults needing 2-5 mg/day and 1-3mg/ day for children (NRC, 1989). Blueberries, nuts, legumes and soy are highly concentrated with Mn (ATDSR, 2008). Upon digestion 95-97% of Mn is cleared through the bile with only 1-5% absorbed into the systemic bloodstream (Davis et al., 1993) and is readily and heterogeneous distributed into the brain.

This essential metal is vital for normal central nervous system (CNS) functions such as Mn metalloenzymes including arginase (Aschner and Aschner, 2005), glutamine synthetase (Takeda, 2003), phosphoenolpyruvate decarboxylase and Mn superoxide dismutase (Hearn et al., 2003). Post-mortem analysis of human adult brains identified the caudate and putamen nuclei as regions normally high in Mn (Markesbery et al., 1984). Interestingly, rodent studies indicate the highest levels of Mn accumulation in the brain occurred at day five postnatal indicating a role for Mn in brain development (Takeda et al., 1999). Mn is also essential for normal immune system functioning, connective tissue and bone growth, blood clotting as well as metabolism of fats, carbohydrates and proteins (Erikson and Aschner, 2003; Gerber et al., 2002). Deficiency in Mn, although rare, leads to growth retardation, reproductive failure,

and alterations in glucose and HDL levels (Gregor et al., 1999). Although Mn deficiency can occurs, in humans a greater issue is the overexposure to Mn, causing Mn toxicity.

SOURCES OF MN EXPOSURE

Mn toxicity in humans is increasingly recognized as an important disease due to enhanced environmental and dietary distribution of Mn. Multiple occupational exposures to Mn can occur, the highest manufactured product containing Mn is steel. Steel consists of ore and alloy consisting of Mn oxide, ferromanganese and silicomanganese which contain up to 70% Mn (Bast-Pettersen et al., 2004; Kaji et al., 1993; Mergler et al., 1994). Along with the manufacturing of steel, Mn dioxide is used in making dry batteries (Bader et al., 1999), welders exposed to welding rods high in Mn (Bowler et al., 2006b) and mining (Montes et al., 2008) (Rodriguez-Agudelo et al., 2006) are just a few of the sources of occupational exposure. Environmental human exposure to Mn has increased since the addition of methylcyclopentadienyl Mn tricarbonyl (MMT) an anti-knock agent in gasoline in both United States and Canada (Aschner and Allen, 2000; Mergler et al., 1999) along with Maneb an organochemical fungicide utilized on crops worldwide. The active ingredient is manganese ethylene-bis-dithiocarbamate leading to concerns of high exposure through contaminated food in rural communities (Ferraz et al., 1988).

Occupational exposure effects factory workers exposed to high levels of Mn-dust via inhalation, up to 3.298 mg/m^3 therefore, the EPA has set maximum

exposure of Mn at 0.05ug/m^3 (ATDSR, 2008) for inhalation. The inhalation of Mn can result in high levels of Mn in the blood, 20-30ug/L (Bader et al., 1999; Kaji et al., 1993; Mergler et al., 1994), while normal levels are 4-12ug/L (ATSDR, 2008). Although controversial, there is some validity in using blood concentrations of Mn to monitor the exposure of factory workers to high levels of Mn, while urine and axillary hair are not good indicators of Mn toxicity (Bader et al., 1999).

Another route of exposure that is problematic is dietary or oral exposure. Dietary intake studies have shown that soy-based infant formula has approximately 200-fold more Mn than breast milk (Krachler et al., 2000) and infants receiving total parental nutrition (TPN) experience increase accumulation of Mn in the basal ganglia (Aschner and Aschner, 2005; Iinuma et al., 2003). In some rural communities children have displayed an increase of cognitive deficits due to high concentrations of Mn in well water (Woolf et al., 2002) increasing the need for future epidemiology studies. Increasing evidence indicates Mn accumulation in the brain is elevated by chronic iron deficiency in both humans and rodents leading to cognitive deficits in children (Garcia et al., 2007), it is recorded that 2 billion children experience iron deficiency worldwide stressing the importance in determining the role of Mn toxicity in the developing brain. Due to the concern of Mn toxicity through oral exposure the EPA has issued a reference dose of 0.14mg of Mn/ kg/ day (IRIS 2008).

MANGANSISM - NEUROLOGICAL AND NEUROPATHOLOGICAL FEATURES

Exposure to toxic levels of Mn through dietary, environmental and occupational routes leads to neurotoxicity and a disorder called Manganism. This disorder was first described in 1837 in France when five ore crushing plant workers experienced symptoms similar to Parkinson's Disease (Aschner and Aschner, 1991). Manganism is a neurodegenerative disease, targeting the globus pallidus, striatum, and substantia nigra pars reticulata, with substantial neuronal loss and reactive gliosis observed in afflicted humans (Yamada et al., 1986). Mn is known to accumulate in the basal ganglia region of the brain in humans, non-human primates and rodent models with variety of exposure routes and concentrations to the metal. The normal concentration of Mn in rodent brains is 0.26ug/g wet-weight (Markesbery et al., 1984) and the half-life of Mn in the brain is 51-74 days (Takeda et al., 1995). Patients exposed to Mn through occupational settings have an increase in Mn in the globus pallidus nuclei identified through MRI imaging (Herrero Hernandez et al., 2002). Multiple non-human primates exposed to Mn intervenously and inhalation routes have found an increase of Mn accumulation in the caudate, putamen, globus pallidus and cerebellum along with the cortical regions and olfactory bulbs due to route of exposure (Dorman et al., 2006a; Dorman et al., 2006b; Guilarte et al., 2006; Struve et al., 2007). Adult rodents administered Mn via drinking water or oral gavage had a significant increase in Mn in the striatum and hypothalamus brain regions as well as some evidence of cerebellum accumulation (Dorman et al., 2000; Lai et al., 1992). Recently, a MRI identified that the uptake of Mn is

greater in the primate brain than the rodent brain and is possibly due to the transport of Mn by cerebral spinal cord (Bock et al., 2008). This evidence of primate brain being more sensitive to Mn exposure may explain why multiple basal ganglia nuclei and cortical brain regions accumulate Mn versus rodent models experiencing accumulation only in a few key nuclei.

The earliest signs of Mn exposure manifest as a psychological disorder that includes aggressiveness, irritability, depression and hallucinations, and has even been identified as "maganese madness" (Mena et al., 1967). More recently, cognitive deficits have been noted such as loss of memory, poor ability to process visospatial recognition and decrease in ability to learn new things (Bowler et al., 2006a; Josephs et al., 2005). Along with early symptoms of reduced cognitive and psychiatric functioning, a decrease in normal motor function and control is observed with Mn toxicity. Late phase clinical symptoms present with neurological and motor deficits that include dystonia, bradykinesia, rigidity, masked facia, and difficulty in walking backwards (Mena et al., 1967). Some of these features are shared with Parkinson's disease, but dystonia and lack of resting tremor are more characteristic of manganism (Rodier, 1955). Interestingly, in a study of alloy factory workers, a decrease in the ability to do coordinated movement tasks was identified (Mergler et al., 1994), which is mediated by the extrapyramidal motor system, damaged with Mn toxicity (Barbeau, 1984; Donaldson et al., 1982).

MECHANISMS OF MN NEUROTOXICITY

MN TRANSPORT

Mn can be found in the bloodstream as Mn^{2+}, a free ion, which is much more stable than the oxidized form, Mn^{3+} found bound to serum transferrin (Aschner et al., 2007). The free ion can be taken up into the brain through several methods. Facilitated diffusion of Mn^{2+} across the blood brain barrier (BBB) and areas of the brain lacking intact BBB such as the circumventricular organs are the most common methods of Mn neural transport. High levels of blood Mn through intervenous injections in mice allowed rapid transport across the choroid plexus as well (Rabin et al., 1993) and store-operated calcium channels allow for the distribution of Mn across the BBB (Crossgrove and Yokel, 2005). In addition, uptake of brain Mn^{2+} can be transported via active transporters with high affinity for Mn such as divalent metal transporter-1 (DMT-1) (Au et al., 2008), NMDA receptor (Itoh et al., 2008), and ZIP-8 dependent transport (Yokel, 2006). DMT-1 has a high affinity for Mn^{2+} along with other divalent metals such as Fe^{2+} and Pb^{2+} (Gunshin et al., 1997), therefore the competition for the transport of Mn versus Fe is of concern in Mn toxicity. In fact, rats receiving an iron deficient diet are more vulnerable to Mn neurotoxicity (Erikson et al., 2002a) and this could be due to the increase uptake of Mn through DMT-1. The trivalent form of Mn, Mn^{3+} can also be found in the bloodstream usually bound to transferrin and then transported across the BBB through transferrin receptors (Aschner and Gannon, 1994), and some evidence suggest this is a major player in the transport of Mn into the brain (Crossgrove

and Yokel, 2004). Interestingly the receptor is increased in regions of the brain that Mn accumulates such as the striatum and globus pallidus. Once Mn is transported into the brain it may stay in the brain for months possibly leading to detrimental effects on neuronal synaptic transmission and modulation of several cellular signaling pathways in glial cells.

Mn EFFECTS ON SYNAPTIC TRANSMISSION

It has been well established that Mn toxicity influences synaptic transmission of GABAergic, dopaminergic, glutaminergic, and cholinergic neurons. Glutaminergic neurons release glutamate, the principal excitatory transmitter in the brain, but with Mn treatment glutamate and Mn are concurrently released indicating a correlation between Mn toxicity and glutamate excitotoxicity (Takeda et al., 2002). Increased glutamate at the synapse in Mn toxicity may be due to decrease glutamate uptake in astrocytes (Crooks et al., 2007) ((Hazell and Norenberg, 1997; Normandin and Hazell, 2002) caused by a reduction in expression of a glutamate transporter located in murine primary astrocytes (Erikson, 2002). Moreover, non-human primates exposed to Mn via inhalation had no change in glutamate production, but a decrease in the two main astrocytic glutamate transporters, GLT and GLAST along with a decrease in glutamine synthetase in the globus pallidus and caudate nucleus (Erikson et al., 2002b). The increase of glutamate in the synapse may lead to neuronal excitotoxicity with subsequent neuronal death (Fitsanakis et al., 2006), thought to be a central neuropathic mechanism in manganism and other neurodegenerative disorders.

A multitude of evidence indicates that the two GABAergic nuclei essential in normal locomotor inhibition, the globus pallidus and striatum, are targets for Mn toxicity (Dorman et al., 2006b; Guilarte et al., 2006; Kim et al., 2005; Liu et al., 2006). An increase in intracellular GABA, an inhibitory neurotransmitter, has been identified in multiple Mn toxicity rodent models in the striatal and pallidum (Gwiazda et al., 2002; Lipe et al., 1999). An *in vitro* model of GABAergic expressing cells had an increase of intracellular but no change in extracellular GABA was identified (Crooks et al., 2007) as well. However, multiple reports indicate no significant change in striatal GABA levels in rodent (Bonilla et al., 1994; Liu et al., 2006) and non-human primate models (Eriksson et al., 1992; Struve et al., 2007) following exposure to Mn. Although controversial, Mn toxicity does seem to render the GABAergic neuronal system vulnerable due to the highly GABAergic nuclei involved in Mn toxicity.

Dopamine is a neurotransmitter essential in motor coordination and cognitive function, and is reduced in diseases afflicting the basal ganglia. It has been well established that loss of dopamine in the striatum occurs with Mn toxicity (Cotzias et al., 1976). The cell bodies of dopaminergic neurons reside in the substantia nigra pars compacta (SNpc) and project up to the striatum, but in Mn toxicity unlike PD, the SNpc is commonly uninjured, though striatal dopamine is decreased in primate (Guilarte et al., 2008a; Perl and Olanow, 2007) and rodent models (Dorman et al., 2000; Liu et al., 2006). Position emission tomography (PET) has revealed a decrease in D2-dopamine receptors (D2R), but fully intact neurons projecting from the SNpc to the striatum (Shinotoh et al.,

1997; Wolters et al., 1989) along with decrease D2R binding sites in young male rodents (Reichel et al., 2006) with exposure to Mn indicating that althought the neurons are intact Mn causes damage to the uptake and distribution of dopamine once it reaches the striatum. Dopamine is a key player in decreased locomotion observed in Mn toxicity, therefore it is imperative to determine any abnormal activity of the dopaminergic system in models of manganism.

Recently, the adverse effects of Mn on the cholinergic neuronal system have become more concerning and evident, due to the system's role in locomotion and emotional behavior. Miners exposed to Mn have an increase in acetylcholinesterase (AChE) leading to a decrease in the neurotransmitter acetylcholine (ACh) (Iashchenko, 1998). A chronic Mn exposure model demonstrated a decrease in choline uptake in striatal synaptosomes only in the developing rat, indicating that the effect on the cholinergic system is age-dependent (Lai et al., 1982). Investigating the specific enzyme cholineacetyltransferase (ChAT), a marker of cholinergic activity, it was ascertained that during development ChAT activity decreases (Lai et al., 1984). More recently, ChAT positive neurons in the striatum were characterized to be undergoing apoptosis in adult mice exposed to Mn (Liu et al., 2006). The role of Mn toxicity on neurotransmitters is still not completely understood, but in most models there is loss of "normal" functioning of most neuronal synaptic transmissions. Therefore it is essential to tease out the mechanism by which Mn acts on synaptic pathways.

Mn EFFECTS ON MITOCHONDRIA AND CELLULAR ENERGY METABOLISM

The primary intracellular organelle targeted by toxic levels of Mn is the mitochondria (Gavin et al., 1999; Gunter et al., 2006). Mn can accumulate in the mitochondria by utilizing the Ca^{2+} uniporter, a divalent cation transporter, and is primarily bound to membrane and matrix proteins (Gavin et al., 1990). Sequestered Mn inhibits complex 2 of mitochondria respiration, disrupting Ca^{2+} homeostasis and ATP synthesis (Brouillet et al., 1993; Gavin et al., 1999; Zhang et al., 2003). Glial Ca^{2+} signaling is essential for glial-neuron communication, functional hyperemia, regulation of synaptic transmission (Araque et al., 1998; Fiacco and McCarthy, 2004) and even prevention of neuronal death (Bezzi et al., 2001; Blanc et al., 1998). Therefore, modulation of Ca^{2+} by Mn toxicity will lead to astrocyte dysfunction leading to a variety of cellular miscommunications. Additionally, Mn induces a decrease in enzymatic activity of α-ketoglutarate dehydrogenase and aconitase (Du, 1997; Malthankar et al., 2004; Zheng et al., 1998) indicating a reduction in normal cellular energy metabolism.

Astrocytic exposure to high concentrations of Mn causes a decrease in ATP synthesis and dysfunction of antioxidant proteins, with subsequent increase in oxidative stress leading to neuronal injury or even death (Chen and Liao, 2002; Malthankar et al., 2004). Astrocytic ATP propogates Ca^{2+} waves in astrocytes, vital for neuronal-glial and glial-glial crosstalk. ATP-dependent Ca^{2+} waves can travel up to 300μm *in vitro*, but after exposure to low-dose Mn, these waves are significantly blunted (Tjalkens et al., 2006). Furthermore, Tjalkens and colleagues determined that low concentrations of Mn caused sequestering of Ca^{2+} in the

mitochondria, reducing the ER pool of releasable Ca^{2+} and therefore inhibiting Ca^{2+} wave activity (Tjalkens et al., 2006). Mn toxicity is a disruptor of normal mitochondrial function, including Ca^{2+} signaling, specifically in astrocytes, which may predispose associated neurons to injury by decreasing astrocytic trophic activities.

NEUROINFLAMMATION AND MN NEUROTOXICITY

A. ASTROCYTES

Astrocytes are the most abundant cells found in the brain making up 50% of total neural cells in humans (Chen and Swanson, 2003). They are located heterogenously in the brain and unlike neurons, astrocytes are a non excitable cell but, communicate to neurons through intra- and intercellular Ca^{2+} signaling (Cornell-Bell et al., 1990). Astrocytes are key players in the formation of the BBB and vital for neurovascular coupling (Metea and Newman, 2006). These glial cells are essential for neuronal survival due to their role in providing electrolytes to surrounding neurons, maintaining pH, releasing of ATP and lactate (Ohgoh et al., 2000; Pellerin and Magistretti, 2004) and providing neurons with glutamine (Westergaard et al., 1995). Other key roles of astrocytes vital for the survival of neurons are the uptake of glutamate, glycine, and γ-aminobutyric acid (GABA), and antioxidant defense, via astrocytic production of glutathione (Aschner, 1998; Aschner et al., 1994; Pekny and Nilsson, 2005). Although astrocytes are vital for normal brain function, upon injury or stress they can become activated and

secrete pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO), leading to neuronal damage (Hirsch et al., 1999).

Astrocytes are thought to be an early target of Mn, in part because uptake of Mn in astrocytes is approximately 50 times that of neurons with basal levels of Mn in astrocytes being 50-70μM due to glutamine synthetase occurring in the astrocytes (Maurizi et al., 1987). Therefore Mn can accumulate to pathologic levels selectively in astrocytes, potentially disrupting key trophic functions. Accordingly, excessive Mn disrupts both glutamate uptake (Hazell and Norenberg, 1997) and mitochondrial function (Gavin et al., 1999; Gunter et al., 2006) in cultured astrocytes. Deprecations in these and other trophic functions in reactive astrocytes may act in concert with overproduction of inflammatory mediators to impair neuronal homeostasis. However, the mechanism by which reactive astrocytes upregulate inflammatory genes in manganism and other neurodegenerative diseases remains obscure. It has been suggested that the activation of astrocytes, characterized by increased expression of glial fibrillary acidic protein (GFAP), is due to secretion of inflammatory cytokines, such TNFα and IFNγ, by the surrounding microglial cells (Hirsch et al., 1998), increasing overall inflammation and leading to tissue damage over time. Prior reports in manganism have shown an increase in GFAP and S100β in rodent models, which precedes overt neuronal injury, revealing astrocytes may be early targets of Mn toxicity (Henriksson and Tjalve, 2000), and possibly having a primary role in induction of subsequent neuronal degradation.

B. MICROGLIA

Microglia are the principal immune cells of the brain and consist of about 12% of the overall neural cell population (del Rio-Hortega, 1932). There are several types of microglial cells, characterized by differences in morphology and location of the cells in the brain. Microglia that migrate into the brain early during development and differentiate from monocytes are called parenchymal microglia (Richardson et al., 1993). Perivascular microglia, another subtype, act as the initial immune responder throughout life, responding to foreign organisms infiltrating into the brain (Lassmann et al., 1993; Rinner et al., 1995). These microglia will ward off any pathogens and respond to traumatic injury by becoming phagocytic and releasing pro-inflammatory cytokines. Interestingly, microglia are concentrated in the midbrain region, which includes the basal ganglia (Kim et al., 2000; Lawson et al., 1990). In neurodegenerative conditions including mutiple sclerosis (MS)(Bo et al., 1994; Prineas et al., 2001), acquired immunodeficiency syndrome (AIDS) (Lane, 1997; Sharer, 1992), Parkinson's disease (PD) (Vila and Przedborski, 2003) and Alzheimer's Disease (AD) (McGeer et al., 1987), microglia- induced production of inflammatory cytokines was found to be detrimental to the surrounding neurons. Activated microglia, like astrocytes, will secrete pro-inflammatory mediators, including cytokines, NO and reactive oxygen species (ROS) (Banati et al., 1993; Gehrmann and Banati, 1995; Hopkins and Rothwell, 1995) upon activation. Therefore the combination of increased microglia and microglia-induced inflammation allows the basal ganglia to be sensitive to Mn toxicity.

Upon exposure to low dose Mn, microglial activation and H_2O_2 production in primary rat microglia occurs along with MAP kinase signaling (Zhang et al., 2007). Cultured microglia exposed to high dose Mn and lipopolysacaride (LPS) experience an increase in cytokine secretion and activation of p38 (Crittenden and Filipov, 2008) providing more evidence that an inflammatory signaling pathway is induced in microglia. Also utilizing the PD model 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) combined with blockade of microglia activation, found a significant reduction in ROS production NOS2 expression along with a decrease in neuronal injury, indicating a role for microglia in inflammatory mediated PD (Wu et al., 2002). Therefore, microglia may be contributing to Mn neurodegeneration, but the signaling mechanism inducing an inflammatory phenotype is simply not known.

C. NF-κB SIGNALING AND MN NEUROTOXICITY

A multitude of studies in neurodegenerative diseases have identified the production of pro-inflammatory cytokines and NO as major culprits in inducing a neuroinflammatory phenotype. Post-mortem brain analysis indicates an increase in cytokines in diseases such as multiple sclerosis, PD and AD (Sheng et al., 1998). Low levels of these inflammatory mediators can be protective through the release of trophic factors and free radical scavengers, but in an extreme inflammatory state, these molecules become deleterious to the brain causing neuronal injury and gliosis. The inflammatory signaling pathways promoting NO

and cytokine production are of great concern for neurodegeneration and specifically Mn toxicity.

Nitric Oxide Signaling. NO is a key signaling molecule in the nervous system for signaling pathways such as cGMP, vasodilation and constriction (Metea and Newman, 2006; Prado et al., 1993), synaptic plasticity (Dinerman et al., 1994) and to combat pathogens. The brain has the highest amount of constitutively expressed nitric oxide synthase (nNOS or NOS1), the enzyme responsible for the production of NO, compared to any other organ in the body (Hibbs et al., 1987; Salter et al., 1991). The NOS reaction occurs when L-arginine is synthesized to L-citrulline, resulting in the production of NO (Stuehr, 1997). There are three types of NOS enzymes, neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). NOS1 and NOS3 are Ca^{2+}/calmodulin- dependent (Bredt and Snyder, 1990) and primarily associated with membranes, while NOS2 is Ca^{2+}/calmodulin- independent and mostly cytosolic (Christopherson and Bredt, 1997; Knowles and Moncada, 1994). NOS1 is constitutively expressed in glia and neurons, primarily in the cerebellum and hippocampus, and is activated through Ca^{2+} influx via voltage-gated Ca^{2+} channels (Bredt et al., 1990; Egberongbe et al., 1994; Endoh et al., 1994; Oka et al., 2004). NOS3 is constitutively expressed in glial and endothelial cells (Colosanti, 1998).

NOS2 is not constitutively expressed, but is rapidly induced by stimuli such as pro-inflammatory cytokines (Stuehr and Griffith, 1992). NOS2 is highly upregulated in activated astrocytes and microglia (Boje and Arora, 1992; Galea

et al., 1992; Simmons and Murphy, 1992), and more recently neuronal upregulation although highly controversial (Moro et al., 1998). Interestingly, L-arginine, the precursor to NOS was identified to be present at highest concentrations in rodent astrocytes both near and away from the vasculature compared to other neural cells (Aoki et al., 1991) lending some insight into the increase of NOS2 expression in astrocytes. Mn strikingly potentiates the capacity of TNFα and IL-1β to induce expression of NOS2 and subsequent overproduction of NO in astrocytes by acting on NF-κB (Liu et al., 2005; Spranger et al., 1998). Previous studies from our laboratory demonstrated that Mn strongly potentiates NO production in cytokine-primed astrocytes, leading to secondary apoptosis in co-cultured neurons (Liu et al., 2005). NO is highly unstable free radical that has a very short half-life (5 seconds) (Meulemans, 1994), but can combine with superoxide, leading to peroxynitrite formation (Beckman et al., 1990). Peroxynitrite can modify nitrotyrosine residues, leading to cellular damage through oxidative stress (Luth et al., 2002) and modulate signaling pathways such as MAP kinases (Go et al., 1999) and protein kinase C (Balafanova et al., 2002) to name a few.

NF-κB Activation and MAPK signaling. NOS2 expression is principally mediated by the transcription factor NF-κB, a Rel protein family member (Xie et al., 1993) involved in inflammation, cell division, apoptosis, and immune responses (Karin and Ben-Neriah, 2000; Perkins, 2000). NF-κB interacting-kinase (NIK) and MAPK signaling pathways are recognized upstream effectors activating the NF-κB pathway. NF-κB is activated following phosphorylation of

the inhibitory subunit (IκBα) by the IκB kinase (IKK) complex, targeting it for degradation by 26S proteosome and allowing the p65-RelA/p50 subunits of NF-κB to translocate into the nucleus (Nakano et al., 1998; Vermeulen et al., 2002). MAP kinases, such as p38, c-Jun N terminal kinase (JNK) and extracellular signal-responsive kinase (ERK) are activated in astrocytes upon exposure to LPS, leading to activation of NF-κB (Xie et al., 2004). Along with astrocytes, it was determined that microglia exposed to a variety of cytokines underwent NF-κB activation preceding NOS2 expression (Cho et al., 2001). A role of both p-38 and ERK activation has been identified in both primary rat micoglia cells and a transformed microglia cell line N9 cells exposed to both low (10μM) and high levels (500mM) of Mn (Crittenden and Filipov, 2008; Zhang et al., 2007). Previous studies from our lab in C6 astroglioma cells demonstrate activation of ERK by Mn, which subsequently promoted activation of NF-κB (Barhoumi et al., 2004; Nakano et al., 1998). These studies implicate MAP kinases in Mn-induced activation of NF-κB, but the precise contribution of various these MAPK family members remains to be determined. Previous studies from our laboratory (Barhoumi et al., 2004; Liu et al., 2005), led us to investigate the IKK/NF-κB signaling complex as a possible convergence point, integrating multiple upstream signals elicited by Mn that further enhance the actions of inflammatory cytokines on induction of NOS2 in glia.

Transcriptional Regulation of NF-κB. NF-κB consists of two primary subunits, p65 and p50, forming a heterodimer (p50/p65) located in the cytoplasm that is inactive until IκBα is phosphorylated by IKKα/β/γ complex at serine 32 and

36 (Maniatis, 1997; Rothwarf et al., 1998; Zandi et al., 1997). The heterodimer, p50/p65, has been shown to bind to the κB response elements on the NOS2 promoter upon exposure to LPS (Pascual et al., 2005), TNFα and IFNγ (Ganster et al., 2005), IL-8 (Hoberg et al., 2004) and Mn with LPS (Barhoumi et al., 2004; Chen et al., 2006). Elegant studies by Pascual et al. (2005), found that in order for the NOS2 gene to be activated by p65, the nuclear corepressor NCoR2 must first be ubiquitinated and degraded at the κB response element identified in astrocytes exposed to Mn (Tjalkens et al., 2008). The identification of the factors promoting transcriptional activation of NOS2 following Mn exposure is essential to understanding the key elements of NO overproduction in Mn neurotoxicity.

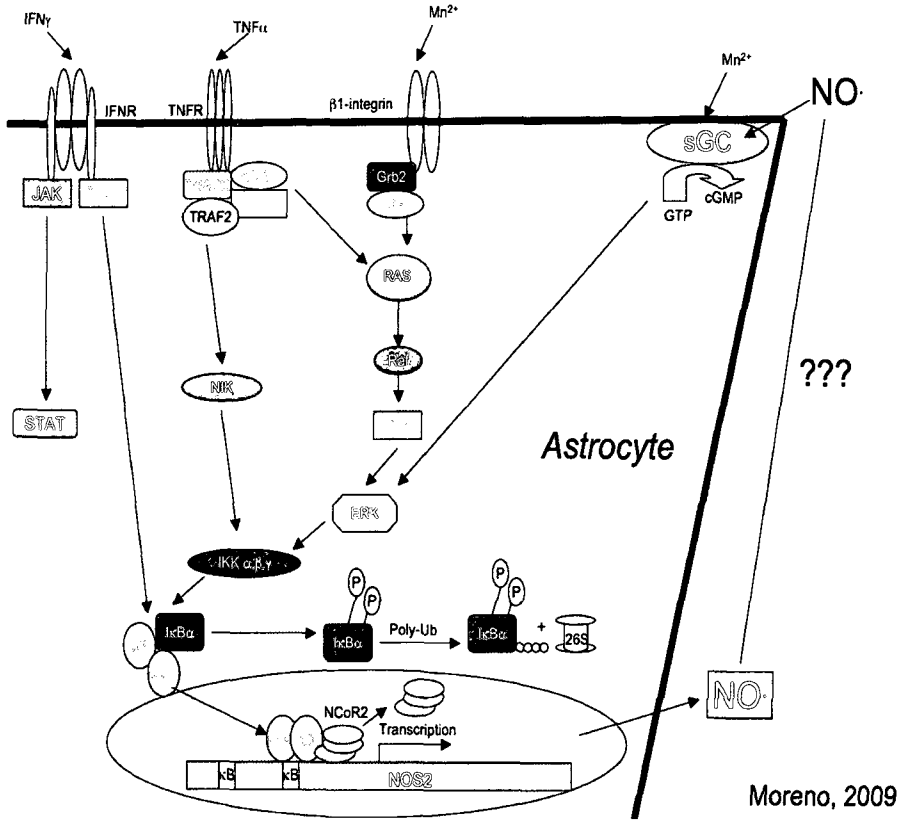


Diagram 1. Molecular Signaling pathway in astrocytes exposed to Mn, TNFα and IFNγ

DEVELOPMENTAL VULNERABILITY TO MN NEUROTOXICITY

A. AGE-DEPENDENT VULNERABILITY

One in every six children in the United States suffers from a developmental disorder, with the most common being a delay in learning and/or attention-deficit/hyperactivity disorder (ADHD) (Boyle, 1994; CDC, 2008). The National Research Council has stated that 25% of developmental delays are due to environmental toxins interacting with genes (NRC, 2000). As with many environmental toxicants such as Mn the developing brain is extremely vulnerable and of great concern. Due to enhanced Mn absorption, decrease BBB formation and very little biliary excretion until weanling age, neonates accumulate more Mn than adults (70% vs. 3%, respectively) (Brenneman et al., 2000). In fetuses and newborns Mn is able to cross the placenta and accumulate in their brain (Tabacova, 1986). Infants receiving total parental nutrition (TPN) accumulate 200% more Mn in the brain compared to children not receiving TPN (Fell et al., 1996). Moreover, neuronal synapses develop in the brain throughout childhood until adolescence, specifically in the prefrontal cortex (Huttenlocher and Dabholkar, 1997; Rapoport and Gogtay, 2008), giving rise to increasing the vulnerability of the developing brain neurotoxicants such as Mn.

Cognitive deficits and hyperactivity were identified in children exposed to moderate levels of Mn in drinking water (Bouchard et al., 2007; Wasserman et al., 2006; Woolf et al., 2002). Moreover, children unknown to be exposed to high

levels of Mn but that suffer from ADHD had high levels of Mn in their hair follicules (Collipp et al., 1983). Other dietary intake studies have shown soy-based infant formula has approximately 200-fold more Mn than breast milk leading to increase accumulation of Mn in the brains of exposed infants (Krachler et al., 2000). Interestingly, children exposed to even non-soy infant formula experience 10 times more Mn in hair follicles compared to age matched controls (Collipp et al., 1983). Iron deficiency is a major world health problem affecting approximately 2 billion infants, children and adolescents (WHO, 2005). Recently it has been identified that lack of iron in the brain will allow for increase uptake of Mn. As mentioned previously, high levels of Mn can lead to ADHD, a condition identified in children with reduced brain iron levels (Konofal et al., 2004). Rodents with iron deficiency and exposed to toxic levels of Mn have increased accumulation of Mn compared to rodents receiving an iron-rich diet (Aschner and Allen, 2000; Chandra and Shukla, 1976; Garcia et al., 2007; Mena et al., 1967). The literature described indicates a cognitive vulnerability of children exposed to Mn and identifies an age-dependent susceptibility to Mn toxicity that warrants future studies.

B. SEX-SPECIFIC VULNERABILITY

PD and many other neurodegenerative diseases have a sex-dependent susceptibility, with a higher incidence in males than females (Kenchappa et al., 2004). The ovarian steroid hormone, 17-β-estradiol (E_2), has many functions both reproductive and non-reproductive. E_2 has been implicated in regulation of synaptic plasticity and involved as a protectant in various neurodegenerative

diseases and ischemic events (Morale et al., 2006; Stirone et al., 2005). Previous studies identified neuroprotection by E_2 replacement therapy in young 4 months old rats by increasing blood brain barrier (BBB) integrity, (Bake and Sohrabji, 2004) reducing brain inflammation (Nordell et al., 2003) and increased trophic support (Jezierski and Sohrabji, 2001) such as increasing transforming growth factor- β (TGF-β) in astrocytes (Dhandapani et al., 2005).

Prior animal studies utilizing MPTP- and 6-hydroxydopamine (6-OHDA)-treated mice show a less pronounced decrease in striatial dopamine and less depletion of SNpc dopaminergic neurons when animals also received E_2 (Callier et al., 2001; Murray et al., 2003; Tripanichkul et al., 2007) indicating a protective role for E_2. Recent evidence demonstrates that E_2 decreases astro- and micro-gliosis following MPTP exposure (Tripanichkul et al., 2006) and that astrocyte cultured media protects against neuronal death when pre-exposed to E_2 (Dhandapani and Brann, 2003). Mn inhalation increased Mn concentrations in more so in young male rats compared to age matching females (Dorman et al., 2004) indicating a possible role for E_2 protection with Mn toxicity. Overall, E_2 may modulate Mn-induced neurotoxicity, although the precise mechanism remains unclear.

E_2 likely inhibits inflammatory signaling, beginning with E_2 binding to both estrogen receptor- α and -β subtypes expressed in astrocytes and microglia. Some evidence indicates E_2 alters inflammatory signaling by inhibiting NOS2 synthesized NO in astrocytes and microglia (Marchetti et al., 2005; Morale et al., 2004). The transcription of NF-κB (Dodel et al., 1999; Vegeto et al., 2003) and

the cellular signaling pathways involving ERK and PI3K/Akt (Dhandapani and Brann, 2007) are modulated by E_2 in the brain, but the specific mechanism by which E_2 modulates Mn toxicity is simply unknown in glial cells.

SUMMARY

The studies cited above demonstrate that Mn, although an essential nutrient and necessary cofactor, can lead to a parkisnsonism-like disorder in adults and cognitive developmental issues in children. Mn toxicity has been viewed as an occupational disorder affecting a minute subset of individuals worldwide. In the last decade, the disorder has become of greater concern due to the recognition that iron deficiency, a worldwide health concern, allows for an increase in Mn brain uptake in primarily the young. Moreover, epidemiology studies around the world have shown that increases in Mn concentrations in well water corresponds to hyperactivity and learning deficits in children. Therefore, manganism as a discreet neurodegenerative disorder is of public health concern. In Mn neurotoxicity and other neurodegenerative diseases, an inflammatory phenotype of the glial cells along with neuronal injury is observed in humans, and these processes are central to disease development. In order to determine the role of glial inflammatory signaling, it is essential to identify signaling mechanisms inducing inflammatory mediators like NO. Prior literature states NF-κB is critical for expression of NOS2 in astrocytes, but the upstream effectors activating this pathway, as well as specific factors mediating p65-dependent transcriptional activation, are not fully understood in activated astroglia or microglia upon exposure to Mn. It is also unknown if there is an age-dependent susceptibility, or if adolescence exposure coupled with later adult exposure causes increased vulnerability of the basal ganglia. Other models of

neurodegenerative diseases have identified that E_2 can modulate inflammation. Therefore, determining if E_2 has the same capacity in Mn toxicity will give insight into sex-dependent mechanism of neurodegeneration. By identifying an age-and sex-dependent vulnerability to Mn along with the intracellular signals involved in the activation of NF-κB and NOS2 gene expression in glial cells will lead to a better understanding of the mechanisms underlying glial inflammatory signaling in Mn neurotoxicity.

CHAPTER 2

MANGANESE POTENTIATES NF-KAPPA BETA-DEPENDENT EXPRESSION OF NITRIC OXIDE SYNTHASE 2 IN ASTROCYTES BY ACTIVATING SOLUBLE GUANYLATE CYCLASE AND EXTRACELLULAR REGULATED KINASE SIGNALING PATHWAYS

ABSTRACT

Inflammatory activation of glial cells is associated with neuronal injury in several degenerative movement disorders of the basal ganglia, including manganese neurotoxicity. Manganese (Mn) potentiates the effects of inflammatory cytokines on NF-κB-dependent expression of nitric oxide synthase 2 (NOS2) in astrocytes but the signaling mechanisms underlying this effect have remained elusive. It was postulated in the present studies that direct stimulation of cGMP synthesis and activation of mitogen activated protein (MAP) kinase signaling pathways underlies the capacity of Mn to augment NF-κB-dependent gene expression in astrocytes. Exposure of primary cortical astrocytes to a low concentration of Mn (10 μM) potentiated expression of NOS2 mRNA, protein, and production of NO in response to interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) that was prevented by overexpression of dominant negative IκBα. Mn also potentiated IFNγ- and TNFα-induced phosphorylation of extracellular response kinase (ERK), p38, and JNK, as well as cytokine-induced activation of a fluorescent NF-κB reporter construct in transgenic astrocytes. Activation of ERK preceded that of NF-κB and was required for maximal activation of NO synthesis. Independently of IFNγ/TNFα, Mn stimulated synthesis of cGMP in astrocytes and inhibition of soluble guanylate cyclase (sGC) abolished the potentiating effect of Mn on MAP kinase phosphorylation, NF-κB activation, and production of NO. These data indicate that near physiologic concentrations of Mn potentiate cytokine-induced expression of NOS2 and

production of NO in astrocytes via activation of sGC that promotes ERK-dependent enhancement of NF-κB signaling.

INTRODUCTION

Manganese (Mn) is an essential cofactor for multiple enzymes critical to metabolic homeostasis in the central nervous system (CNS), including (Hearn et al., 2003; Takeda, 2003) and glutamine synthetase (Takeda, 2003). However, excessive accumulation of Mn can cause injury to neurons in the basal ganglia, resulting in a progressive neurodegenerative disorder known as manganism, that is accompanied by reactive astrogliosis (Yamada et al., 1986). Studies using rodent models of manganism suggest that astrocytes are affected early in Mn neurotoxicity, based upon changes in expression of the phenotypic markers of astrocyte activation, glial fibrillary acidic protein (GFAP) and S100β, that precede overt neuronal injury (Henriksson and Tjalve, 2000). Additionally, astrocytes accumulate much higher levels of Mn than neurons (Maurizi et al., 1987) that may disrupt critical neurotrophic functions and promote an inflammatory phenotype. Mn enhances the release of inflammatory cytokines interleukin-6 and TNFα (Yamada et al., 1986) from microglial cells (Chang and Liu, 1999; Filipov et al., 2005) that can promote the activation of astrocytes and subsequent release of inflammatory mediators such as prostaglandin E_2 and nitric oxide (NO) (Chen et al., 2006; Hirsch et al., 1998; Spranger et al., 1998). Previous studies from our laboratory (Liu et al., 2005) and others (Spranger et al., 1998) demonstrated that Mn strongly potentiates NO production in cytokine-stimulated astrocytes, leading to apoptosis in co-cultured neurons. Low concentrations of Mn can potentiate the capacity of TNFα and IL-1β to induce expression of NOS2 and production of NO in astrocytes by acting on NF-κB (Liu et al., 2005; Spranger et al., 1998) but the

signaling mechanisms responsible have not been elucidated. NO is produced from L-arginine by nitric oxide synthases (NOS1, 2, and 3), of which the inducible isoform (NOS2) is not constitutively expressed but is highly upregulated in activated astrocytes and microglia by stimuli such as pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS) (Stuehr and Griffith, 1992).

Expression of NOS2 is principally regulated by the transcription factor NF-κB (Xie et al., 1993), a Rel protein family member involved in inflammation, cell division, apoptosis, and immune responses (Karin and Ben-Neriah, 2000; Perkins, 2000). Signals that activate NF-κB converge through the IKappaB Kinase (IKK) complex to phosphorylate IκBα, targeting it for degradation by 26S proteasome and allowing the p65-RelA/p50 subunits of NF-κB to translocate into the nucleus (Nakano et al., 1998; Vermeulen et al., 2002). Upstream activators of NF-κB include mitogen activated protein (MAP) kinases, such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-responsive kinase (ERK) (Fernandes et al., 2007; Kim et al., 2006). We previously demonstrated that Mn-dependent activation of ERK in C6 glioma cells augments LPS-induced expression of NOS2 through the NF-κB pathway (Barhoumi et al., 2004) but a similar mechanism has not been reported in astrocytes. One possible target of Mn in astrocytes is the NO-sensitive soluble guanylate cyclase (sGC) that catalyzes the conversion of GTP to cyclic GMP (cGMP) (Sardon et al., 2004; Shen et al., 2005). It has been reported in other systems that the enzymatic activity of sGC is strongly increased in the presence of Mn^{2+} to a greater extent than its native co-factor, Mg^{2+} (Winger and Marletta, 2005). Interestingly,

expression of sGC is increased in the striatum of mice exposed to the parkinsonian neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Chalimoniuk et al., 2004). These data are intriguing given the established role for glial inflammation in the neurotoxicity of MPTP but a similar role for cGMP in Mn neurotoxicity remains to be determined. Based upon these findings and those of previous studies from our laboratory implicating NF-κB in Mn-dependent expression of NOS2 (Barhoumi et al., 2004; Liu et al., 2005), we postulated that Mn potentiates cytokine-induced activation of NF-κB and expression of NOS2 in astrocytes by directly enhancing production of cGMP that stimulates MAP kinase signaling.

MATERIALS AND METHODS

Reagents-All chemical reagents were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise indicated. C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Fluorescent dyes, cell culture media, antibiotics, and Alexa-Fluor secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Cell Culture- Primary cortical murine astrocytes were isolated from cortices of day 1 old C57/Bl6 and transgenic GFP mice as described in (Aschner et al., 1992) and grown 18 days to maturity before use in experiments. Astrocytes were maintained in Minimum Essential Medium (MEM) with L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin, 50 ng/mL streptomycin, and 100 ng/mL neomycin (PSN) at 37 °C, 5% CO_2 in a humidified atmosphere. Immunostaining for glial fibrillary acidic protein (GFAP) indicated that cultures were consistently > 96% astrocytes.

NOS2 mRNA and protein expression- RNA was isolated using the Qiagen RNeasy Kit, with an on-column DNase digestion, and reverse transcribed via iScript (Biorad, Hercules CA). Semi-quantitative real-time PCR was used to measure relative *Nos2* expression normalized to *β-actin* mRNA. The following primers designed from mouse *Nos2* and *β-actin* transcript were used: (*Nos2; NCBI Accession # P29477*) 5'-TCACGCTTGGGTCTTGTT-3'(forward), 5'-CAGGTCACTTTGGTAGGATTTG-3'(reverse); (*β-actin*) 5'-GCTGTGCTATGTTGCTCTAG-3' (forward), 5'-CGCTCGTTGCCAATAGTG-3' (reverse). NOS2 protein expression levels in astrocytes were measured using

immunofluorescence. Briefly, astrocytes plated on glass coverslips underwent methanol fixation, were permeabilized with 0.01% Triton X-100 and then probed for the protein of interest. Primary monoclonal antibody for GFAP and polyclonal antibodies for NOS2 (BD Pharmingen, SanDeigo CA) and phosphorylated ERK (Cell Signaling, Danvers MA) were used at 1:1000, 1:400 and 1:100 respectively. Protein levels were visualized via fluorescence microscopy following hybridization of Alexa Fluor- 488 or 647- labeled secondary antibodies.

Determination of NO steady state levels- NO production was assessed by wide-field live cell fluorescence imaging in primary astrocytes exposed to treatment groups for 8 hours and then incubated with the fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate prepared as a 5 mM stock in DMSO and diluted to a final concentration of 5 μM in medium for 10 min. Images were then collected at 490 nm excitation/ 520 nm emission at intervals of 5 min for 60 min using a Zeiss Axiovert 200M microscope equipped with a Hammatsu ORCA-ER cooled charge-coupled device camera.

Adenoviral expression in astrocytes- In order to determine the upstream role of NF-κB with Mn and cytokines exposure, activation of NF-κB was suppressed by a mutant NF-κB inhibitory subunit (IκBα) containing serine-to-alanine mutations at amino acids 32 and 36. This construct has been subcloned into an adenoviral vector (provided by Dr. David Brenner, M.D., Professor of Medicine at Columbia University) and is expressed in 90-100% of infected astrocytes at $1x10^3$ viral particles per mL of culture media for 24 hours. Following incubation of virus for 24 hours media was removed, cells washed with PBS to

remove viral particles, and culture medium replaced for 24 hours prior to treatment. Parallel control experiments were performed using the adenoviral vector lacking the mtIκBα insert as described previously (Liu et al., 2005).

Immunoblotting of phosphorylated MAP kinases- Mature astrocytes were serum starved for two hours followed by treatment with Mn and cytokines. Protein was then isolated by lysing cells using RIPA buffer containing sodium orthovanadate (0.2mM) and Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis IN). Protein, 10 or 20 μg was resolved by 10% SDS-PAGE. Resolved proteins were transferred to polyvinylidene fluoride membranes and incubated with primary polyclonal antibodies, phospho-ERK, phospho-JNK or phospho-p38 at 1:4000, 1:1000 and 1:1000 respectively (Cell Signaling, Danvers MA). Horseradish peroxidase-conjugated secondary antibody (1:5000; Cell Signaling, Danvers MA) was applied and protein was visualized using chemiluminescence (Amersham Biosciences, Piscataway NJ). The membrane was then re-probed with a protein loading control, total ERK 1:5000, total JNK 1:1000 and total p38 1:1000 (Cell Signaling, Danvers MA).

cGMP and ERK Activation Assays- To assay guanosine 3', 5' cyclic monophosphate (cGMP) levels with a competitive enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor MI), astrocytes were treated and then lysed with 0.1 M hydrogen chloride. Samples were acetylated in order to achieve quantification of cGMP at concentrations less than 1 pmol/mL. Each treatment group had three biological replicates and each replicate was assayed in triplicate.

The assay was analyzed using a Thermomax™ microplate reader (Molecular Devices, Sunnyvale, CA) measuring absorbance at 420 nm. Cellular Activation of Signaling ELISA (CASE™) kit, a colorimetric assay, was utilized to measure total ERK and ERK phosphorylation (Superarray, Frederick MD). Cells were plated on a 96-well tissue culture plate at 5×10^4 cells per well then treated in duplicate representing three biological replicate. Quantitative analysis of both phosphorylated and total ERK was performed using the aforementioned microplate reader at absorbance 450 nm.

Statistical analysis- At least 3 repetitions per isolation of each sample group were performed for each study consisting of three separate biological replicates. Two-group comparison was performed utilizing Student's t-test and one-way ANOVA with Neuman-Keuls post-test was used for comparing differences between more than three means. Differences between groups were considered significant at $p<0.05$, and indicated through assignment of a unique character (eg. a-d).

RESULTS

Expression of *Nos2* mRNA was examined in primary mouse cortical astrocytes exposed to low concentrations of Mn and IFNγ/TNFα to identify a threshold dose that potentiated cytokine-mediated induction of this gene. Treatment with 10 μM Mn led to a slight increase in levels of *Nos2* mRNA (2.5 ± 0.8 -fold greater than control) (Fig. 1A). Combinatorial treatment with IFNγ and TNFα resulted in a dose-dependent increase in *Nos2* mRNA levels (Fig. 1B), with 1000 pg/mL IFNγ + 10pg/mL TNFα causing a 8.0 ± 3.0 - fold increase over control that was selected as a 'sub-optimal' dose corresponding to mild activation of astrocytes. Exposure of astrocytes to 10 μM Mn in the presence of 1000 pg/mL IFNγ + 10 pg/mL TNFα strongly potentiated induction of *Nos2* mRNA levels (21.3 ± 9 - fold increase over control at 10 μM Mn + 1000 pg/mL IFNγ + 10 pg/mL TNFα; Fig. 1C). Based upon these data, this dose of Mn and cytokines was used in subsequent experiments to induce an inflammatory phenotype and expression of *Nos2*. Time-dependent expression of *Nos2* mRNA levels in astrocytes exposed to 10 μM Mn + IFNγ/TNFα for 0, 2, 4, 6, and 8 hours indicated that expression of message significantly increased by 8 hrs (Fig. 1D).

Expression of NOS2 protein was determined in astrocytes by immunofluorescence following 8 hrs exposure to 10 μM Mn + IFNγ/TNFα (Figure 2). As shown in Fig. 2A, expression of NOS2 (red fluorescence) was not detected in control cells but was induced upon exposure to Mn + IFNγ/TNFα. In all fields examined, expression of NOS2 was localized to cells expressing GFAP (green fluorescence). Production of NO was assessed by live cell imaging using

the fluorescent NO indicator DAF-FM diacetate in primary astrocytes exposed to Mn + IFNγ/TNFα for 8 hours. Intracellular levels of NO were increased by 6 ± 0.2% and 4 ± 0.06% in astrocytes exposed to 10 μM Mn or 1000 pg/mL IFNγ + 10pg/mL TNFα, respectively, but increased to 18.7 ± 5.7% over control in cells exposed to Mn + IFNγ/TNFα (Fig. 2C).

The role of NF-κB in Mn-induced expression of NOS2 was examined in Figure 3. Primary astrocytes were transfected with a control adenoviral vector or with adenovirus expressing a phosphorylation deficient mutant of IκBα (mtIκBα; IκBα S32/36A) and exposed to Mn + IFNγ/TNFα. Expression of mtIκBα partially abrogated induction of *Nos2* mRNA upon treatment with Mn + IFNγ/TNFα, whereas *Nos2* mRNA levels were increased similar to untransfected astrocytes in cells exposed to Mn + IFNγ/TNFα in the presence of control adenoviral vector (Fig. 3A). Exposure to Mn + IFNγ/TNFα increased NOS2 protein in control-transfected astrocytes and this induction was completely prevented in cells expressing mtIκBα (Fig. 3B). Similarly, overexpression of mtIκBα prevented Mn- and IFNγ/TNFα-induced increases in steady state intracellular levels of NO, as determined by live-cell imaging (Fig. 3C). Treatment with Mn + IFNγ/TNFα also directly increased activation of NF-κB in transgenic astrocytes expressing an NF-κB-GFP reporter construct; green fluorescence in these cells represents functional transactivation by p65 (Fig. 3D inset and graph). As a control, transgenic astrocytes were transfected with mtIκBα or control vector and assessed for increased GFP fluorescence (Fig. 3E). Treatment with Mn + IFNγ/TNFα strongly enhanced GFP fluorescence in transgenic astrocytes

transfected with control vector whereas expression of mtIκBα prevented both basal and inducible induction of GFP.

Mn induced rapid phosphorylation of the MAP kinase family members ERK, JNK, and p38 (Figure 4). Phosphorylation of ERK increased only slightly over time in astrocytes treated with either 10 μM Mn or IFNγ + TNFα individually but co-exposure potentiated phosphorylation of ERK that was maximal at 120 min (Fig. 4A). Treatment with Mn + IFNγ/TNFα also increased time-dependent phosphorylation of JNK and p38 (Fig. 4B-C). To determine the functional significance of Mn-dependent MAP kinase activation, production of NO was determined by live cell fluorescence imaging in astrocytes exposed to Mn + IFNγ/TNFα in the presence of inhibitors of ERK, p38 and JNK (Fig. 4D). Inhibition of ERK (U0126, 10 μM) and p38 (SB203580, 30 μM) attenuated NO production following exposure to Mn + IFNγ/TNFα but inhibition of JNK (SP600250, 10 μM) failed to decrease NO production induced by Mn + IFNγ/TNFα. NO levels were increased in the presence of vehicle control (DMSO) comparably to treatment with Mn + IFNγ/TNFα.

The temporal sequence of ERK and NF-κB activation was determined in transgenic NF-κB reporter astrocytes by co-immunofluorescence following exposure to Mn and IFNγ/TNFα (Figure 5). The fluorescence intensity of phosporylated ERK in transgenic NF-κB-GFP astrocytes peaked at 4 hrs, followed by maximal NF-κB activation at 6 hrs (Fig. 5A). Quantitation of fluorescence imaging data (Fig. 5B) indicated significant differences from control for both ERK and NF-κB upon exposure to Mn and IFNγ/TNFα. By 2 hrs, levels

of phosphorylated ERK increased, peaking at 4 hrs, and subsiding to baseline by 6 hrs. NF-κB activity, as determined by GFP fluorescence, was not increased until 6 hrs and decreased to a level still greater than control by 8 hrs. The functional correlation between Mn-dependent activation of ERK and NF-κB was determined by immunoblotting for phosphorylated IκBα in astrocytes exposed to concentrations of Mn from 0 – 50 μM in the absence or presence of the ERK inhibitor U0126 (Fig. 5C). Mn directly enhanced the phosphorylation of IκBα at all concentrations examined and this effect was prevented by U0126 (10 μM).

Synthesis of cGMP by soluble guanylate cyclase (sGC) was next examined as a potential target of Mn leading to activation of ERK and NF-κB (Figure 6). Levels of cGMP were determined by enzyme-linked immunosorbent assay (ELISA) in astrocytes treated with 0, 1, 10, and 100 μM $MnCl_2$ in the absence or presence of IFNγ/TNFα (Fig. 6A). Mn caused a dose-dependent increase in levels of cGMP at concentrations as low as 1 μM (218 ± 53 pmol/mL vs. 124 ± 11 pmol/ml, control) that were near maximal at 10 μM (380 ± 44 pmol/mL), with 100 μM Mn increasing cGMP levels to only 456 ± 15 pmol/mL. Treatment with IFNγ/TNFα in the absence of Mn increased cGMP over control levels but did not enhance Mn-induced increases in cGMP (Fig 6A, final two columns). The EC_{50} for Mn-dependent activation of cGMP production in astrocytes was 1.1 ± 0.4 μM (Fig. 6A, inset). The sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; EMD Biosciences), prevented increases in cGMP induced by 10 μM Mn + IFNγ/TNFα (412 ± 43 pmol/mL, Mn + IFNγ/TNFα vs. 122 ± 8 pmol/mL, Mn + IFNγ/TNFα + ODQ) but vehicle control

(DMSO) had no effect on Mn-dependent activation of sGC (Fig. 6B). Inhibition of sGC with ODQ also prevented Mn-dependent activation of ERK, as determined using ELISA to measure the amount of phosphorylated and total ERK in cellular lysates (Fig. 6C). Levels of phosphorylated ERK were suppressed by ODQ in astrocytes exposed to Mn and IFNγ/TNFα (Fig. 6B), whereas levels of total ERK remained unchanged (Fig. 6D). Finally, co-treatment with ODQ but not DMSO significantly inhibited NO production (Fig. 6E) and NF-κB activation (Fig. 6F) in live astrocytes following exposure to Mn and IFNγ/TNFα.

DISCUSSION

Increased production of NO by reactive glia has been linked to the progression of neurodegenerative diseases including Alzheimer's, Parkinson's, and manganism (Gomez-Isla et al., 2003; Hirsch et al., 2003; Hunot et al., 1999). Accordingly, gene deletion of *Nos2* in mice is neuroprotective in models of ischemia (Sugimoto and Iadecola, 2002) and parkinsonian neurodegeneration (Liberatore et al., 1999), but a direct role for NO manganism is less clear. We reported recently that loss of striatal interneurons in Mn-exposed mice was most prominent near reactive astrocytes expressing NOS2 and was associated with increased immunoreactivity for 3-nitrotyrosine protein adducts, a marker of peroxynitrite ($ONOO^-$) formation (Liu et al., 2006). Additionally, we reported that expression of NOS2 and overproduction of NO in activated astrocytes exposed to Mn causes apoptosis in co-cultured PC12 cells (Liu et al., 2005).

NF-κB is directly associated with overproduction of NO in Mn-treated astrocytes (Barhoumi et al., 2004; Liu et al., 2005) but the mechanism underlying the capacity of Mn to potentiate the effects of inflammatory cytokines and LPS on NF-κB-dependent induction of NOS2 has remained elusive. The MAP kinase family of stress activated protein kinases can directly activate NF-κB through the IKK signaling complex (Lee et al., 1998; Nakano et al., 1998) and represent a potential upstream signaling target underlying Mn-dependent activation of NF-κB. The data presented here indicate that Mn enhances cytokine-induced activation of NF-κB and expression of NOS2 through the ERK pathway. Additionally, these data reveal that the mechanism by which Mn stimulates ERK requires increases

in cGMP. Physiological concentrations of Mn range from 2-8 μM in brain tissue (Pal et al., 1999) and at 10 μM, Mn significantly elevated intracellular levels of cGMP in astrocytes that resulted in rapid activation of ERK (Fig 6).

These observations provide a mechanistic explanation for earlier findings describing the ability of Mn to enhance NF-κB-dependent expression of NOS2 in activated astrocytes (Liu et al., 2005; Spranger et al., 1998). Genetic inhibition of NF-κB through overexpression of mtIκBα completely prevented Mn-dependent increases in NOS2 protein and NO (Figure 3) while only partly preventing increases in *Nos2* mRNA. This partial abrogation of *Nos2* mRNA levels may be attributed to the possible contribution of other transcription factors that can induce expression of *Nos2*, such as Signal Transducer and Activator of Transcription-1 alpha (STAT-1α), cAMP-responsive element binding protein (CREB), and activating protein-1 (AP-1) (Blanchette et al., 2007; De Stefano et al., 2006; Lee et al., 2003). A prior study by Chen et al., 2006 (Chen et al., 2006) demonstrated that Mn enhanced AP-1 activity in mixed glia cultures primed with LPS and IFN-γ, indicating that this transcription factor could also be involved in Mn-induced expression of *Nos 2*. However, the observation that steady state levels of NOS2 protein and NO were both decreased to control levels by mtIκBα despite the incomplete suppression of transcript levels, suggests that NF-κB may also influence post-transcriptional regulation of NOS2 expression. This is supported by data demonstrating that NOS2 requires the co-factor tetrahydrobiopterin for full protein assembly and activity (Chiarini et al., 2005) and by studies reporting that the rate-limiting enzyme in the synthesis of

tetrahydrobiopterin, GTP cyclohydrolase, is coordinately regulated with NOS2 by an NF-κB-dependent mechanism (Cho et al., 2001; Togari et al., 1998). Additional preliminary studies from our laboratory indicate that Mn potentiates cytokine-induced expression of GTP cyclohydrolase mRNA and that this induction is suppressed by mtIκBα (data not shown).

The present studies indicate that concentrations of Mn only slightly above physiologic levels can strongly potentiate inflammatory signaling pathways in astrocytes primed with low levels of cytokines. This may provide insight into why astrogliosis is so prominently associated with neurodegeneration in humans (Yamada et al., 1986) and experimental animals (Liu et al., 2006; Spranger et al., 1998) exposed to Mn. Brain Mn levels increase overall only 2 – 3 fold within the basal ganglia in models of manganism (Liu et al., 2006) but astrocytes can accumulate concentrations 50-fold greater than neurons (Aschner et al., 1992; Maurizi et al., 1987) due to active transport systems (Aschner et al., 1992), easily within the range shown here that stimulates sGC activity and MAP kinase signaling. Through the use of transgenic astrocytes expressing an NF-κB reporter construct, the data in Figure 3 demonstrate not only that NF-κB is required for expression of NOS2 but also that low concentrations of Mn enhance functional transactivation of NF-κB through p65 consensus binding elements. Previous data from our laboratory suggested that ERK was a potential target of Mn and that convergant signaling from this pathway enhanced NF-κB activation (Barhoumi et al., 2004). The data presented here demonstrate both that activation of ERK precedes the peak of NF-κB activity (Figure 5) and that cGMP-

dependent activation of ERK is required for Mn to potentiate expression of NOS2 and production of NO in cytokine-primed astrocytes (Figure 6). Experiments examining activity of MAP kinases and production of NO in the presence of various kinase inhibitors (Figure 4) indicated that p38 has some capacity to stimulate NO production independently of ERK. However, the efficacy of mtIκBα in completely preventing Mn-dependent increases in NOS2 protein and NO levels suggests that activation of p38 in this system also likely conveys Mn-dependent signals directly to the IKK/NF-κB complex (Fig. 4D). This is consistent with other studies reporting direct activation of NF-κB by p38 (Kim et al., 2006) and with reports indicating that inhibitors of p38 prevent Mn-dependent expression of TNFα in microglial cells (Filipov et al., 2005), a gene also strongly induced by NF-κB. However, p38-dependent activation of other transcription factors, such as JAK/STAT proteins, could partially explain the slightly elevated levels of *Nos2* mRNA that persist even in the presence of overexpressed mtIκBα, despite complete suppression of NOS2 protein.

The data in Figure 6 identifying sGC as a direct target of Mn in astrocytes provides a critical link explaining how only slightly elevated concentrations of Mn augment stress kinase signaling that subsequently potentiates activation of NF-κB. The mechanism by which Mn activates sGC is reported to involve replacement of the native cofactor Mg^{2+} that increases activity of the enzyme (Braughler, 1980; Winger and Marletta, 2005). The relevance of increased sGC to glial activation and neurodegeneration in manganism has not been reported. However, an association is suggested by indirect evidence from studies in

MPTP-treated mice reporting an increase in expression of sGC and levels of cGMP activity in the striatum (Chalimoniuk et al., 2004). Concentrations of Mn as low as 10 μM increased cGMP levels in cultured astrocytes (Fig. 6A). The addition of low concentrations of IFNγ and TNFα did not further enhance cGMP levels upon combined exposure with Mn (Fig. 6B), indicating that the observed increase in cGMP was Mn-dependent and distinct from signaling pathways activated by inflammatory cytokines. Additionally, inhibition of sGC prevented activation of both ERK and NF-κB, as well as production of NO (Fig. 6C-F), in Mn-treated astrocytes, indicating a sequence of signaling events involving sGC/cGMP/ERK that converges on the IKK/NF-κB pathway to potentiate expression of NOS2 in astrocytes.

In conclusion, the present studies demonstrate that near physiological levels of Mn strongly induce NOS2 and production of NO in astrocytes primed by a mild inflammatory insult. The mechanism underlying the striking effect of low-level Mn on expression of NOS2 in astrocytes appears to reside in the capacity of the divalent metal to potently stimulate sGC, leading to elevated intracellular levels of cGMP and ERK-dependent activation of NF-κB. Further dissection of the signaling pathways leading to activation of MAP kinases in Mn-exposed astrocytes, as well as the downstream transcriptional mechanisms regulating *trans*-activation by NF-κB, will likely improve our understanding of how NOS2 and other inflammatory genes are inappropriately expressed in activated astroglia during degenerative conditions of the basal ganglia such as manganism.

CHAPTER 2

FIGURES

FIGURE 1
NOS2 mRNA expression

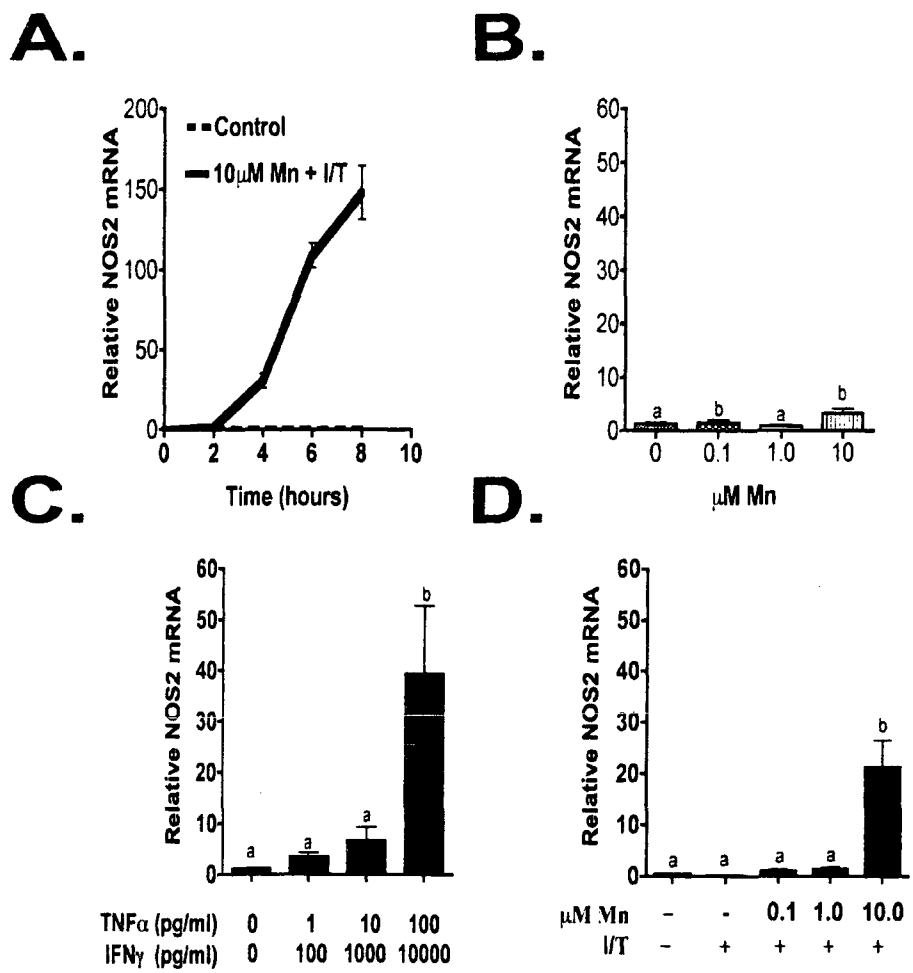


Figure 1. Manganese potentiates cytokine-induced expression of *Nos2* mRNA in astrocytes. (A) *Nos2* mRNA expression was measured using real-time PCR in astrocytes exposed to saline, 0.1 μM, 1 μM, or 10 μM $MnCl_2$ (Mn) for 8 hours. *Nos2* mRNA expression was significantly increased when astrocytes were treated with 10 μM Mn compared to control. (B) *Nos2* mRNA expression in cells exposed to saline, 100 pg/mL IFNγ +1 pg/mL TNFα, 1000 pg/mL IFNγ + 10 pg/mL TNFα or 10000 pg/mL IFNγ+ 100 pg/mL TNFα for 8 hours indicated that 1000 pg/mL IFNγ + 10pg/mL TNFα concentration is adequate to induce moderate expression. (C) Astrocytes were exposed to 1000 pg/mL IFNγ + 10 pg/mL TNFα (I/T) and 0 – 10 μM of Mn for 8 hours, showing potentiation of *Nos2* mRNA expression with exposure to 10 μM Mn + I/T. (D) Astrocytes exposed to saline or 10 μM Mn + I/T over time (0 - 8 hrs), with increased *Nos2* mRNA expression observed at 4, 6, and 8 hrs.

FIGURE 2
NOS2 protein and NO signalinig

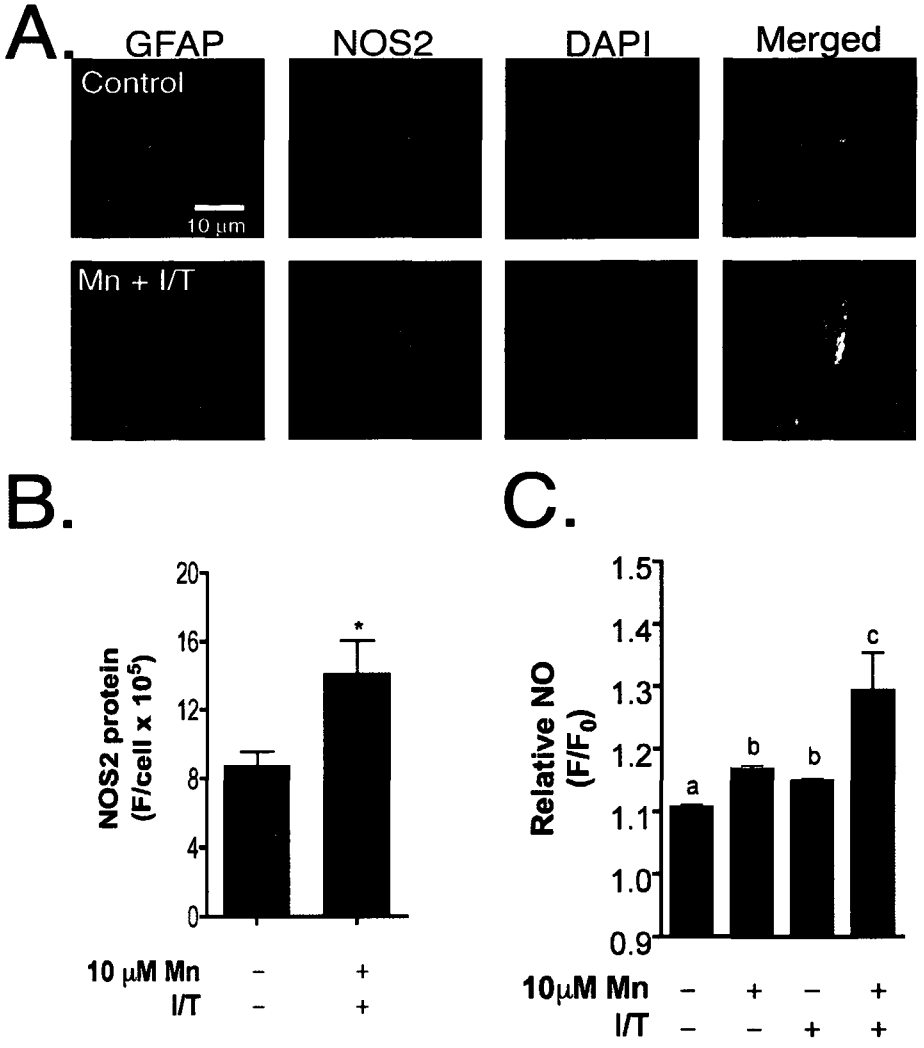


Figure 2. Manganese potentiates cytokine-induced expression of NOS2 protein and production of NO in astrocytes. (A) Astrocytes were exposed to saline, 10 μM Mn, 1000 pg/mL IFNγ + 10 pg/mL TNFα (I/T), or Mn + I/T for 8 hours and analyzed by immunofluoresence for expression of NOS2 protein. Representative images showing GFAP (green) and NOS2 (red) protein levels. Scale bar = 10 μm (B) Quantification of NOS2 fluorescence indicating significantly increased protein expression upon exposure to Mn + I/T compared to control in GFAP positive cells. (C) Astrocytes were exposed to Mn + I/T as in (A) and NO levels were determined by live-cell fluorescence imaging using the NO indicator DAF-FM. NO production was significantly increased by Mn and I/T individually and strongly potentiated by combinatorial treatment with Mn + I/T.

FIGURE 3
NF-κB activation

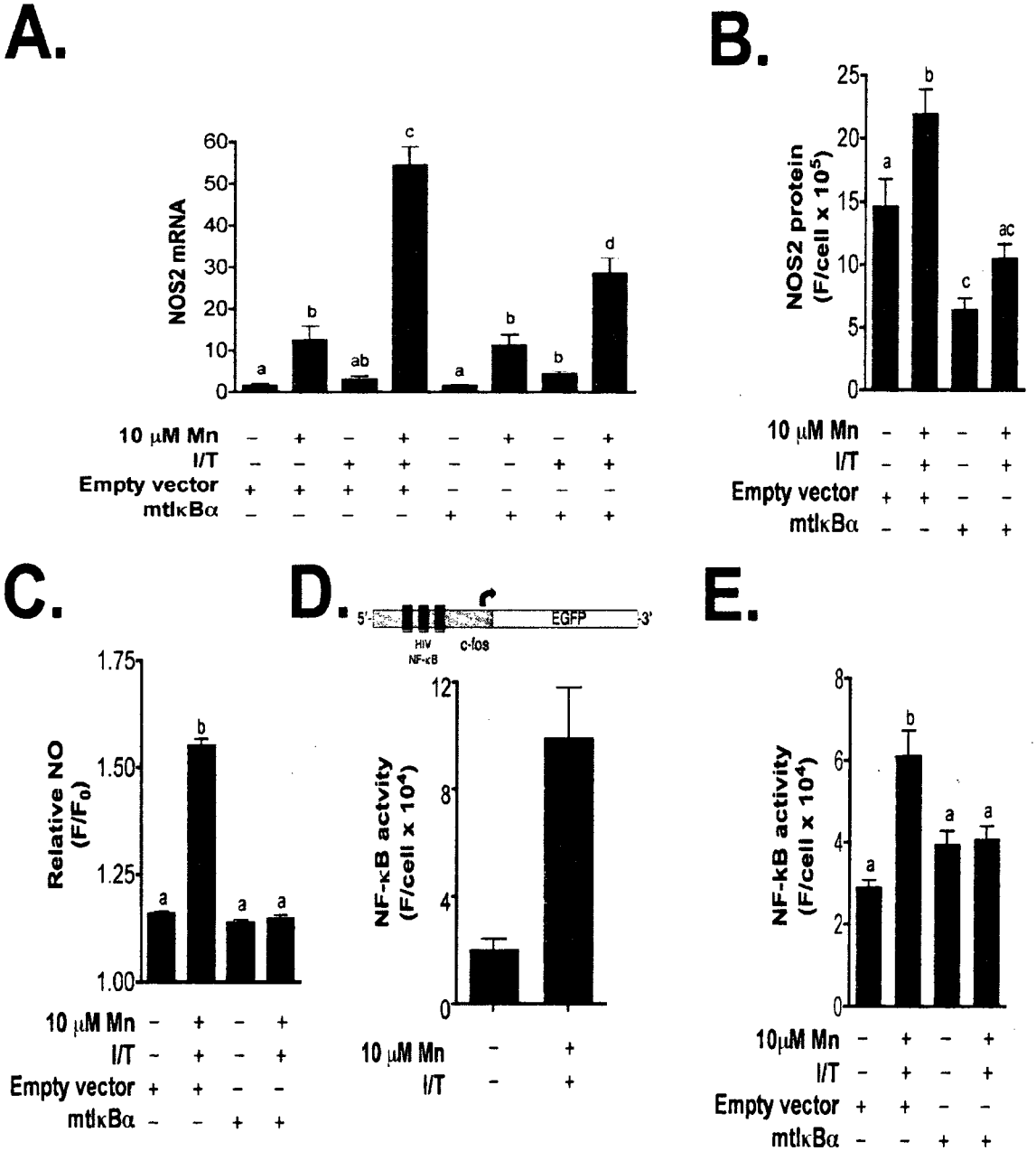


Figure 3. Manganese-induced potentiation of NOS2 expression and production of NO requires activation of NF-κB. Astrocytes were exposed for 8 hours to saline, 10 μM Mn, 1000 pg/mL IFNγ + 10 pg/mL TNFα (I/T), or Mn + I/T in the absence or presence of a dominant negative mutant of IκBα and examined for NOS2 expression and NO production. (A) Mutant IκBα significantly decreased expression of *Nos2* mRNA in astrocytes exposed to Mn + I/T compared to control vector. (B) Expression of NOS2 protein was prevented by mutant IκBα following exposure to Mn + I/T compared to the control vector treatment group. (C) Astrocytes were exposed to Mn + I/T and NO levels determined by live cell fluorescence imaging using the NO indicator DAF-FM. Expression of mutant IκBα prevented increases in NO in astrocytes exposed to Mn + I/T compared to control vector. (D) Inset; schematic representation of NF-κB-enhanced Green Fluorescent Protein (EGFP) reporter construct in transgenic astrocytes. Astrocytes were exposed to Mn + I/T and the total fluorescence intensity per cell was determined by live cell imaging. NF-κB activation is significantly increased by Mn + I/T. (E) NF-κB activation is decreased upon exposure of Mn + I/T in the presence of mutant IκBα compared to empty vector. Data represent at least three independent experiments. Different letters denote significant differences between groups. In panel (A), *ab* in column 3 denotes no significant difference from columns 1 and 2. In panel (B), *ac* in column 4 denotes no significant difference from columns 1 and 3.

FIGURE 4
MAPK signaling

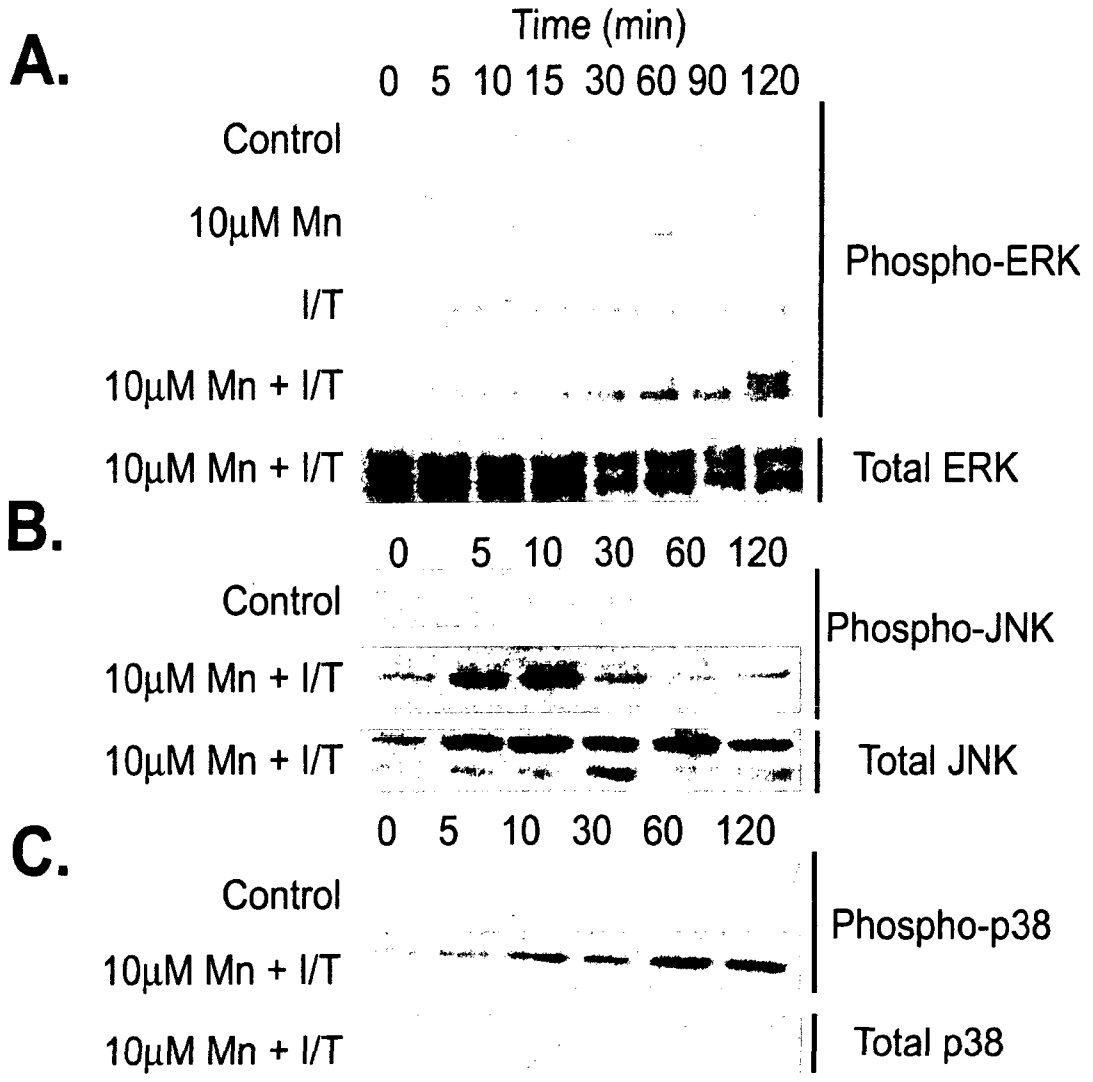


Figure 4. MAP kinase activation occurs in manganese-treated astrocytes. Phosphorylation of ERK, p38, and JNK was measured by immunoblotting in primary astrocytes treated with saline control, 10 μM Mn, 1000 pg/mL IFNγ + 10 pg/mL TNFα (I/T), or Mn + I/T in serum-free media (A) Phoshphorylation of ERK (P-ERK) was assessed in astrocyte exposure to saline, Mn, I/T or Mn + I/T over time (0-120 min). (B) Phospho-p38 (P-p38) in astrocytes treated with saline and Mn + I/T. (C) Phospho-JNK levels (P-JNK) in astrocytes exposure to saline or Mn + I/T. Mn enhanced time-dependent phosporylation of ERK, JNK, and p38. Membranes were re-probed for total ERK, p38 and JNK to control for protein loading.

FIGURE 5
MAPK signaling role on NO production

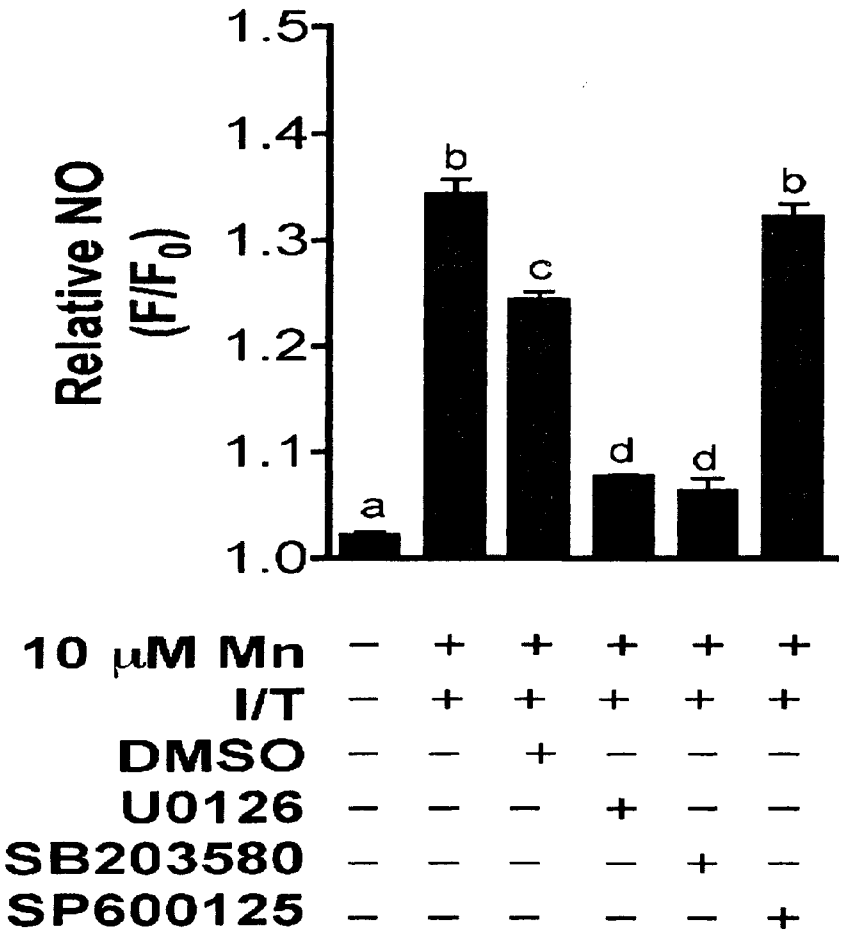


Figure 5. MAP kinase activation in manganese-treated astrocytes is required for potentiation of NO production.) NO levels in astrocytes exposed to Mn + I/T were determined in the presence of inhibitors for ERK (U0126;10 μM), p38 (SB203580; 30 μM) and JNK (SP600125; 10 μM). Inhibition of ERK and p38, significantly inhibited NO production.

FIGURE 6
ERK activation proceeds NF-κB activation

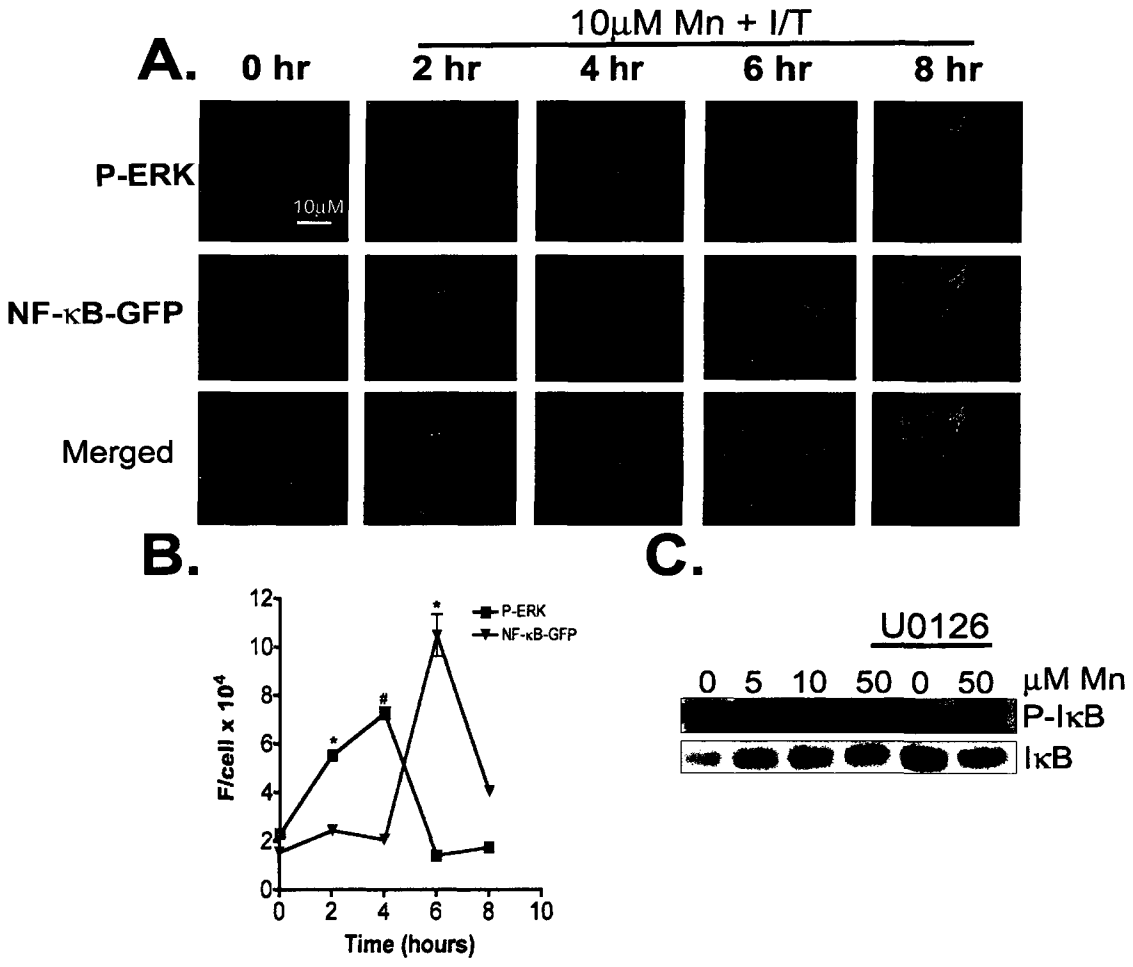
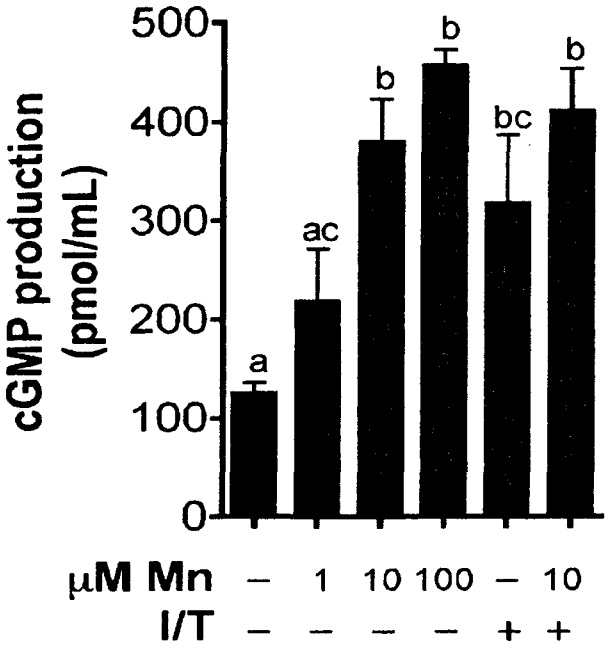


Figure 6. ERK is required for manganese-dependent activation of NF-κB. (A) Activation of ERK and NF-κB was simultaneously determined over time in NF-κB-GFP transgenic astrocytes by immunofluoresence. Images of transgenic NF-κB-GFP astrocytes exposed to 10 μM Mn and 1000 pg/ml IFNγ + 10 pg/ml TNFα (Mn + I/T) at 0, 2, 4, 6, or 8 hours depicting activation of NF-κB (green), phosphorylation of ERK (P-ERK, red), nuclear staining (DAPI, blue) and merged images. (B) Quantitative analysis of P-ERK and NF-κB-GFP over time indicates that phosphorylation of ERK peaks at 4 hrs, preceding the peak of NF-κB activation at 6 hrs. Groups were compared within each fluorescence channel by one-way ANOVA followed by Neuman-Keuls test ($p<0.05$). (C) Immunoblot for phosphorylation of IκBα (P-IκBα) in astrocytes exposed to 0 - 50 μM Mn, U0126 (10 μM), or 50 μM Mn + U0126 (10 μM). Blots were reprobed for total IκBα to control for protein loading and are representative of three independent experiments.

FIGURE 7
cGMP production



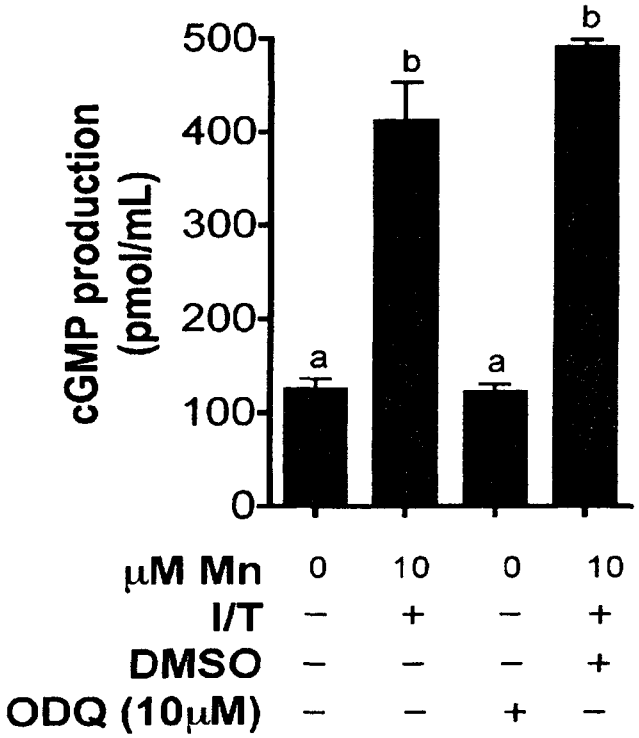
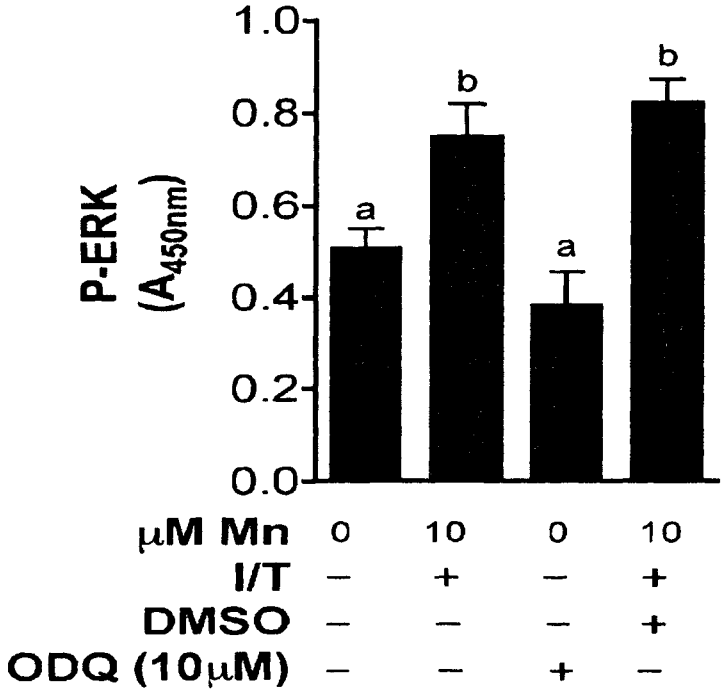


FIGURE 8
sGC and ERK activation



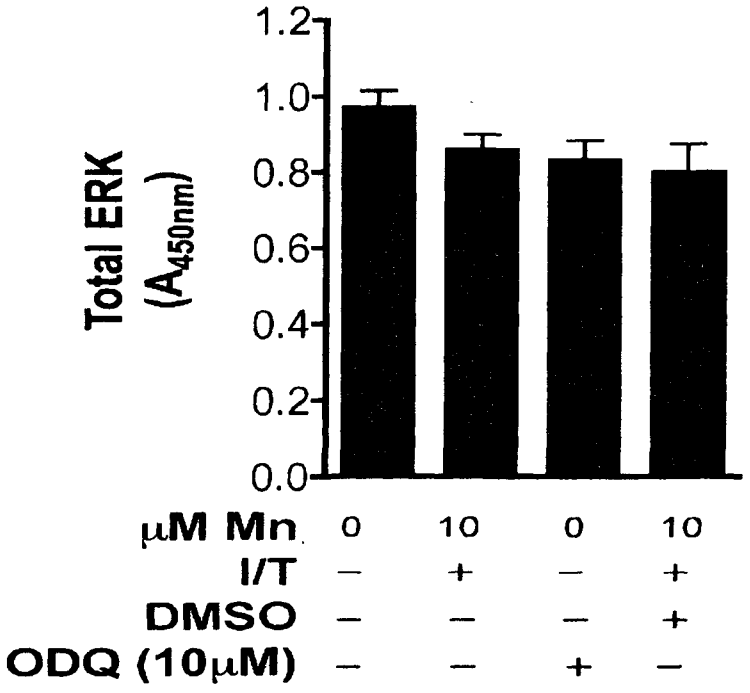
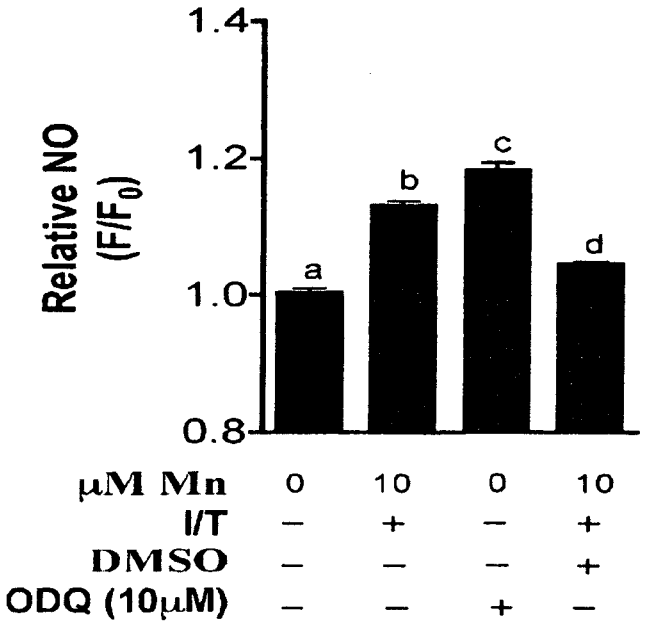


FIGURE 9
sGC on NF-κB activation and NO signaling



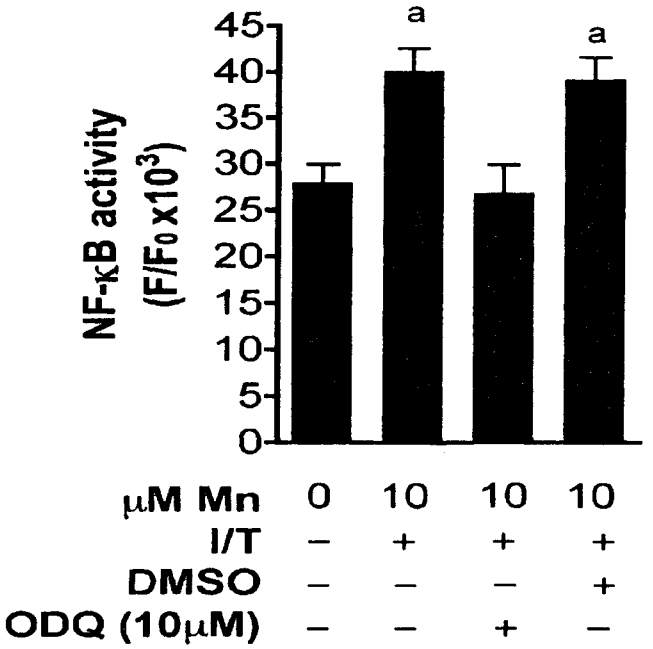


Figure 7. cGMP production increases with Mn exposure independent t of cytokines in astrocytes. (A) Astrocytes were treated for 8 hrs with 0, 1, 10, and 100 μM Mn or 10 and 100 μM Mn + 1000 pg/mL IFNγ and 10 pg/mL TNFα (I/T) and cGMP levels determined by ELISA. Mn significantly inceased intracellular cGMP at 10 and 100 μM that was not further enhanced by co-treatment with I/T. The EC_{50} of Mn-induced cGMP production in astrocytes was 1.1 ± 0.4 pmol/mL (inset). (B) Mn-induced increases in cGMP were inhibited by the sGC inhibitor ODQ (10 μM) following treatment with 10 μM Mn + I/T but not by vehicle control (DMSO). Significance is denoted by differing letters. $p < 0.05$

Fig. 8 Soluble guanylate cyclase (sGC) relays signals to ERK in Mn-treated astrocytes. Phosphorylation of ERK was determined by ELISA and was significantly decreased in astrocytes exposed to 10 μM Mn + I/T + ODQ compared to 10 μM Mn + I/T alone or 10 μM Mn + I/T + DMSO. (A) Total levels of ERK did not change between the treatment groups in (B). Significance is denoted by differing letters. $p < 0.05$

Figure 9. NF-kB and NOS are regulated by soluble guanylate cyclase (sGC) in astrocytes exposed to Mn and cytokines. (A)ODQ significantly decreased NO levels in astrocytes exposed to 10 μM Mn + I/T compared to 10 μM Mn + I/T alone or 10 μM Mn + I/T + DMSO. (B) Transgenic NF-κB-GFP astrocytes were exposed to 10 μM Mn + I/T with and without DMSO or ODQ; ODQ prevented activation of NF-κB upon treatment with Mn + I/T. Significance is denoted by differing letters. $p < 0.05$

FIGURE 10

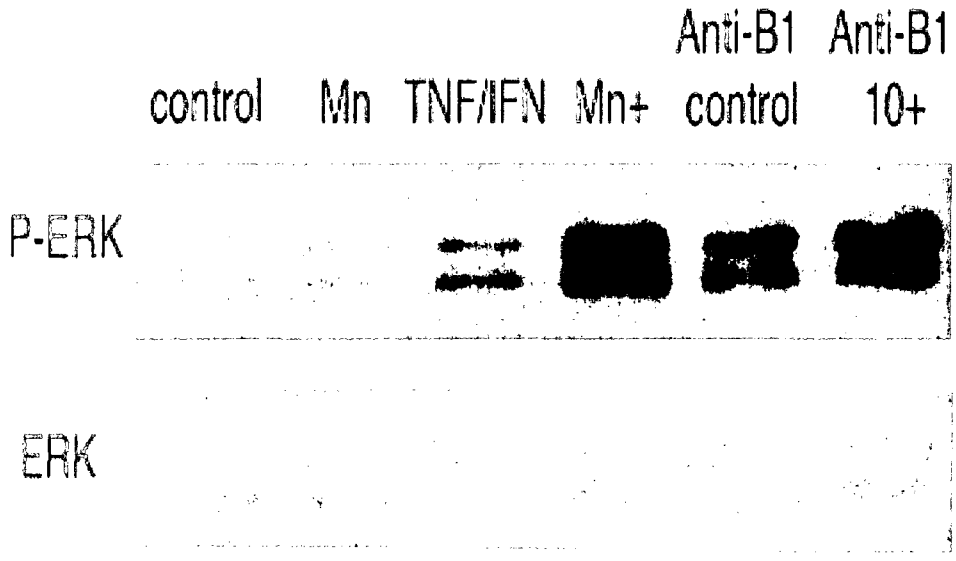


Figure 10. β1 integrin may play a role in activation of ERK with Mn and cytokine treatment of cortical astrocytes. Phosphorylation of ERK rapidly increased upon exposure to Mn and TNFα/IFNγ but was slightly abrogated by pretreatment with a blocking antibody to β1 integrin . Total ERK also shown for a loading control.

CHAPTER 3

AGE-DEPENDENT SUSCEPTIBILITY TO MANGANESE-INDUCED NEUROTOXICITY: THE ROLE OF GLIAL INFLAMMATORY RESPONSE

ABSTRACT

Chronic exposure to manganese (Mn) produces a neurodegenerative condition of the basal ganglia characterized by gliosis and expression of inducible nitric oxide synthase (NOS2). Induction of NOS2 causes overproduction of nitric oxide (NO) and injury to surrounding neurons, resulting in parkinsonian-like motor deficits. Inflammatory activation of astrocytes is believed to be an early event in Mn neurotoxicity but glial responses to Mn in developing animals remains poorly understood. In this study we investigated the effect of juvenile exposure to Mn in C57Bl/6 mice on the activation of astrocytes and microglia and production of NO, postulating that developmental Mn exposure would lead to selective sensitivity to gliosis and increased expression of NOS2 with concomitant overproduction of NO in adult mice exposed again later in life. Mice were exposed to saline, 10 mg/Kg or 30 mg/Kg Mn by daily intragastric gavage. Immunohistochemical analysis revealed increases in expression of IBA-1, a marker for microgliosis, proceeding GFAP, marker for astrogliosis, in the striatum (St), globus pallidus (Gp), and substantia nigra pars reticulata (SNpr) of treated mice compared to controls. Co-immunofluorescence studies demonstrated increased expression of NOS2 in both astrocytes and microglia upon treatment, as well as an increase of 3-nitro-tyrosine protein adducts, a marker for NO/ONOO- formation, in neurons of the in the striatal-pallidal pathway of Mn-treated adult mice pre-exposed as juveniles. These data indicate that sub-chronic exposure to Mn during development leads to inflammatory activation of microglia prior to astrogliosis with exposure leading to NOS2 expression,

resulting in elevated nitrosylation of neurons in the basal ganglia of juvenile and adult mice.

Keywords: manganese, glia, nitric oxide, juvenile exposure

INTRODUCTION

Manganese (Mn) is an essential nutrient and a cofactor for multiple enzymes required for normal cellular function in the central nervous system (CNS), including glutamine synthetase, mitochondrial superoxide dismutase, and pyruvate decarboxyase (Hearn et al., 2003; Martinez-Hernandez et al., 1977; Takeda, 2003). However, increased exposure to Mn through both dietary and inhalation routes leads to neurotoxicity with subsequent damage to the extrapyramidal motor nuclei of the basal ganglia. In scenarios of sustained high exposure, this neurotoxicity can lead to a degenerative motor disorder, termed manganism, that presents with neurological symptoms resembling those of Parkinson's disease (Yamada et al., 1986). It has been well described in adults that Mn toxicity progresses from cognitive and psychological deficits at lower exposures to irreversible neurodegeneration and motor dysfunction with higher exposures (Albin, 2000; Calne et al., 1994; Pal et al., 1999; Rodier, 1955). Occupational exposure to toxic levels of Mn has been recognized in factory workers, smelters, and more recently, welders (Bowler et al., 2006b). Environmental human exposure to Mn has also increased since the addition of methylcyclopentadienyl Mn tricarbonyl (MMT) an anti-knock agent in gasoline in both United States and Canada (Aschner and Allen, 2000; Mergler et al., 1999).

Much less is known regarding the effects of Mn in the developing CNS, particularly with respect to the potential risk posed by exposures early in life that may predispose to later neurological injury. Varying reports indicate that children exposed to moderate levels of Mn in drinking water present with cognitive deficits

and hyperactivity (Bouchard et al., 2007; Wasserman et al., 2006; Woolf et al., 2002), consistent with recent studies in non-human primates demonstrating neuronal degeneration and glial activation in multiple cortical and subcortical regions following Mn exposure (Guilarte et al., 2008b). Dietary intake studies have shown soy-based infant formula has approximately 200-fold more Mn than breast milk (Krachler et al., 2000) and brain Mn levels are also elevated by chronic iron deficiency, a major world health problem (Garcia et al., 2007) that is implicated in attention-deficit/hyperactivity disorder (ADHD) in children (Konofal et al., 2004). Although increased environmental and dietary exposures to Mn are recognized, the fundamental mechanisms leading to damage of the basal ganglia remain poorly understood.

Mn toxicity leads to an activation of both microglia and astrocytes that promote neuronal injury (Henriksson and Tjalve, 2000; Verity, 1999) but the mechanisms underlying Mn-induced gliosis are not fully understood. Prior studies of Mn neuropathology have shown an altered interaction between neurons and glial cells that contributes to neuronal injury, in part due to increased production of inflammatory mediators such as prostaglandins, inflammatory cytokines, and nitric oxide (NO) by reactive glia (Gonzalez-Scarano and Baltuch, 1999). NO is produced from L-arginine by nitric oxide synthases (NOS1, 2, and 3), of which the inducible isoform (NOS2) is highly upregulated in activated astrocytes and microglia. NOS2 is not constitutively expressed but is rapidly induced by stimuli such as pro-inflammatory cytokines (Stuehr and Griffith, 1992). Enhanced expression of NOS2 is a prototypic inflammatory response of activated

astrocytes and previous studies from our laboratory demonstrated that Mn strongly potentiates expression of NOS2 and production of NO in cytokine primed astrocytes, leading to apoptosis in co-cultured neurons *in vitro* and during Mn exposure *in vivo* (Liu et al., 2005; Liu et al., 2006).

Less is known regarding the kinetics of microglial activation in models of Mn neurotoxicity, as well as interactions between microlia and astrocytes that may promote reactive astrogliosis. Microglia are not uniformly distributed in the brain and are enriched in several regions including the basal ganglia (Kim et al., 2000; Lawson et al., 1990). Activated microglia also produce proinflammatory mediators such as NO, TNFα, and IL-1β (Carreno-Muller et al., 2003; Giovannini et al., 2002; Wu et al., 2002) and numerous *in vitro* studies in cultured microglia exposed to Mn have examined the signaling pathways responsible for regulation of these and other inflammatory genes, including cycloxgenase-2 (COX2) (Bae et al., 2006; Chang and Liu, 1999). However, the relative contribution of microglia and astroglia to increased expression of NOS2 and induction of nitrosative stress in neurons following exposure to Mn *in vivo* is not well understood.

We therefore postulated that exposure to Mn during development would induce distinct patterns of microglial and astroglial reactivity that would sensitize nuclei of the basal ganglia to greater expression of NOS2 and induction of nitrosative stress upon subsequent adult exposures. To address this hypothesis, we exposed C57Bl/6 mice to Mn by intragastric gavage as juveniles, adults, or both as juveniles and adults and examined multiple indices of glial reactivity and neuropathology. These studies revealed a striking sensitivity of the developing

basal ganglia to Mn-induced activation of both microglia and astrocytes that correlated with induction of NOS2 and increased nitration of neuronal proteins in the striatum, globus pallidus, and substantia nigra pars reticulata. Early exposure to Mn did not greatly increase glial reactivity following subsequent adult exposures, suggesting that juvenile development represents a critical window of sensitivity to inflammatory activation of glia and induction of nitrosative stress.

MATERIALS AND METHODS

Reagents. All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Primary antibodies for glial fibrillary acidic protein (GFAP) were from Dako Cytomation (Denmark) and Sigma (St. Louis, MO). Antibodies for ionizing Ca^{2+} binding adaptor molecule-1 (IBA-1) and inducible nitric oxide synthase (NOS2) were from Wako Chemicals Inc. (Osaka, Japan), and BD Biosciences (San Jose, CA), respectively. Primary antibodies to 3-nitrotyrosine (3-NTyr) were from Upstate, (Charlottesville, VA), dopamine cAMP regulated phosphoprotein- 32 DARPP32 from Chemicon (Burlington, MA), and Major Microtuble Association Protein 2 (MAP2) from Abcam (Cambridge, MA). Horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine reagents were part of the Vectastain ABC kit from Vector Labs (Burlingame, CA). AlexaFluor-488 and 568–labeled secondary antibodies were from Invitrogen (Eugene, OR).

Animal exposure model. Male and female C57Bl/6J mice were housed in microisolator cages (five animals per cage) and kept on 12-h light/ dark cycles with access to laboratory chow and water *ad libitum*. Littermates from timed pregnant dams were paired in control and Mn-exposed groups and received 0.9% normal saline, 10mg/Kg $MnCl_2$, or 30mg/Kg $MnCl_2$ by gastric gavage daily during the following time periods: juvenile exposure, day 20-34 postnatal; adult

exposure, from week 12-20; and juvenile + adult exposure day 20-34 postnatal and week 12-20. Animals were weighed prior to each gavage and the amount of Mn delivered was adjusted accordingly. Additionally, the amount of Mn delivered was adjusted for the molar concentration in the tetrahydrate form so as to achieve a precise dose of 10 or 30 mg/Kg. All procedures were performed under the supervision of the Animal Care and Use Committee at the Colorado State University and were approved prior to onset of the studies.

Immunohistochemistry and immunofluorescence. Mice were anesthetized by inhalation of isofluorane and perfused intracardially with 4% paraformaldehyde in 0.1M $NaKPO_4$ buffer (pH 7.4) and tissue processed for immunohistochemical analysis as published previously by our laboratory (Liu et al., 2006; Moreno et al., 2008b). Briefly, brains were collected and kept in 4% paraformaldehyde overnight and stored in 0.1M $NaKPO_4$ buffer at 4°C. Paraffin embedded 10 µM coronal serial sections were examined for protein expression in substantia nigra pars reticulata (SNpr), globus pallidus (Gp), and striatum (St) by immnunohistochemistry using primary antibodies to GFAP (1:400) and IBA-1 (1:500). Sections were developed using horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine reagents from the Vectastain ABC Kit. For co-immunofluorescence studies, sections were incubated with anti-IBA1 (1:1000) or anti-GFAP (1:500) combined with anti-NOS2 (1:100) to examine gliosis. Anti-MAP-2 (1:500) and DARPP-32 (1:300) antibodies were used in combination with anti-3-nitrotyrosine (1:100) to levels of protein nitration in

neurons. Specific protein epitopes were visualized with secondary antibodies labeled with AlexaFluor- 488 or -568 and slides were mounted in media containing DAPI to identify cell nuclei. Images were acquired using either a Zeiss 20X or 40X air PlanApochromat objective and 6 – 8 microscopic fields were examined per treatment group in 2 – 4 animals per group.

Statistical Analysis. Comparison of two means was performed by Student's *t*-test. Comparisons three or more means was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison *post-hoc* test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). For all experiments, $p < 0.05$ was considered significant.

RESULTS

Astroglial activation in juvenile and adult mice exposed to Mn was determined by pathological scoring based upon expression of glial fibrillary acidic protein (GFAP) and astrocyte morphology in 10 μm serial sections through the striatum (St), globus pallidus (Gp), and substantia nigra pars reticulata (SNpr), the three basal ganglia nuclei most vulnerable to Mn exposure (Josephs et al., 2005; Liu et al., 2006). As in other Mn models there was no change in tryosine hydroxylase (TH) expression in the SN or St (data not shown). Immunohistochemistry slides were scored by a veterinary pathologist blinded to the experimental treatment groups. The elevation in expression of GFAP and change in astrocyte morphology was scored on a scale of 1-5. A baseline level of GFAP activation was observed in the components of all the basal ganglia analyzed; St, Gp, SNpr (Fig. 12). Juvenile controls showed an equivalent level of activation in the control and treatment groups (10 mg/Kg and 30 mg/Kg $MnCl_2$) in St and Gp brain regions and a slight decrease in activation in the SNpr. In contrast to juveniles, mice treated only as adults had a significant increase in activation of GFAP in both the SNpr and St at both treatment groups (0.5±0.16, control vs. 4.1±0.04, 10 mg/Kg $MnCl_2$ and 3.4±0.24 30 mg/Kg $MnCl_2$ for SNpr; 0.3±0.13, control vs. 1.8±0.08, 10 mg/Kg $MnCl_2$ and 1.3±0.05 30 mg/Kg $MnCl_2$ for St). Mice exposed as juveniles and adults had a significant increase in activation of astroglia in the Gp at 30 mg/kg $MnCl_2$ (2.5±0.12 vs. 3.9±0.15) and SNpr at both exposure levels (1.0±0.0 control, vs. 1.8±0.04 10 mg/Kg $MnCl_2$ and 2.8±0.06 30 mg/Kg $MnCl_2$) (Table 2). No activation of astrocytes was observed

in the substantia nigra pars compacta in any treatment group (data not shown). Intense GFAP staining was detected in both the Gp and SNpr of 30 mg/kg Mn-treated mice exposed as juveniles and adults, with brain regions containing astrocytes undergoing hyperplasia (Figure 12). In contrast to the St and SNpr, the Gp brain region in control animals had well defined, fibrous astrocytes, with more intense GFAP expression and was scored accordingly 2.5±0.12 (Gp) vs. 1.3±0.06 (St) and 1.0±0.0 (SNpr) (Table 1).

Activation of microglia was similarly examined by pathological scoring in the St, Gp, and SNpr, based upon expression of the ionizing Ca^{2+} binding adaptor molecule-1 (IBA-1) and cellular morphology of microglia. The relative degree of microglial activation was assigned a numerical value on a scale of 0 - 3. An increase in activation of Iba-1-positive microglia was observed only in juvenile mice exposed to both 10 mg/Kg and 30 mg/Kg Mn (Table 1). The microglia underwent a morphology change, a retraction and thickening of the processes occurred upon treatment with Mn (Figure 11). Juvenile animals exposed to 10 mg/Kg and 30 mg/Kg $MnCl_2$ had significantly higher pathology scores than control animals in all three brain regions (0.33±0.09, control vs. 1.5±0.06 10 mg/Kg $MnCl_2$, and 1.4±0.08 30 mg/Kg $MnCl_2$ for Gp; 0.0±0.0, control vs. 1.3±0.0410 mg/Kg $MnCl_2$, and 1.2±0.06 30 mg/Kg $MnCl_2$ for St; 0.67±0.06, control vs. 1.6±0.06 mg/Kg $MnCl_2$, and 1.3±0.13 30 mg/Kg $MnCl_2$ for SNpr). Mice exposed only has adults had significant IBA-1 expression at the highest exposure, 30 mg/kg $MnCl_2$ compared to controls (Gp: 0.8±0.13 vs. 2.8±0.10, St: 0.8±0.16 vs. 2.7±0.1, SNpr: 0.86±0.05 vs. 2.2±0.07). Finally, no microglia

activation was observed in the exposure group treated as juveniles and adults (Table 1).

The expression of NOS2 was examined in activated astrocytes by co-immunofluorescence (Figures 15 and 16). Expression of NOS2 was increased in GFAP-positive cells in Mn-treated animals compared to controls in the SNpr of all 3 exposure groups; juvenile, adult and juvenile + adult exposure groups (Figure 3A-C). The treatment of 30 mg/Kg $MnCl_2$ caused processes of the astrocytes to thicken in all three exposure groups (Figure 15A-C). Quantification of the number of NOS2-positive astrocytes as a percentage of the total number of GFAP-positive cells indicated that NOS2 expression increased significantly in activated *astrocytes upon juvenile exposure to 30 mg/Kg $MnCl_2$ in the St, Gp and SNpr* (Figure 16A). In contrast to mice exposed to 30 mg/Kg $MnCl_2$ as both juveniles and adults, mice exposed to this dose of $MnCl_2$ solely as adults had an increase in astrocytic NOS2 expression only in the Gp and SNpr brain regions but not in St (Fig. 16B). Interestingly, mice exposed as both juveniles and adults had significantly increased NOS2 expression in GFAP positive cells at both 10 mg/Kg and 30mg/Kg $MnCl_2$ (Fig 16C), whereas mice exposed only as adults displayed no change in astrocytic expression of NOS2 at 10 mg/Kg $MnCl_2$.

IBA-1-positive microglia were also examined for expression of NOS2 in the Gp, St, and SNpr of control and Mn-treated juvenile and adult mice (Figures 13 and 14). Representative images from the SNpr are presented in Figure 13 for each group of mice following exposure to 0 or 30mg/kg $MnCl_2$. Microglia in sections from control mice had a ramified phenotype with multiple fine processes

characteristic of resting cells and were without evident expression of NOS2 (Figure 13 A – C). In contrast to microglia in control mice, Iba-1-positive cells in mice exposed to 30 mg/Kg $MnCl_2$ displayed an activated phenotype characterized by a thickened, ameboid appearance with fewer cytoplasmic processes and marked expression of NOS2. Quantification of the percent of total Iba-1-positive cells co-expressing NOS2 in each treatment group is presented in Figure 14. Mice exposed to Mn as juveniles had increased numbers of IBA-1-positive cells in the St at 30 mg/Kg $MnCl_2$ and in the Gp and SNpr at both 10 mg/Kg and 30 mg/Kg $MnCl_2$ (Fig 14A). In mice exposed to Mn only as adults, the percent of IBA-1 positive cells co-expressing NOS2 was increased only in the SNpr at 30 mg/Kg Mn (Fig. 14B). In the absence of juvenile exposure, adult exposure to Mn did not result in increased microglial expression of NOS2 at either 10 mg/Kg or 30 mg/Kg $MnCl_2$ in the St and Gp. Mice receiving Mn exposure as juveniles + adults displayed an increase in NOS2 expression in IBA-1-positive cells at both 10mg/Kg and 30mg/Kg $MnCl_2$ in all three brain regions examined, the St, Gp and SNpr (Fig. 14C).

To determine effects of increased expression of NOS2 in asrocytes and microglia, formation of neuronal 3-nitrotyrosine (3-NTyr) protein adducts was evaluated by immunofluorescence as an indicator of NO production and $ONOO^-$ (peroxynitrite) formation, a general measure of nitrosative stress. Neurons were identified by staining with the general neuronal marker, MAP-2, and co-localization was determined by overlaying images of 3-NTyr and MAP-2. Figure 17 depicts representative images from the SNpr of each treatment group of mice

at 0 mg/Kg and 30 mg/Kg $MnCl_2$. Only low levels of 3-NTyr adducts were detected in control juvenile or adult animals (Figure 17 A – C, 0 $MnCl_2$ panels), whereas exposure to 30 mg/Kg $MnCl_2$ resulted in large increases in immunofluorescence staining for 3-NTyr protein adducts in MAP-2-positive neurons in the SNpr, primarily in soma but also in the neuropil and in dendrites (Figure 17 A – C, 30 $MnCl_2$ panels). Although some level 3-NTyr staining was detected even in control sections, levels of 3-NTyr protein adducts were increased in the Gp and SNpr of juvenile mice exposed to 30 mg/Kg Mn (Figure 8A), in the Gp of mice exposed only as adults to 30 mg/Kg Mn (Figure 18B), and in the SNpr of mice exposed as both juveniles and adults (Figure 18C). The regional pattern of neuronal 3-NTyr formation therefore appeared to depend upon the age at which the animals were exposed to Mn, as well as the time-point at which analysis of protein nitration was performed.

Protein nitration was also examined in the St by co-immunofluoresence for 3-NTyr protein adducts and DARPP-32, a marker for striatal interneurons expressing D1 and D2 dopamine receptors (Reiner et al., 1998) (Fig. 19 & 20). No change in protein nitration was detected in DARPP-32-positve striatal neurons in either juvenile or adult mice exposed to 30 mg/Kg $MnCl_2$ (Fig. 20A,B) but increases in 3-NTyr adducts were evident in mice exposed to 30 mg/Kg $MnCl_2$ as both juveniles and adults (Fig. 20C).

DISCUSSION

Inflammation of the brain is of great concern in many motor disorders with injury in the basal ganglia such as manganese toxicity and Parkinson's disease. Within the basal ganglia, the striatum receives most of the motor neuronal input while the Gp and SNpr are the two major output nuclei (Saka et al., 2002.) Prior studies in our laboratory demonstrated that Mn causes neuronal injury in the striatal-pallidal system in adult mice (Liu et al., 2006) but the role of gliosis in developmental vulnerability to neuroinflammatory injury from Mn has yet to be determined. The present studies activation of microglia and astrocytes and the role of the inflammatory mediator, nitric oxide (NO), on neuronal nitrosative stress in developing and adult mice. Developmental exposure to Mn resulted in an increase in activation of both microglia and astrocytes in young mice and enhanced the glial response to Mn in adult mice that were pre-exposed as juveniles, indicated by greater expression of NOS2 and extent of neuronal protein nitration in the basal ganglia.

Juvenile exposure to Mn causes an increase in microgliosis at both 10 and 30 mg/Kg $MnCl_2$, whereas increased activation of astrocytes was only detected in adult animals that had been previously exposed as juveniles, suggesting a requirement for prior activation of microglia in promoting astroglial activation following Mn exposure (Table 1 and 2). It was previsously reported that microglia divide prior to astrocytes during brain injury (Norton, 1999). The data reported here seemed at first to be at odds with this model, because mice exposed only as adults did have activated microglia at 30 mg/Kg $MnCl_2$ but the juvenile + adult

exposure group did not. However, it is important to note the temporal nature of microglial activation, which occurs rapidly after stress and injury and then subsides (Liberatore et. al., 1999). Thus, it is likely that the initial stress response to Mn in adult animals without prior exposure as juveniles resulted in a robust microglial responses, as well as detectable astrogliosis, whereas adult mice with previous exposure to Mn as juveniles were not only sensitized to exposure, but also that the Mn-induced lesion had likely progressed beyond the initial phases of injury and microglial activation to a later stage with only activated astroglia remaining detectably increased. This phenomenon of microglia activation proceeding astrocyte activation has been shown in previous *in vivo* MPTP studies where microglia activation occurred between days 1 and 14 proceeding exposure while astrocyte activation was not found until day 21 after exposure (Kohutnicka et al., 1998) along with activated microglial secretion of inflammatory cytokines leading to astroglial scarring found *in vitro* (Giulian and Lachman, 1985). While microgliosis did not occur upon exposure as a juvenile + adult, astrogliosis was seen in both the SNpr and Gp. Previous literature indicates that the Gp is the most susceptible brain region in Mn neurotoxcity (Josephs et al., 2005; Olanow and Tatton, 1999; Yamada et al., 1986) explaining the increase in gliosis, NOS2, and nitration observed during this study with treatment in the Gp region. A decrease in activation of astrocytes is observed in the SNpr more than likely a cause of exposure to Mn inhibiting the normal proliferation of astrocytes (Table 2).

Multiple *in vitro* and *in vivo* studies identify NOS2 as a key inflammatory gene involved in Mn neurotoxcity (Bae et al., 2006; Liu et al., 2006; Moreno et al., 2008a) but little is known on the role of glial cells and the specific brain regions expressing the gene as well as NOS2 role in developmental sensitivity. An increase of NOS2 protein expression in GFAP positive cells indicate that the activation of astrocytes through Mn exposure leads to inflammatory signaling in brain regions involved in manganism. Young mice exposed to 30 mg/Kg $MnCl_2$ had a significant amount of NOS2 protein expression in astrocytes that were GFAP positive (Fig. 16A) in the three brain regions, but only the Gp and SNpr were vulnerable for NOS2 protein expression in the adult exposure group (Figure 16B). Unlike the juvenile and adult only groups mice exposed as juveniles and again as adults had a significant increase in NOS2 expression in GFAP positive cells in both the 10 mg/Kg and 30 mg/Kg $MnCl_2$ treatment groups indicating that prior exposure to Mn does make mice more susceptible to NOS2 expression in astrocytes at a lower dose of Mn (ie. 10 mg/Kg) (Fig. 16C).

Activated microglia were shown to have an increase in NOS2 protein expression in juvenile exposure group with the SNpr being the most susceptible brain region due to both doses showing an increase of NOS2 expression. SNpr is a nuclei of the basal ganglia that is known to accumulate Mn and involved in the neuronal motor pathway, therefore an increase in microglia NOS2 expression leads to inflammatory signaling in a vital area of the brain needed for normal motor movement (Albin et al., 1989; Grillner et al., 2005). The adult only exposure group had a significant change with the SNpr as well, but the number

of IBA-1 positive cells was extremely small (approx. 5-6 cells) in the control groups and of those IBA-1 positive cells ~35-50% were NOS2 positive, making our control percents increased for the adult exposure group leading to no significant change in the St and Gp brain regions (Fig. 14B). The percent of IBA-1 positive cells co-expressing NOS2 was significantly increased with Mn exposure equally for 10 mg/Kg and 30 mg/Kg $MnCl_2$ doses in the juvenile + adult group unlike the GFAP positive cells with only the 30 mg/Kg $MnCl_2$ expressing NOS2. It is well known that microglia exposed to Mn secrete inflammatory cytokines (Chang and Liu, 1999; Filipov et al., 2005) which leads to activation of astrocytes with subsequent release of NO and prostaglandins (Hirsch et al., 1998; Moreno et al., 2008a; Spranger et al., 1998), therefore at the lower dose of Mn (10 mg/Kg) the microglia are activated and expressing NOS2 while the astrocytes are not affected.

Glia cells are essential for the survival of neurons, play a vital role in the uptake of glutamate, glycine, and g-aminobutyric acid (GABA), and antioxidant defense, via astrocytic production of glutathione (Aschner, 1998; Aschner et al., 1994; Pekny and Nilsson, 2005). Accordingly, excessive Mn disrupts both glutamate uptake (Hazell and Norenberg, 1997) and mitochondrial function (Gavin et al., 1999; Gunter et al., 2006) in cultured astrocytes. Deprecations in these and other trophic functions in reactive glia may act in concert with overproduction of inflammatory mediators to impair neuronal homeostasis. The accumulation of NO in the extracellular space produced by the glia, combined with superoxide, leads to the formation of the neurotoxin, peroxynitrite (Huie and

Padmaja, 1993; Koppenol et al., 1992). Once peroxynitrite is formed its well known that the molecule has a strong affinity to nitriate tyrosine residues in proteins (Ischiropoulos, 2003) and has been shown to lead to neuronal injury in a number of models of neurological disorders, including ischemia, Alzheimer's and Parkinson's disease (Carbone et al., 2008; Hensley et al., 1998; Ma et al., 2007). In order to examine neuronal injury induced by glial-derived NO, the general neuronal marker, MAP2, was utilized in combination with 3-NTyr to examine the role of the subsequent NO produced by the glial expressing NOS2. Co-immunofluorescence indicated that juvenile exposure group was the most susceptible to neuronal 3-NTyr protein formation in the Gp and SNpr while only the SNpr was vulnerable in the pre-exposed adult group. These results give insight into the brain regions most susceptible to glial induced injury to the neurons and that young mice were more vulnerable than adults pre-exposed as juveniles.

To address a more specific neuronal subtype projecting in the pathway well known to be damaged in Mn toxicity we examined the amount of 3-NTyr protein expression in neurons involved in the striatal-pallidal pathway. The neuronal marker, DARRP32 associates with dopamine 1 (D1) receptors on neurons innervating two major striatal projections neurons going to the pallidum and SNpr (Reiner et al., 1998). Therefore our results of an increase NOS2 expression in neurons that are projecting to the SNpr and Gp in our pre-exposed adult group, indicate that the neuronal injury is occurring in neurons essential to motor pathways affected in Manganism. It is also expressed in a review Kuhn et

al., that the nitration process of dopaminergic neurons maybe a late occurring event which could be why the increase of 3-NTyr expression in DARPP32 positive neurons did not occur until the mice who were pre-exposed as juveniles were exposed again as adults (Kuhn et al., 2004).

These studies demonstrate that exposure of mice to moderate levels of Mn by intragastric gavage at a young age, approximately equilivant to a 2 year old child, increases the vulnerability to glia induced NOS2 expression and subsequent nitration of neuronal proteins during a period of life vital for neuronal development (Davison and Dobbing, 1966; Tong et al., 2002). In addition to juvenile susceptibility it was shown that adults pre-exposed as juveniles and then again as adults were not more vulnerable for glia activated induced NOS2 expression but were more susceptible to neuronal tyrosine nitration in the key neurons in the striatial-pallidal pathway. Through these observations we have identified a critical window of sensitivity to Mn exposure and the role of gliosis and glial-derived NO on neuronal pathology in the basal ganglia.

CHAPTER 3

FIGURES AND TABLES

TABLE 1

Table 1. Pathological scoring of microgliosis in specific brain regions exposed to Mn *in vivo*.

		Pathology Score (*IBA-1)		
Time of Exposure	**Treatment ($MnCl_2$)**	**Gp**	**St**	**SNpr**
Juvenile	0 mg/kg	0.33±0.09	0.0±0.0	0.67±0.06
	10 mg/kg	1.5±0.06†	1.3±0.04†	1.6±0.06†
	30mg/kg	1.4±0.08†	1.2±0.06†	1.3±0.13†
Adults	0 mg/kg	0.8±0.13	0.8±0.16	0.86±0.05
	10 mg/kg	1.5±0.17	1.7±0.08	0.6±0.08
	30 mg/kg	2.8±0.10†	2.7±0.1†	2.2±0.07†
Juvenile + Adult	0 mg/kg	0.8±0.08	0.5±0.05	1.1±0.03
	10 mg/kg	0.6±0.11	0.5±0.11	1.1±0.03
	30 mg/kg	0.5±0.30	1.10±0.09	1.7±0.09

*No microglia= 0 *Activated microglia= 3

Data shown are means ± SEM ($n \geq 3$). Significance compared to controls within a specific brain region (GP, ST, or SNpr) per exposure group is denoted by †.

FIGURE 11
Microgliosis

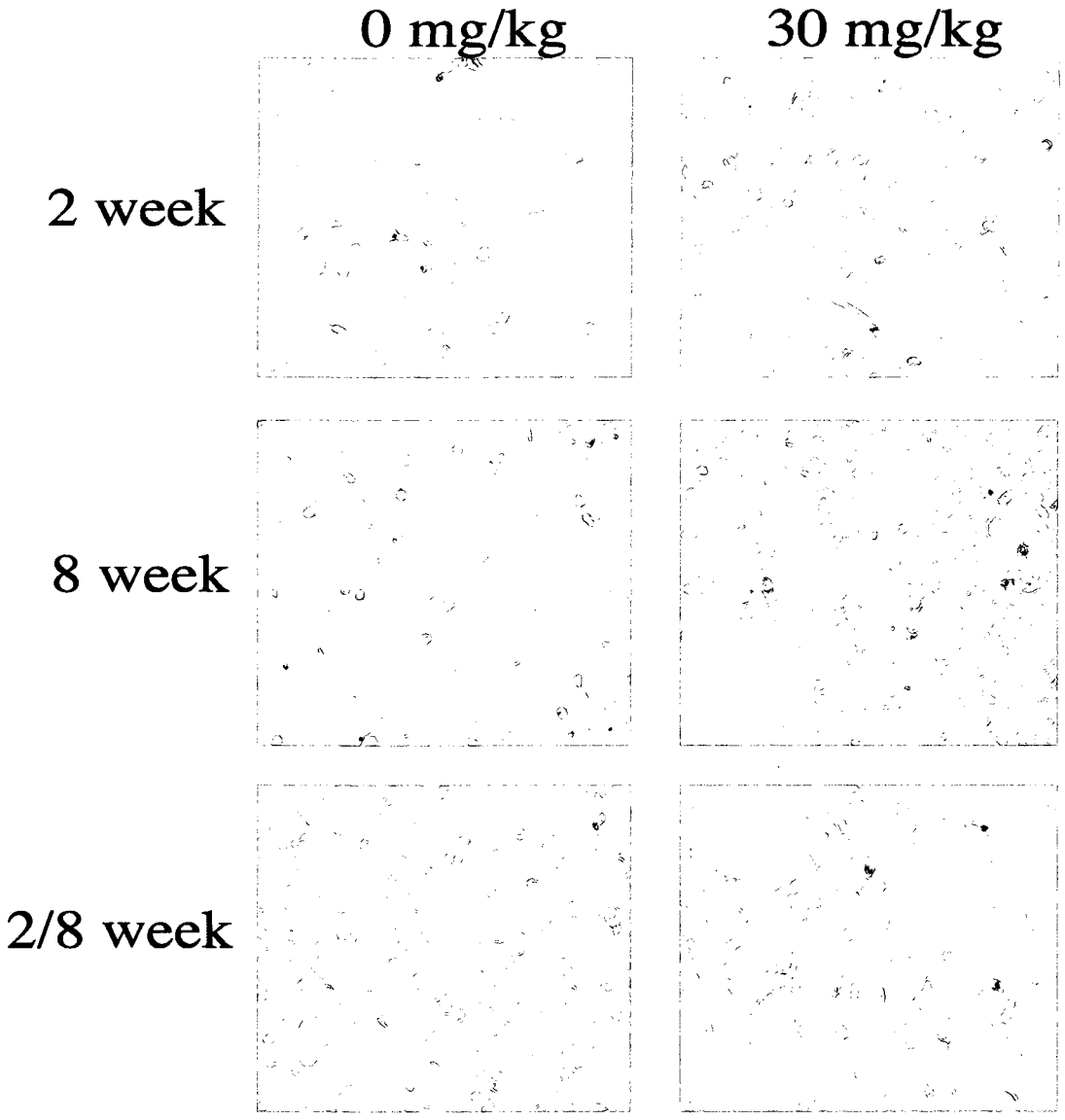


Figure 11. Differential exposure to Mn in juvenile and adult C57Bl/6J mice induces distinct patterns of microgliosis in the basal ganglia. Mice were exposed to 0, 10, or 30 mg/Kg $MnCl_2$ by daily intragastric gavage from day 21 – 34 postnatal (juvenile), week 12 – 20 (adult), or day 21 –34 postnatal plus week 12 – 20 (juvenile + adult). Multiple brain regions in the basal ganglia were assessed for activation of microglia by immunohistochemical staining for Iba-1 including the striatum (St), globus pallidus (Gp), and and substantia nigra pars reticulata (SNpr). Representative images of the SNpr are depicted for control mice and though exposed to 30mg/kg $MnCl_2$ as juveniles (A), adults and (B), or both juveniles and adults. Scale bar = 10 μm.

TABLE 2

Table 2. Pathological scoring of astrogliosis in specific brain regions exposed to Mn *in vivo*.

(GFAP*)

Time of Exposure	Treatment ($MnCl_2$)	Gp	St	SNpr
Juvenile	0 mg/kg	3.5±0.26	1±0.33	3.5±0.07
	10 mg/kg	3.9±0.07	1.8±0.11	3.2±0.06
	30 mg/kg	3.6±0.11	1.4±0.06	2.4±0.04†
Adults	0 mg/kg	2.6±0.19	0.3±0.13	0.5±0.16
	10 mg/kg	3.5±0.35	1.8±0.08†	4.1±0.04†
	30 mg/kg	3.2±0.23	1.3±0.05†	3.4±0.24†
Juvenile + Adult	0 mg/kg	2.5±0.12	1.3±0.06	1.0±0.0
	10 mg/kg	2.1±0.04	1.0±0.02	1.8±0.04†
	30 mg/kg	3.9±0.15†	1.8±0.07	2.8±0.06†

*Normal Astrocyte= 1 *Activated Astrocyte= 5

Data shown are means ± SEM ($n \geq 3$). Significance compared to controls within a specific brain region (Gp, St, or SNpr) per exposure group is denoted by †.

FIGURE 12
Astrogliosis

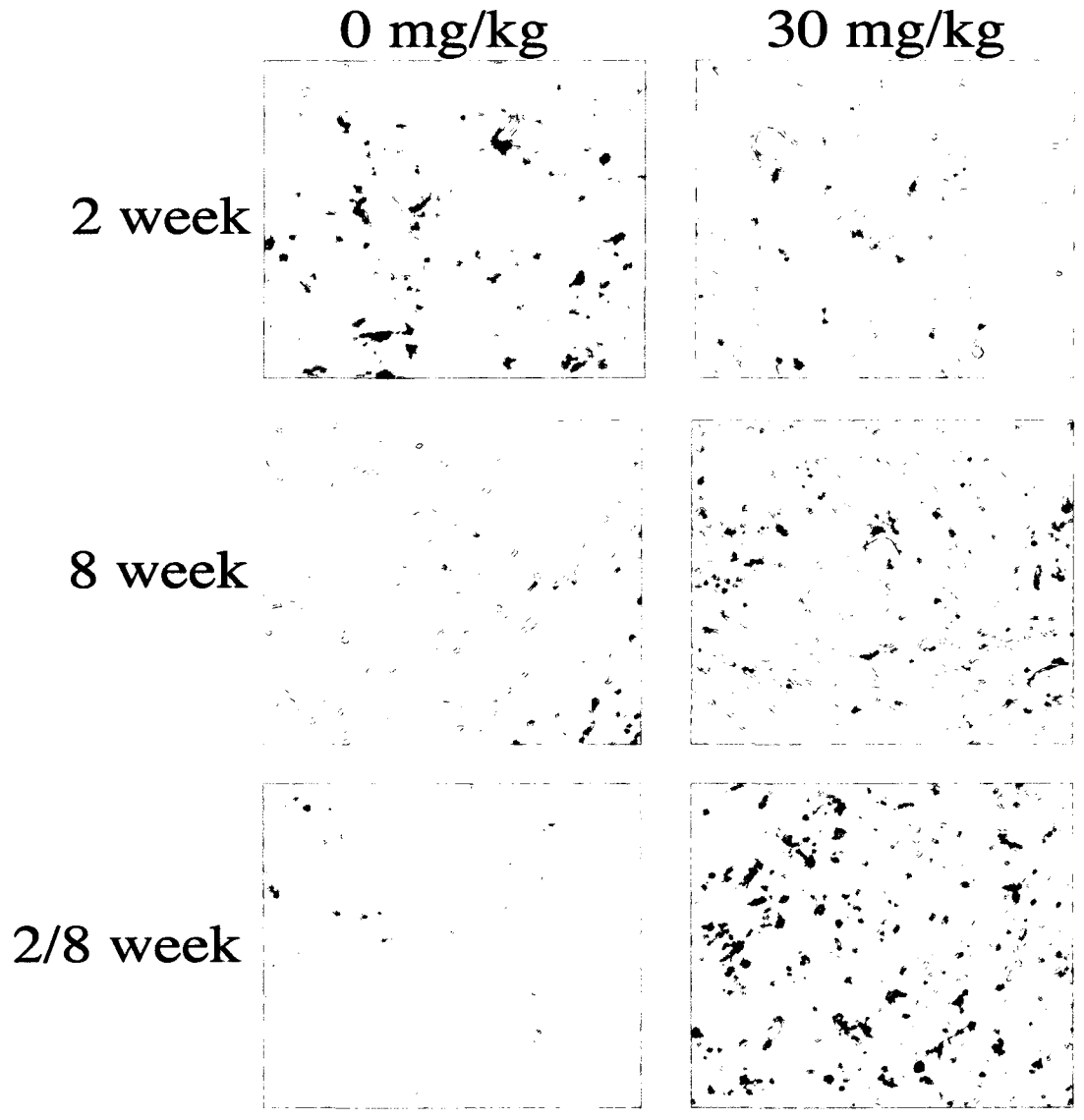


Figure 12. Differential exposure to Mn in juvenile and adult C57Bl/6J mice induces distinct patterns of astrogliosis in the basal ganglia. Mice were exposed to 0, 10, or 30 mg/Kg $MnCl_2$ by daily intragastric gavage from day 21 – 34 postnatal (juvenile), week 12 – 20 (adult), or day 21 –34 postnatal plus week 12 – 20 (juvenile + adult). Multiple brain regions in the basal ganglia were assessed for activation of astroglia by immunohistochemical staining for GFAP including the striatum (St), globus pallidus (Gp), and and substantia nigra pars reticulata (SNpr). Representative images of the SNpr are depicted for control mice and though exposed to 30mg/kg $MnCl_2$ as juveniles (A), adults and (B), or both juveniles and adults. Scale bar = 10 μm.

FIGURE 13
Microglia expressing NOS2

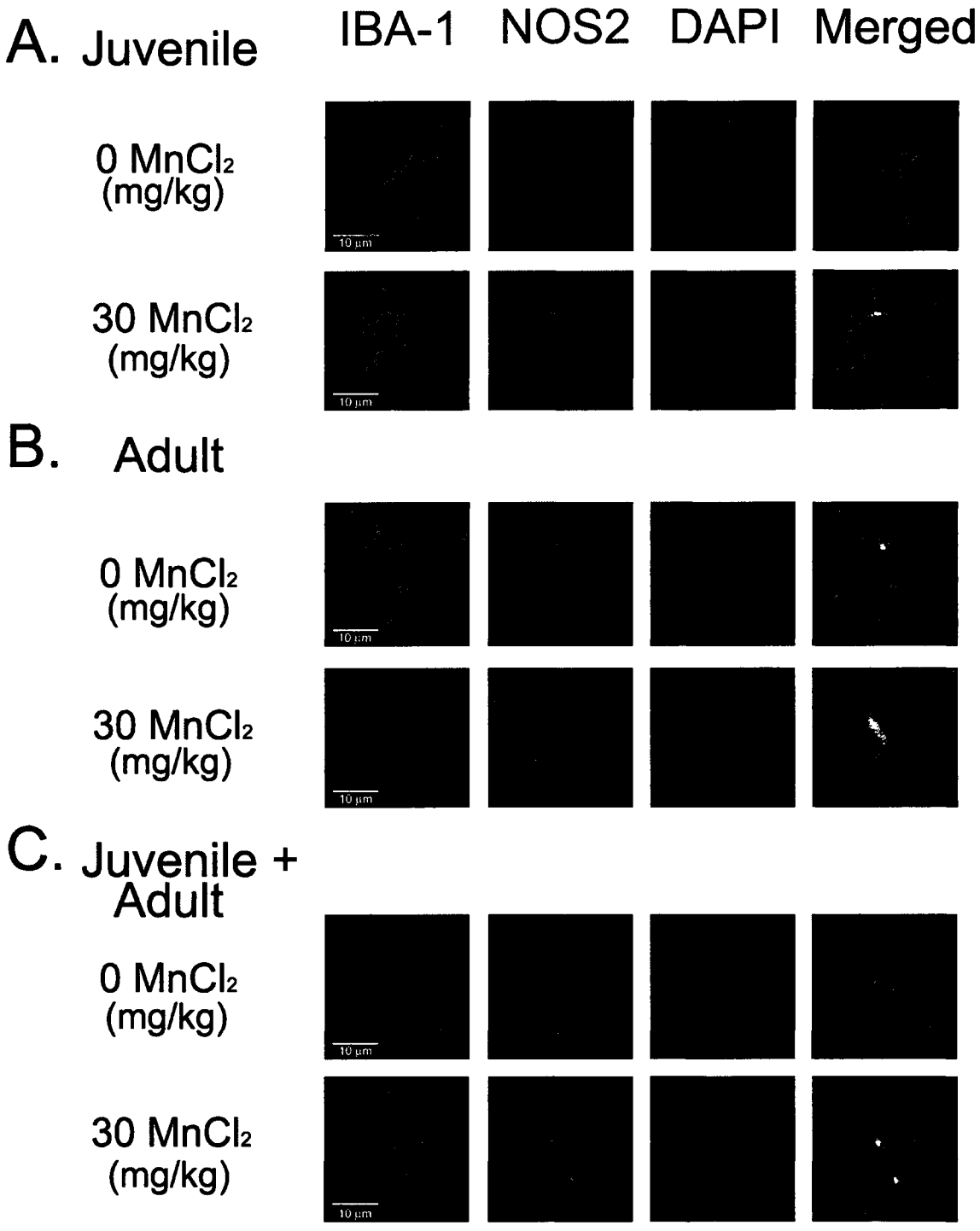


FIGURE 14
Microglia expressing NOS2

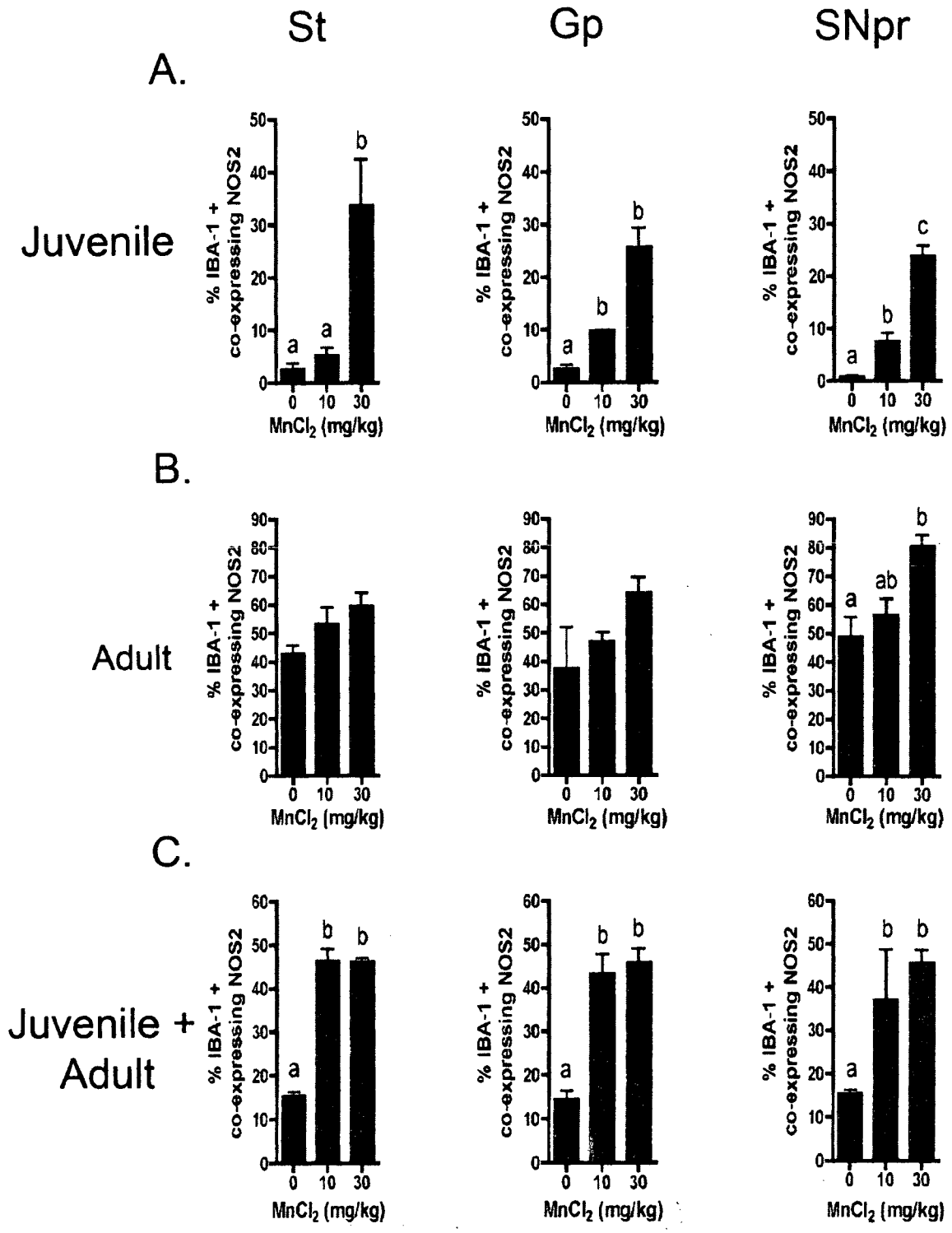


Figure 13. Activated microglial cells in C57Bl/6J mice express NOS2 following differential exposure to Mn as juveniles and adults. Microglial expression of NOS2 was assessed via co-immunofluorescence in multiple regions of the basal ganglia from mice exposed to Mn. Representative images of the SNpr are presented from control mice and those treated with 30 mg/kg $MnCl_2$ as juveniles (A), adults (B), or both juveniles and adults (C). Images of Iba-1 and NOS2 expression are shown in green and red channels, respectively, and cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Merged images indicate co-localization of NOS2 expression in Iba-1-positive microglia in mice exposed to Mn. Scale bar = 10 μm.

Figure 14. Quantitative analysis of NOS2 expression in Iba-1-positive microglial cells reveals regionally and developmentally distinct patterns of microglial reactivity following exposure to Mn. The percent of Iba-1-positive microglia expressing NOS2 was determined in mice exposed to exposed to 0, 10, or 30 mg/Kg $MnCl_2$ by daily intragastric gavage as as juveniles (A), adults (B), or both juveniles and adults (C) in the striatum (St), globus pallidus (Gp), and substantia nigra pars reticulata (SNpr). Three serial sections were stained for each brain region per hemisphere from an average of 2 – 4 mice per treatment group. For quantitative cell counts, images were acquired using a 20X Zeiss Planapochromat air objective and a minimum of 100 cells per microscopic field were counted from three microscopic fields per brain region for each serial section. Data represent the mean percentage of Iba-1-positive microglia expressing NOS2. Different letters denote significant differences between treatment groups, $p>0.05$.

FIGURE 15
Astrocytes expressing NOS2

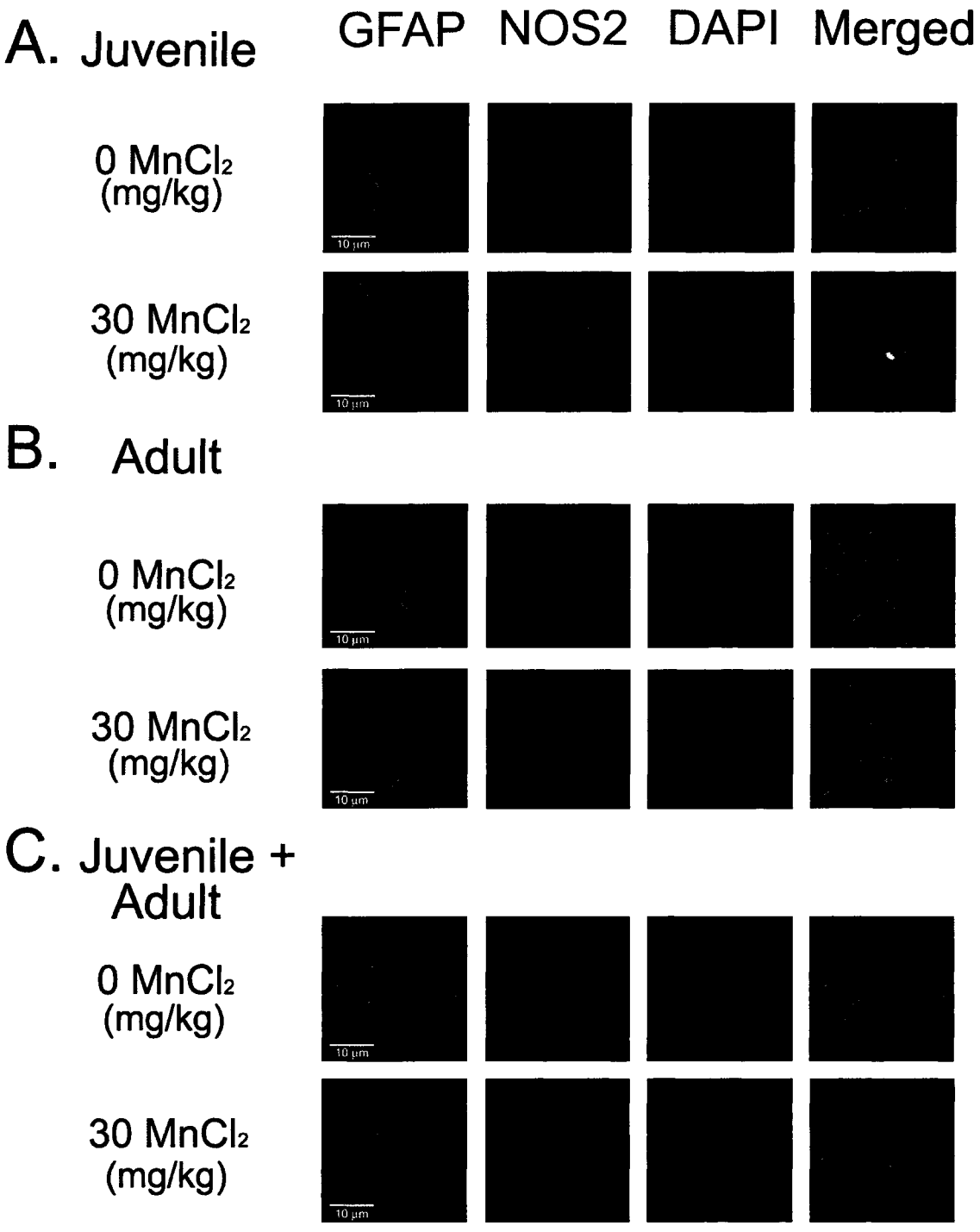


FIGURE 16
Astrocytes expressing NOS2

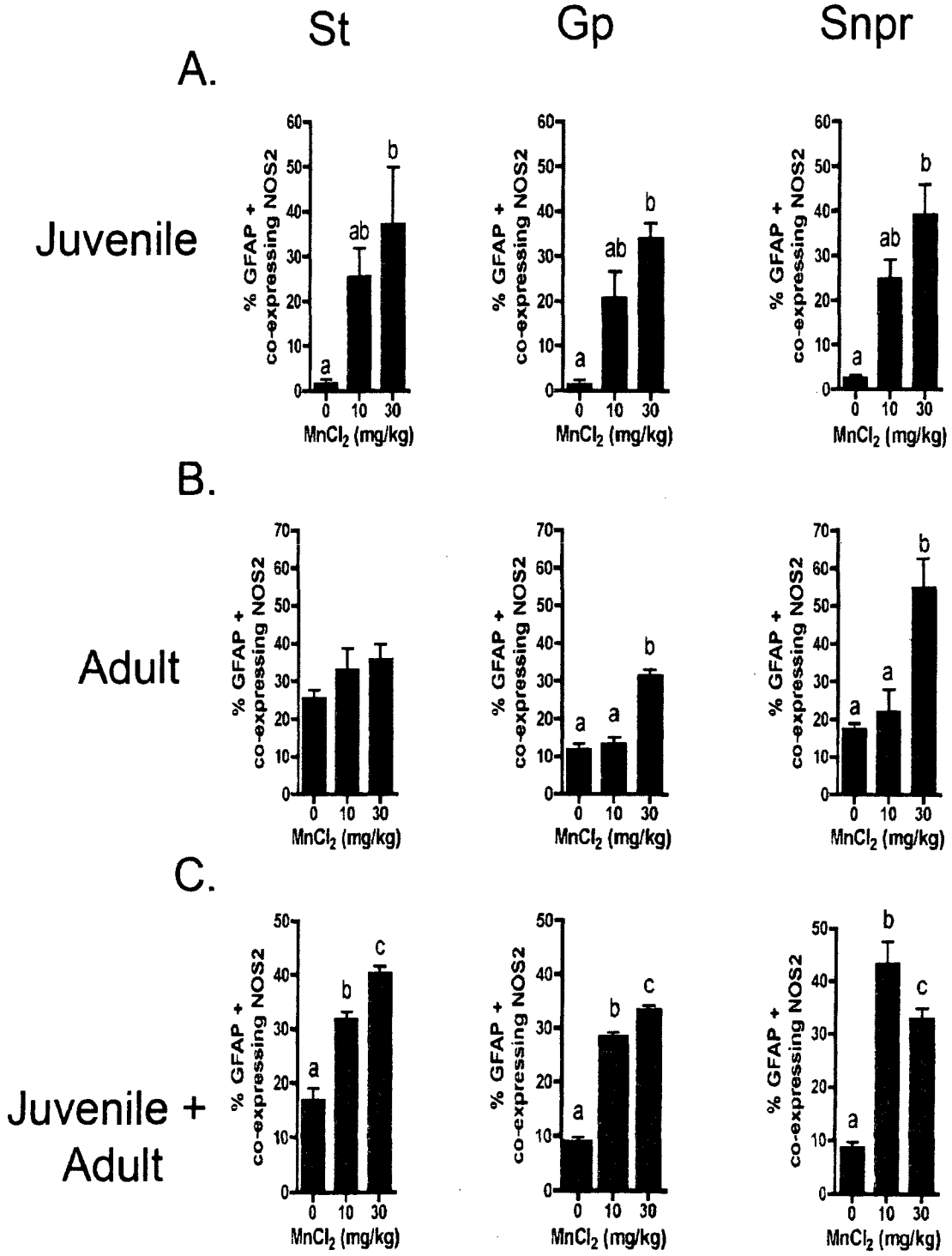


Figure 15. Activated astrocytes in C57Bl/6J mice express NOS2 following differential exposure to Mn as juveniles and adults. Astroglial expression of NOS2 was assessed via co-immunofluorescence in multiple regions of the basal ganglia from mice exposed to Mn. Representative images of the SNpr are presented from control mice and those treated with 30 mg/kg $MnCl_2$ as juveniles (A), adults(B), or both juveniles and adults (C). Images of GFAP and NOS2 expression are shown in green and red channels, respectively, and cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Merged images indicate co-localization of NOS2 expression in GFAP-positive astrocytes in mice exposed to Mn. Scale bar = 10 μm.

Figure 16. Quantitative analysis of NOS2 expression in GFAP-positive astrocytes reveals regionally and developmentally distinct patterns of astroglial reactivity following exposure to Mn. The percent of GFAP-positive astrocytes expressing NOS2 was determined in mice exposed to exposed to 0, 10, or 30 mg/Kg $MnCl_2$ by daily intragastric gavage as as juveniles (A), adults (B), or both juveniles and adults (C) in the striatum (St), globus pallidus (Gp), and substantia nigra pars reticulata (SNpr). Three serial sections were stained for each brain region per hemisphere from an average of 2 – 4 mice per treatment group. For quantitative cell counts, images were acquired using a 20X Zeiss Planapochromat air objective and a minimum of 100 cells per microscopic field were counted from three microscopic fields per brain region for each serial section. Data represent the mean percentage of GFAP-positive astrocytes expressing NOS2. Different letters denote significant differences between treatment groups, $p>0.05$.

FIGURE 17
Neuronal 3-NT expression

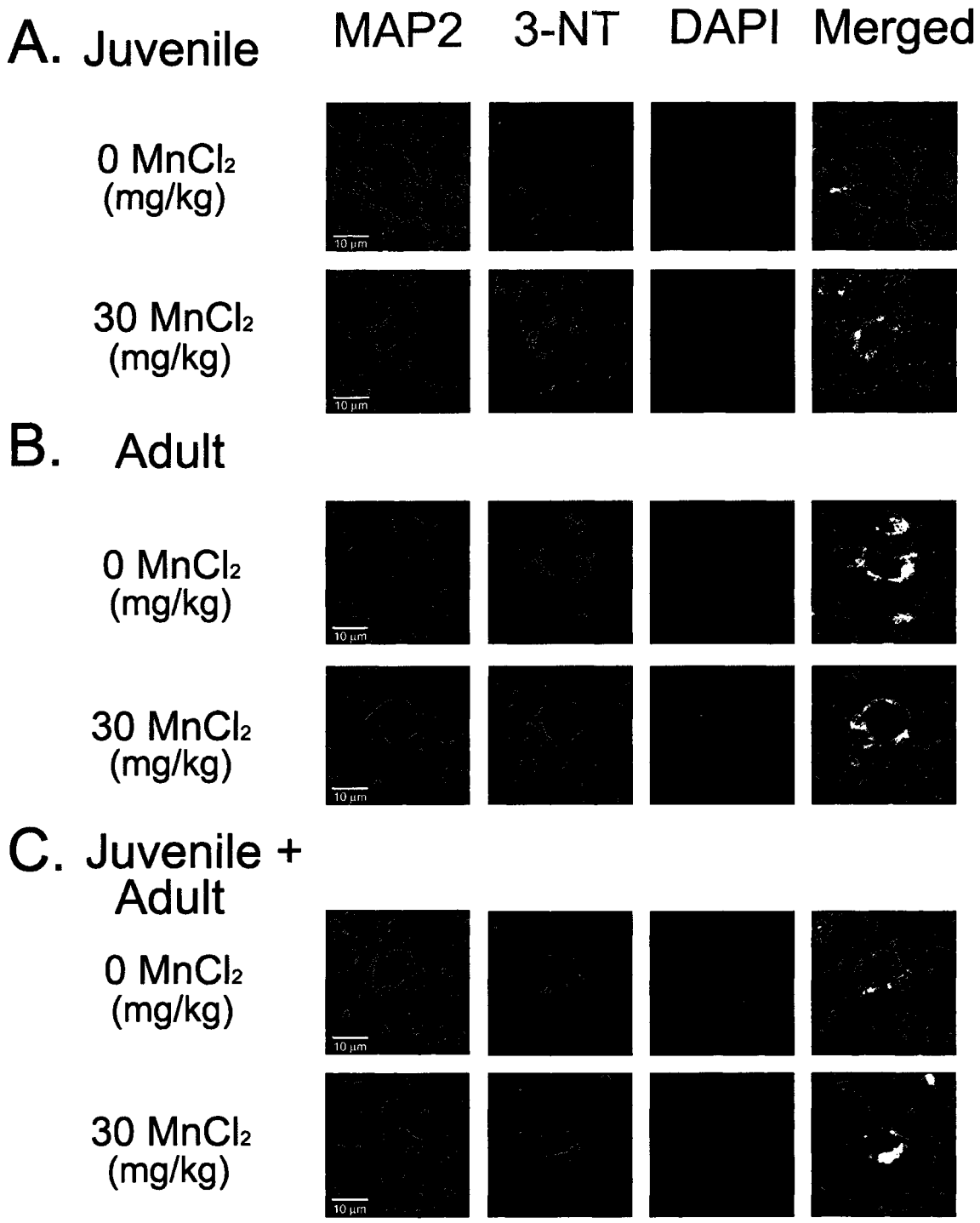


FIGURE 18
Neuronal 3-NT expression

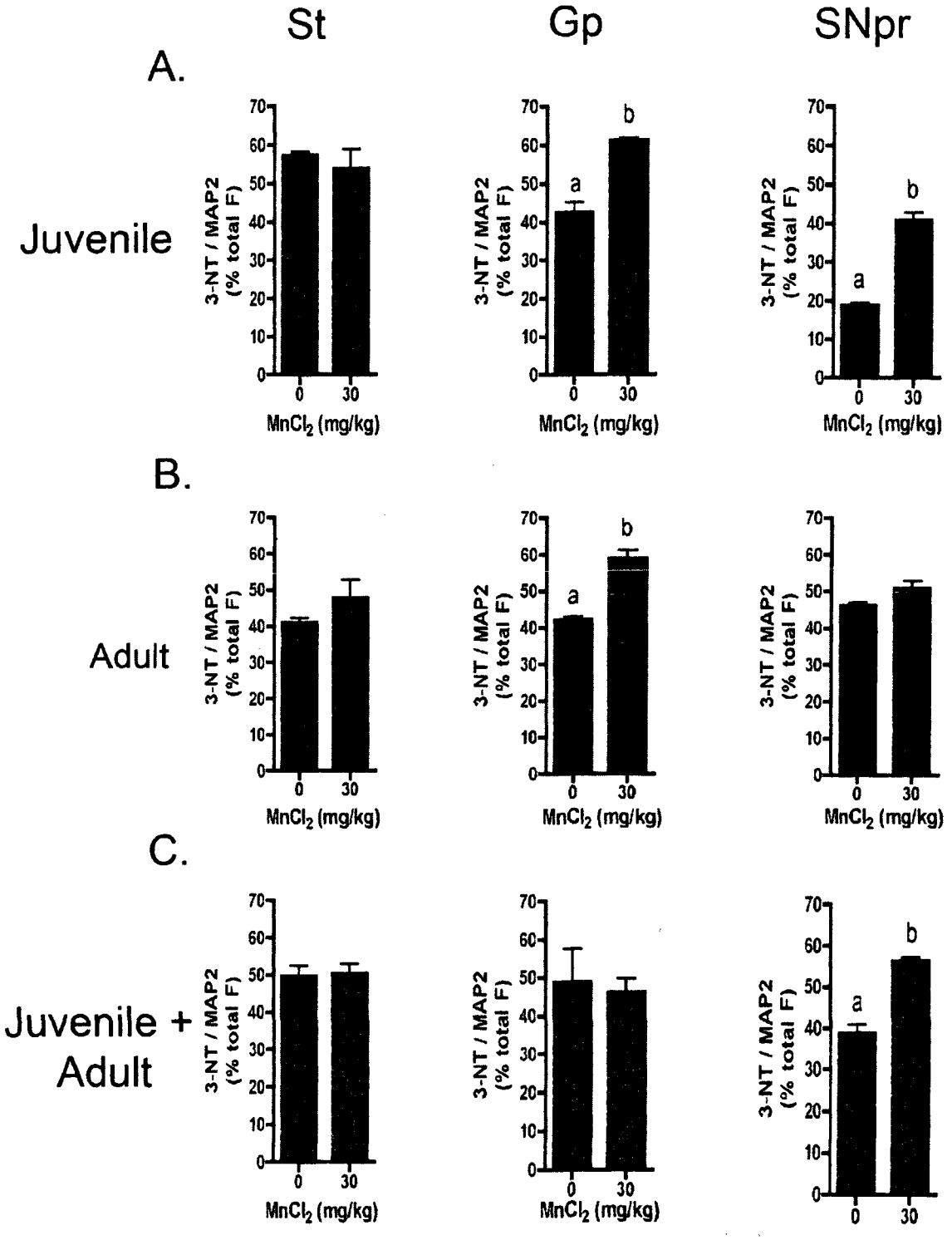


Figure 17. Manganese exposure increases levels of 3-nitrotyrosine (3-NTyr) protein adducts in basal ganglia neurons. To detect modification of neuronal proteins by peroxynitrite ($ONOO^-$) derived from increased production of NO by activated glia, serial sections from the SNpr of juvenile mice exposed to 1 and 30 mg/Kg $MnCl_2$ from day 20 – 34 postnatal were stained with antibodies against the general neuronal marker Microtuble Association Protein 2 (MAP2; green) and 3-NTyr (red) and were counterstained with DAPI to identify cell nuclei (blue). Representative images of 3-NTyr-modified proteins indicate co-localization of 3-NTyr adducts with both neuronal soma and dendrites in the SNpr of Mn-treated juvenile mice. Scale bar = 10 μm.

Figure 18. Quantitative analysis of 3-nitrotyrosine adducts indicates that modification of neuronal proteins by peroxynitrite correlates with regional patterns of glial activation and NOS2 expression. Specific brain regions were evaluated for co-localization of Microtuble Association Protein 2 (MAP2) and 3-NTyr by immunofluorescence in mice exposed to control and 30mg/Kg $MnCl_2$. Graphs indicate quantification of levels of 3-NTyr protein adducts in neurons from striatum (St), globus pallidus (Gp), and (C) Substantia nigra pars reticulata (SNpr) in mice exposed as juveniles (A) adults(B) or juveniles + adults (C). Data indicate increased protein nitration in the neurons of the Gp and SNpr. Different letters denote significant differences between treatments, $p>0.05$. Scale bar = 10 μm.

FIGURE 19
Striatal-pallidal neurons 3-NT

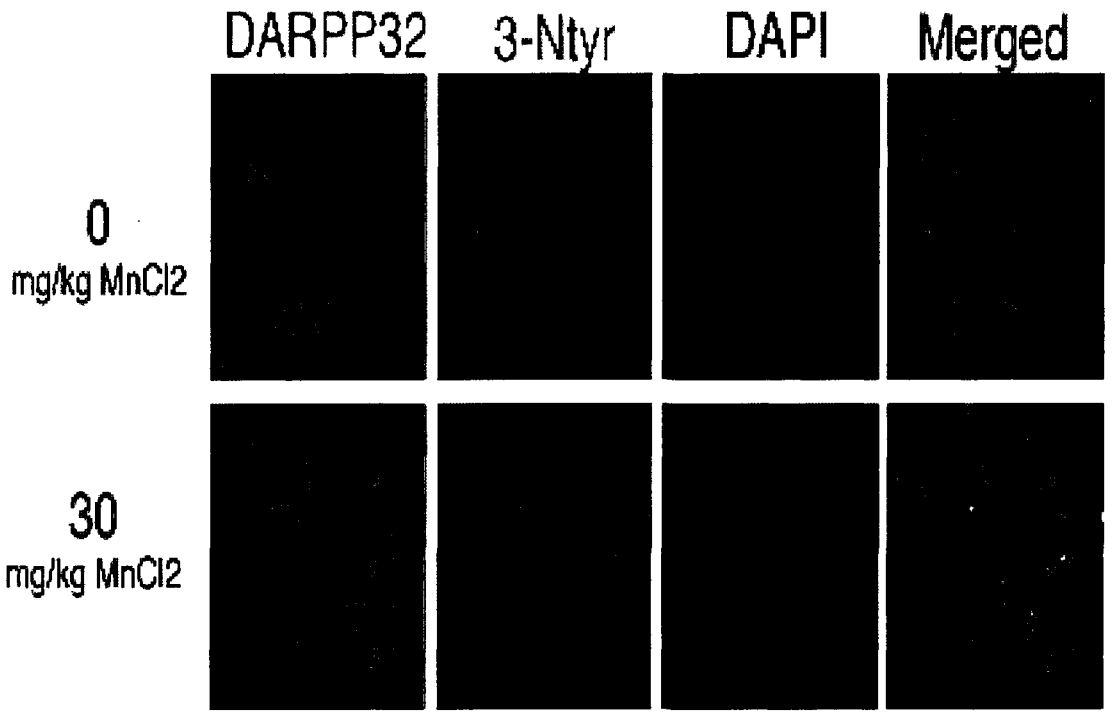


Figure 19. 3-Nytrotryosine occurred in striatal-pallidal neurons with exposure to 30mg/kg $MnCl_2$ mice exposed to Mn as a juvenile and an adult. In order to detect neuronal protein modification by glial secreted NO which forms ONOO- damaging the neurons projecting from the striatum to the pallidum the following antibodies were utilized. The striatum was assessed for dopamine cAMP regulated phosphoprotein- 32 (DARPP-32; green) and 3-NTyr (red) and were counterstained with DAPI to identify cell nuclei (blue). Representaive images of co-localization of DARPP32 and 3-Ntyr expression in the striatum of control and 30mg/ kg $MnCl_2$ animals.

FIGURE 20
Striatal-pallidal neurons 3-NT

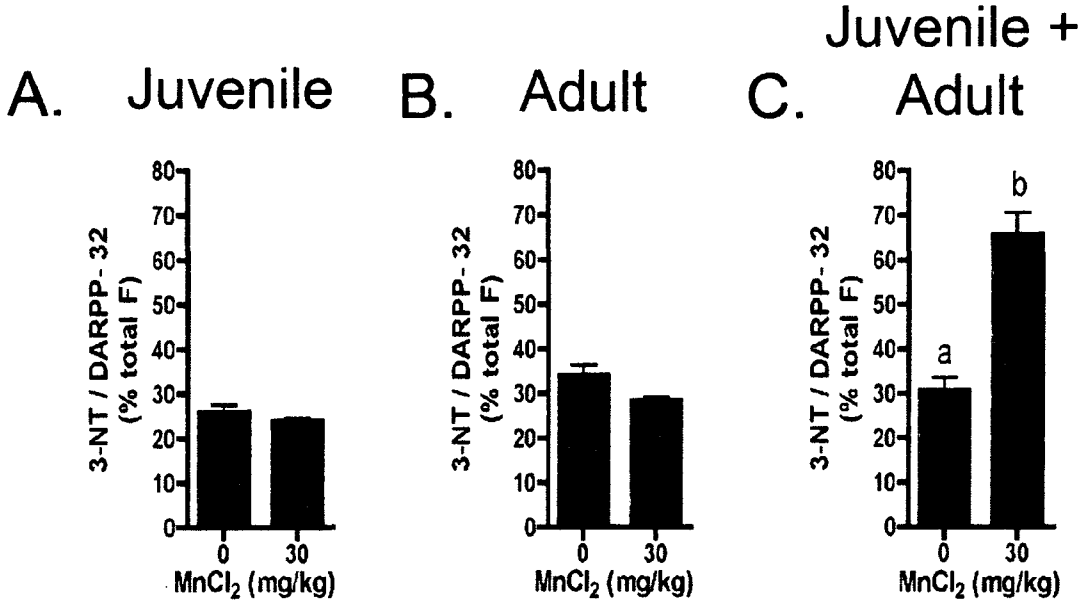


Figure 20. Quantitative analysis of 3-nitrotyrosine adducts indicates that modification of specific striatal neuronal proteins by peroxynitrite correlates with regional patterns of glial activation and NOS2 expression. Striatal neurons were evaluated for co-localization of Dopamine cAMP regulated phosphoprotein- 32 (DARPP32) and 3-NTyr by immunofluorescence in mice exposed to control and 30mg/Kg $MnCl_2$. Graphs indicate quantification of levels of 3-NTyr protein adducts in neurons from striatum (St) in mice exposed as juveniles (A) adults (B) or juveniles + adults (C). Data indicate increased protein nitration in the neurons of the juveniles + adult exposure group. Different letters denote significant differences between treatments, $p>0.05$. Scale bar = 10 μm.

FIGURE 21

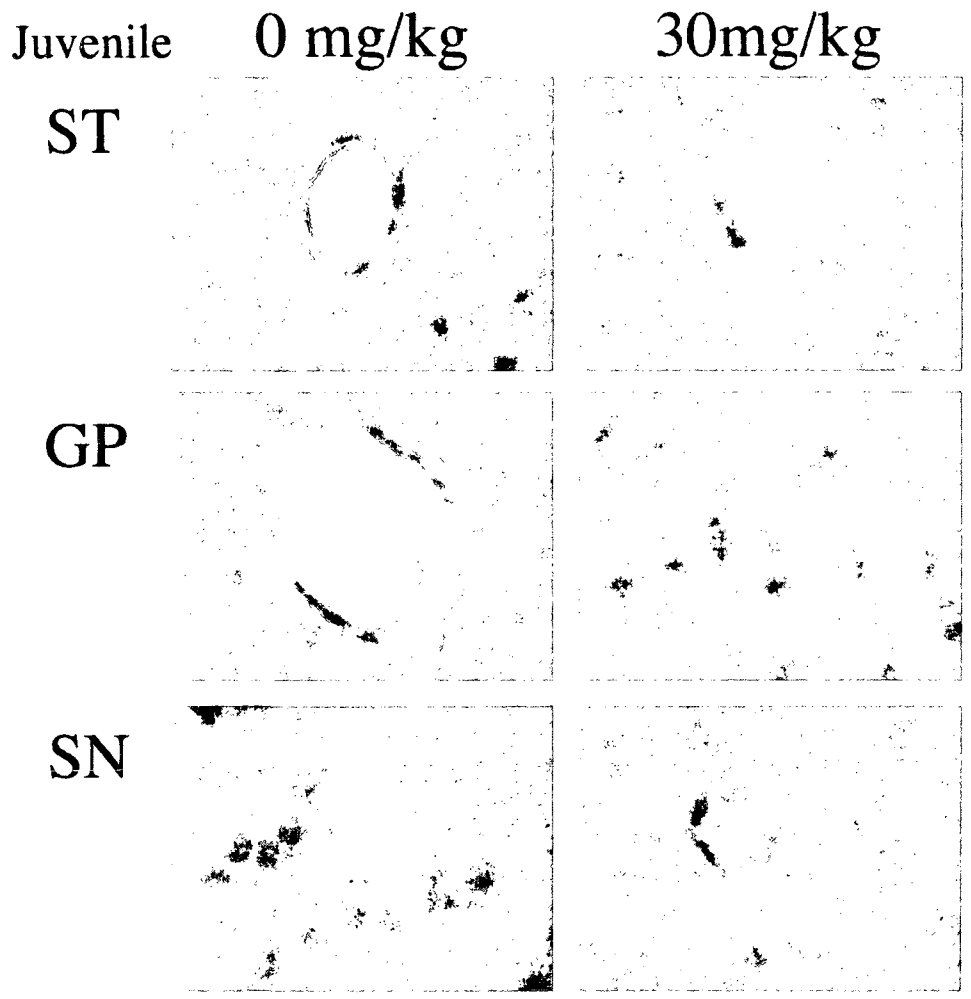


Figure 21. Mouse albumin serum staining in mice exposed to 30mg/kg $MnCl_2$.

CHAPTER 4

AGE-DEPENDENT SUSCEPTIBILITY TO MANGANESE-INDUCED NEUROTOXICITY: BEHAVIORAL AND NEUROCHEMICAL LEVELS

Abstract

Chronic exposure to manganese (Mn) can result in Manganism, a neurodegenerative disorder of the basal ganglia, the area of the brain that modulates motor function. Prior studies have shown increased basal ganglia dysfunction in adult mice exposed to manganese, but the relevance of developmental vulnerability in manganese-induced basal ganglia dysfunction is unknown. Therefore, the following study will assess developmental vulnerability by determining whether Mn-induced dysfunction in mice exposed to Mn as juveniles and adults is different than that of mice exposed only as adults or only as a juvenile. In this study we postulated that mice exposed as juveniles and then again as an adult will exhibit greater behavioral dysfunction and abnormal neurotransmitter release than mice that were only exposed as adults or juvneiles. Juvenile, twenty-day old C57BL/6 mice received gastric gavage daily with saline, 10 mg/kg, or 30 mg/kg of MnCl2 for two weeks. At age 12 weeks, the same mice were again exposed to the same regiment, this time for an eight-week period, while a group of 20 day and 12 week old mice went through the same protocol as that of the re-exposed adults. Locomotor actvity of the mice was determined utilizing chambers that monitor movement on the x-, y- and z-planes. Identification of catacholamine and monomaine levels was also assessed in the striatum and metal accumulation was determined in a variety of brain regions. The results of the locomotor activity indicated an age and sex-dependent vulnerability to Mn toxicity with the juvenile males experiencing an increase in novelty seeking or hyperactivity while the males only exposed as adults

experienced no change in activities measured. The juvenile + adult male exposure group had a decrease in total movement and novety seeking. Furthermore, Mn exposure rendered the dopaminergic system to be more vlnerable to dysfunction as an adult with prior exposure as a juvenile. The juvenile only group had an increase in dopamine and a decrease in serotonin metabolite, 5-HIAA, indicating both the dopaminergic and serotonergic systems are involved in Mn toxicity in the young. Interestingly a sex-dependence was observed as well, with no change in measured activities in the female groups. These results reveal that exposure to Mn during development leads to the dysfunction of normal activity and neurochemical production not only if exposed early in life but as well as if exposed again later in life.

Introduction

Manganese (Mn) is an essential nutrient with an adequate daily intake of 2-5mg/ day for adults and 1-3mg/ day for children. Mn is necessary for central nervous system (CNS) homeostasis involved in the metabolism of proteins, carbohydrates, and lipids (Keen, 1984). Mn also serves as a cofactor in enzymatic pathways, such as mitochondrial superoxide dismutase (Hearn et al., 2003) and glutamine synthase (Takeda, 2003). Routes of exposure to Mn include inhalation of Mn through occupational settings such as steel workers, welding or mining (Bader et al., 1999; Bowler et al. 2006; Josephs et al., 2005; Montes et al., 2008), and dietary intake by drinking Mn contaminated water, and soy-infant formula (Krachler et al., 2000; Wasserman et al., 2006; Woolf et al., 2002) leading to Mn accumulation in the brain. Prior studies in rats and Rhesus monkeys indicate that with Mn exposure the globus pallidus (GP), striatum (St) and cortex accumulate Mn more than other brain regions (Morello et al., 2007; Struve et al., 2007).

Excessive exposure can cause Mn toxicity, termed Manganism a neurodegenerative disease, targeting the basal ganglia, specifically the GP, St, and substantia nigra pars reticulata (SNpr) nuclei in humans exposed to high levels of Mn in respirable air in occupational settings (Yamada et al., 1986). Early neuropsychological symptoms of the disorder include aggressiveness, anxiety, hallucinations and slowing of cognitive function (Halliwell and Gutteridge, 2005). Followed by psychological disturbances, patients also experience neurological and motor deficits that include dystonia, bradykinesia, rigidity, masked facia, and

difficulty in walking backwards (Wolters et al., 1989). Some of these clinical features are shared with Parkinson's disease (PD); however, dystonia and lack of resting tremor are more characteristic of manganism (Rodier, 1955).

In order to assess behavioral abnormalities in models of Mn toxicity a variety of locomotor tests have been utilized. Aged mice were exposed to Mn subcutaneously and had a decrease in horizontal movement (Dodd et al., 2005) while primates experienced gait dysfunction, rigidity, bradykinesia, and facial grimacing with chornic exposure to $MnCl_2$ (Mella et. al., 1924) both indicating Mn is acting on the extrapyramidal system. Human workers exposed to MnO_2 dust in a dry alkaline battery factory experienced decrease in visual reaction time, eye-hand coordination & hand steadiness (Roels et al., 1992) all early signs of neuronal damage in the basal ganglia brain region.

Mn toxicity causes adverse motor symptoms similar to PD that are primarily attributed to loss of dopamine in the striatum and to decreased GABAergic output from from the globus pallidus but, unlike PD, dopaminergic are largely spared (Perl and Olanow, 2007). Dopaminergic neurons project from the SN pars compacta (SNpc) to the caudate nucleus and putamen in the striatum where the catecholamine neurotransmitter dopamine is released and transported back into the presynaptic neuron by the dopamine transporter (DAT). A variety of Mn toxicity studies have found a decrease in striatal dopamine in rats (Dorman et al., 2000; Hirata et al., 2001), mice (Liu et al., 2006), rabbits (Mustafa and Chandra, 1971), and non-human primates (Bird et al., 1984; Eriksson et al., 1987; Neff et al., 1969). A decrease in D2-dopamine receptors (D2R) was

identified in Mn exposed non-human primates through Position emission tomography (PET) but fully intact neurons were projecting from the nigra to the striatum (Shinotoh et al., 1997; Wolters et al., 1989). In young male rodents exposed to Mn a decrease in D2R binding sites was determined (Reichel et al., 2006) indicating a decrease in functional dopamine binding leading to a decrease in normal motor movement. Dopamine is a key player in decreased locomotion observed in Mn toxicity therefore it is imperative to determine any abnormal activity of the dopaminergic system in models of manganism.

Serotonin (5-HT) is a monoamine neurotransmitter that is involved in maintaining emotional stability in the CNS; however, modulation of the serotonergic neurons leads to lack of emotional stability resulting in anger, depression, sleeplessness and loss of memory, (Lesch et al., 1996) all early symptoms of manganism. 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) and was found to be decreased in the GP of primates exposed to aerosol $MnSO_4$ (Struve et al., 2007) while a decrease in 5-HT was identified in rats with high Mn diet (Kimura et al., 1978). Although there is some evidence of the sertonergic pathway modulation with Mn exposure, the involvement of serotonin its is not fully understood and highly dependent on age of primate or rodent and dose of Mn.

Although metal accumulation in the brain, behavior modulations and neurotransmitter concentrations have been assessed in rodent models in the past, it is simply unknown what occurs if the basal ganglia is exposed to Mn during development and then again later in life. Therefore we hypothesize that

exposure to Mn during development will cause greater basal ganglia sensitivity to Mn toxicity upon subsequent adult exposures. To address this hypothesis, we exposed C57Bl/6 mice to Mn by intragastric gavage as juveniles, adults, or as juveniles and again as adults and examined metal accumulation in various brain regions along with neurotransmitters release and behavior abnormalities. The results indicate that the developing basal ganglia is relatively sensitive to Mn exposure leading to an increase in dopamine release and hyperactivity in juvenile mice. Subsequent exposure to Mn again as an adult resulted in a decrease in overall movement along with deceased dopamine and it metabolites. This study has not only identified increased basal ganglia vulnerability to Mn if exposed as a juvenile and then again later in life but has also determined a sensitive time point between weanling and puberty that is highly susceptible to hyperactivity in synaptic transmission and behavior.

MATERIALS AND METHODS

Reagents. All chemical reagents were obtained from Sigma Chemical Co (St. Louis, MO) unless otherwise stated. C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Animal exposure model. Male and female C57Bl/6J mice were housed in microisolator cages (five animals per cage) and kept on 12-h light/ dark cycles with access to laboratory chow and water *ad libitum*. Littermates from timed pregnant dams were paired in control and Mn-exposed groups and received 0.9% normal saline, 10mg/Kg $MnCl_2$, or 30mg/Kg $MnCl_2$ by gastric gavage daily during the following time periods: juvenile exposure, day 20-34 postnatal; adult exposure, from week 12-20; and juvenile + adult exposure day 20-34 postnatal and week 12-20. Animals were weighed prior to each gavage and the amount of Mn delivered was adjusted accordingly. Additionally, the amount of Mn delivered was adjusted for the molar concentration in the tetrahydrate form so as to achieve a precise dose of 10 or 30 mg/Kg. All procedures were performed under the supervision of the Animal Care and Use Committee at the Colorado State University and were approved prior to onset of the studies.

Determination of catecholamines and monoamine neurotransmitters. The following method and equipment has been modified from Champney et. al., in order to assess levels of five metabolites from brain samples exposed to MPTP; Dopamine, 2-(3,4-dihydroxyphenyl)acetic acid (DOPAC), Homovanillic acid (HVA), Serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) (Champney et al., 1992). The Schimadzu LC-10AS high performance liquid

chromotograph was utilized along with Schimadzu LC-10A pump that circulated the 7% methanol mobile phase isocratically at a 1.0 mL/min flow rate. The LED-6A electrochemical detector working electrode was set at +0.67 V, recorder output set at 8nA, response output at STD or 0.5 seconds, negative polarity for the output and polarity of electrode set at reduction. Experiments were performed through a Microsorb-MV C-18 reverse phase column with a pore size of 100A, 5mm size and 25cm in length (Varian; Walnut Creek, CA). The mobile phase consisted of 0.946g Na_2HPO_4, 2.8g of citric acid, 18.6mg of EDTA and 20mg of sodium ocytl sulfate in 930 of Milli-Q water maintained at a pH of 4.6. The buffer was filtered through 0.45mM inorganic sterile filter followed by the addition of 70 mL of HPLC grade methanol was added to the buffer followed by gentle mixing of the buffer.

Brain samples were snap frozen with liquid nitrogen after extraction from the brain and stored in -80°C for up to two years. The samples were then removed and kept on ice, weighed, and 300ul of 0.2M perchloric acid (PCA) containing 0.5 mg/ml of Deoxyephinephrine (EPN) was applied per 10mg of wet weight brain. Tissue samples were then disrupted by 2 sec sonication and placed directly onto ice. 20uL of sample was saved for total protein concentration via BCA protein assay. The tissue sample was then centrifuged at 14,000 x g for 10 min at 4°C. Aliquots of the sample were loaded into glass HPLC vials and ran within eight hours of sonication. Standards for all 5 compounds were processed in the same manner as the samples and 1.0 mg/mL, 0.6 mg/mL, 0.3 mg/mL and 0.1 mg/mL concentrations of standards concluded the runs for each day. The

internal standard EPN was included in all runs in order to account for minute changes in detector sensitivity. The sample peaks areas were compared to the standard peaks area and values are expressed as ng of neurotransmitter/ mg of total protein.

Locomotor activity chambers. Versamax behavior chambers were utilized to assess activity of the mice on X, Y, and Z planes. Activity was measured in the juvenile exposure group every other day for two weeks and for the adult exposure group once a week for two months proceeding oral gavage. Recording of the activity was acquired every minute for a total of ten minutes and then analyzed using the Versadat software.

Levels of Mn, Cu and Fe in brain tissue. Brain regions were collected and stored at -80°C until sample preparation. Brain samples were weighed and transferred to acid-cleaned polypropylene tubes, 100ml of Nitric Acid was applied to each sample and cooked for 2 hours at 95°C to digest the tissue. Lipids were then digested with 50ml of 30% Hydrogen Peroxide for 15 min at 80°C followed by 50ml of Hydrochloric Acid incubation to complex the metals for 15 min at 80°C. Samples were then brought up in 1mL of Milli-Q water. Analysis of the samples was performed on a Perkin-Elmer Elan DRC-II ICP-MS. Throughout the digestion, standards and blank tubes underwent the same digestion procedures as the samples. Concentration of metals analyzed in brain samples are expressed a ppm of metal.

Statistical Analysis. Comparison of two means was performed by Student's *t*-test. Comparisons three or more means was performed using one-

way ANOVA followed by the Tukey-Kramer multiple comparison *post-hoc* test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). For all experiments, $p < 0.05$ was considered significant.

RESULTS

Neurobehavior data was collected to determine the overall locomotor activity of the mice by examining parameters that assess basal ganglia dysfunction such as total movement and time spent near the margin of the chamber, an indicator of extrapyramidal function and novelty seeking, respectively. Assessment of total movement number in the open-field activity chambers for the juvenile + adult exposure group indicated that the females had no significant change (Fig. 22E) while a significant change was observed in males exposed to 30mg/kg $MnCl_2$ (Fig. 22F). Total movement number for the mice exposed only as juveniles (Fig. 22 A-B) and only as adults (Fig. 22 C-D) had no significant change with both doses of $MnCl_2$. Novelty seeking behavior, as indicated by time spent in the margin, was significantly decreased in juvenile males for treatment parameters, 10 and 30mg/kg of $MnCl_2$ while juvenile females had no change (Fig. 23A-B). Male mice exposed to 10 and 30 mg/kg $MnCl_2$ as a juvenile and again as an adult had a significant increase in time spent in the margin compared to control mice while females exposed as juveniles and adults had no change (Fig. 23E-F). The adult only study mice experienced no change in time spent in the margin when treated with $MnCl_2$(Fig. 23C-D).

Neurochemical analysis of transmitters involved in dopaminergic and serotonergic pathways were assessed using high-pressure liquid chromatography (HPLC). Two neurotransmitters involved in the dopamine pathway, dopamine and DOPAC and the seroternergic neurotransmitters 5-HT and 5-HIAA were analyzed from snap frozen brain samples. In mice exposed to

the high dose of Mn, 30mg/kg, during the juvenile period had a significant increase of dopamine (Fig. 24A) compared to both the control and 10mg/kg $MnCl_2$. The opposite affect occurred in the mice exposed to Mn as adults where a decrease of dopamine was observed in the 30mg/kg $MnCl_2$ adult only group (Fig. 24B) while dopamine decreased significantly in both 10 and 30mg/kg $MnCl_2$ in juvenile + adult group (Fig. 24C). The assessment of the dopamine metabolite, DOPAC, identified a decrease of DOPAC at 30 mg/kg $MnCl_2$ dose with all three exposure groups but the adults pre-exposed to Mn as juveniles also had a decrease in DOPAC synthesis at the low dose of 10mg/kg $MnCl_2$ (Fig. 25A-C). Next, the ratio of DOPAC to dopamine production, a marker of dopamine turnover, was measured identifying only a significant change with the 30mg/kg juvenile exposure group (Fig. 26A) while both the adult and juvenile + adult animal groups had no significant change (Fig. 26B-C). Finally the serotionergic synthesis was analyzed and only the juvenile exposure group had a significant increase in 5-HIAA production in the high dose of Mn (Fig. 27A) while no change was seen in other exposure groups (Fig. 27B-C). No significant change in 5-HT production was identified in any of the animals exposed to Mn (Fig. 28A-C).

DISCUSSION

Manganism or Mn toxicity has been well defined as a disorder that affects the basal ganglia brain region with Mn accumulating specifically in the striatum (St), globus pallidus (GP) and substantia nigra pars reticulata (SNpr) nuclei. In order to fully understand the phenotype of the disease neurobehavioral and neurochemical analysis of animals exposed to Mn during development and adulthood was assessed. Mice were separated into three groups: mice exposed to Mn early in life as juveniles, mice exposed during adulthood and mice exposed as juveniles plus adults.

Locomotor activity was assessed in all mice throughout the study and it was identified that male mice exposed as a juvenile and an adult experienced a decrease in overall movement number (Fig. 22F) while no significant changes were observed with any other groups (Fig.22). Decrease activity or movement in neurodegenerative models like manganism and Parkinson's disease has been well characterized. Bradykinesia was identified in factory workers in Taiwan who were exposed to Mn at high levels for two years (Wolters et al., 1989) and in a non-human primate model gait dysfunction, rigidity, and bradykinesia were reported with chronic exposure to Mn via inhalation (Mella, 1924). Adult rats also experienced a decrease in horizontal movement with just one injection of 100mg/kg $MnCl_2$ subcutaneously (Dodd et al., 2005).

The other movement parameter acquired that significantly changed in mice treated with Mn was margin time, a behavior also called "wall hugging." Staying near the margin (ie. an increase in margin time) of the chamber signifies

dysfunction or a lack of novelty seeking by the animal. Moreover, if the animal spends less time in the margin or a decrease in time spent in the margin then the mouse is characterized as having increased novelty seeking. It was ascertained in this study that male mice exposed only as juveniles to both low and high dose of Mn experienced a decrease in time spent in the margin (Fig. 23B) compared to controls. Increase in novelty seeking behavior is a sign of hyperactivity, a symptom also displayed by children that are exposed to Mn through contaminated well water (Wasserman et al., 2006; Woolf et al., 2002).

Multiple studies on Mn-induced neurobehavioral dysfunction during development usually are performed approximately post-natal day 1- 20 in rodents (Dorman et al., 2000; Kontur and Fechter, 1985; Tran et al., 2002) but none have observed significant locomotor or cognitive deficits at this developmental time point. However, Pappas and colleagues did identify increases in hyperactivity at post-natal day 17 in rats exposed to 10mg/ml Mn in drinking water (Pappas et al., 1997). The correlation between this juvenile study and young school aged children is possible due to the mice being exposed after weaning and proceeding puberty identifying a critical window of sensitivity. The adult male mice that were exposed as juveniles and as adults did experience an increase in time spent in the margin, a strong indicator of locomotor dysfunction with exposure to Mn at both 10 and 30 mg/kg (Fig. 23F). The mice exposed only as adults had a trend of decreasing time spent in the margin (Fig. 23D). Although this change was not significant, it does reveal that when mice are exposed early and then again later in life, an increase susceptibility of neurobehavior dysfunction is evident.

The neurotransmitters released in the striatum that are essential for normal motor activity and emotional stability were assessed and analyzed to have better understanding of dopaminergic and serotoneric dysfunction during Mn toxicity. Dopamine is the key neurotransmitter synthesized for controlled movement and is secreted from dopaminergic neurons into the striatum. These dopaminergic neurons were found to be intact through tyrosine hydroxylase (TH) staining of the cell bodies in the substantia nigra pars compacta (SNpc) and assessment of striatal dopaminergic projections in juvenile exposed animals at the 10 and 30 mg/kg dose. Striatal dopamine concentrations were acquired for each exposure parameter. Both the adult only and juvenile plus adult exposure groups had a significant decrease in dopamine levels (Fig 24B-C) at 30mg/kg $MnCl_2$. Interestingly, the juvenile plus adult exposure group at the low dose 10mg/kg had a decrease of striatal dopamine indicating an increased vulnerability of the dopaminergic system due to the pre-exposure as a juvenile (Fig. 24C). Dopamine is synthesized to its metabolite DOPAC by monoamine oxidase-B (MAO-B) (Lai et al., 1992). Mice exposed to high doses of Mn as adults had a significant decrease in DOPAC concentrations, and like the trend for dopamine, the mice exposed as juvenile and then as adults not only had a decrease in DOPAC at the high and at the low dose lending more evidence that the dopaminergic system becomes more sensitive to Mn toxicity upon re-exposure later in life.

The decrease in dopamine upon Mn exposure in the adult brain is well established in other Mn toxicity models and is a pathological indicator of basal

ganglia dysfunction. In monkeys and rabbits exposed to Mn, a significant decrease in dopamine levels was characterized in the striatum (Mustafa and Chandra, 1971; Neff et al., 1969) while previous literature form our laboratory also determined a significant decrease of dopamine in adult female mice exposed to Mn (Liu et al., 2006). Actual changes in dopamine and its metabolites with Mn toxicity is somewhat controversial as many studies show no change in the dopaminergic system with Mn treatment. For instance, no changes in dopamine or DOPAC were ascertained in adult mice exposed to moderate doses of Mn via oral gavage (Dorman et al., 2000). Additionally, data from PET scans in human and non-human primates showed no change in striatal dopamine levels (Wolters et al., 1989) (Mella, 1924), but this could be due to the variety of routes of Mn exposure and concentrations and types of Mn utilized.

The juvenile only group when exposed to Mn showed an opposite effect on the dopaminergic system with a significant increase of dopamine production at 30mg/kg (Fig. 24A) while DOPAC data actually decreased (Fig. 25A). To asses that proper turnover of dopamine to DOPAC occurred we analyzed the ratio of DOPAC to dopamine. This DOPAC to dopamine ratio decreased in the 30mg/kg juvenile while no significant change was observed in both adult groups, indicating that the basal ganglia is sensitive to Mn toxicity early in life as well. Previous studies have seen similar effects on the dopaminergic system when neonates were exposed to Mn through oral gavage with an increase in striatal dopamine; however, unlike this study, an increase of DOPAC was observed as well (Cotzias et al., 1976; Dorman et al., 2000; Shukla et al., 1980). Since the

ratio of DOPAC to dopamine decreased, this indicates a decrease in overall dopamine turnorver which could lead to overall decrease in DOPAC production as seen in these juvenile mice.

Previous reports of Mn toxicity revealed a decrease in 5-HIAA in the pallidum of monkeys exposed to Mn via inhalation (Struve et al., 2007) and oral exposure to milk supplemented with soy and Mn in monkeys (Golub et al., 2005) indicating a role for the serotonergic pathway in Mn toxicity. Following analysis of the dopaminergic system, we then determined the role of serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) in the striatum. It was identified that the mice exposed to 30mg/kg of $MnCl_2$ as juveniles were the only group that had significant decrease in the metabolite 5-HIAA (Fig. 26A). No change was observed in adult mice exposed to Mn (Fig. 26B-C). A change in the serotonergic metabolites is indicative of emotional instability and anxiety in humans, which is analogous to rodent models as well. This observable change in 5-HIAA levels may explain the increase in hyperactivity in the juvenile mice as a sign of high anxiety.

In conclusion we have identified that pre-exposure to low dose Mn leads to neurobehavioral and neurochemical dysfunction within the basal ganglia, which is analogous to the increase in pathological activation of glial leading to NOS2 expression and neuronal injury assessed in Chapter 3. Interestingly, not only the pre-exposed adults were sensitive to Mn toxicity as mice exposed to Mn early in life experienced an increase in hyperactivity and dysfunction of both the dopaminergic and serotonergic systems. Gender susceptibility was also

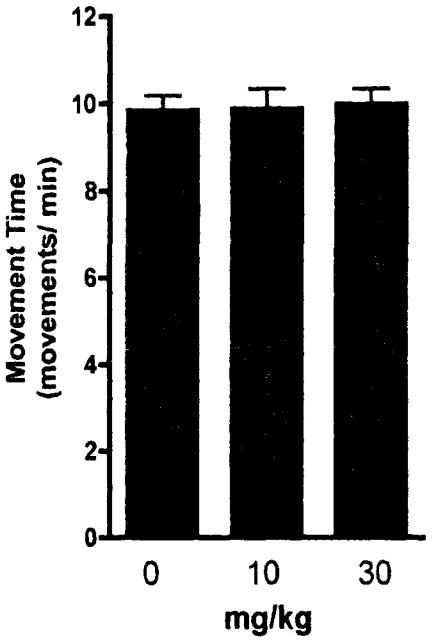
observed with only the males having neurobehavior abnormalities compared to the females. Therefore future studies will need to be done in order to fully understand the mechanism behind the sex-dependent sensitivity with Mn toxicity. Through these findings it seems that we have identified a key point in development where the basal ganglia is vulnerable to Mn toxicity as well as a sex-dependent vulnerability.

CHAPTER 4

FIGURES

FIGURE 22- Movement Number

A. Juvenile exposure- Female



B. Juvenile exposure- Male

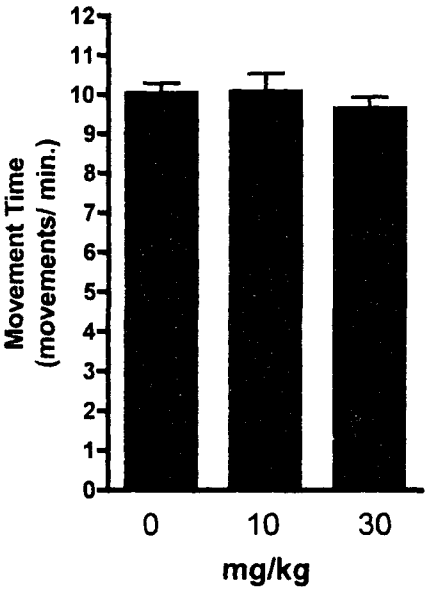
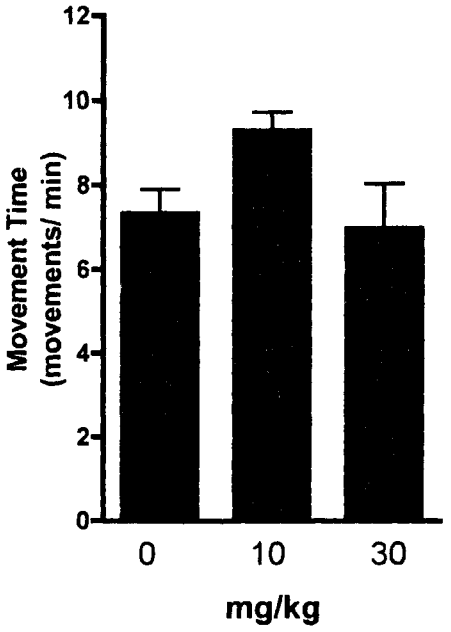


FIGURE 22- Movement Number

C. Adult exposure- Female



D. Adult exposure- Male

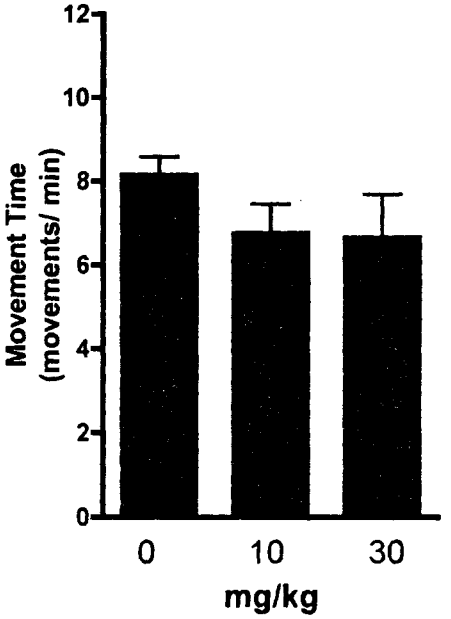
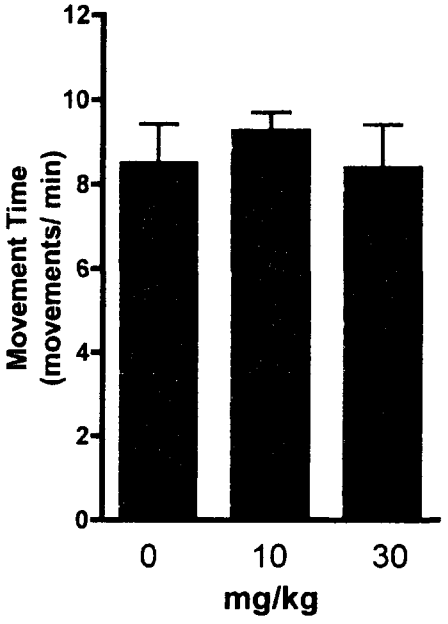


FIGURE 22- Movement Number

E. Juvenile + Adult exposure- Female



F. Juvenile + Adult exposure- Male

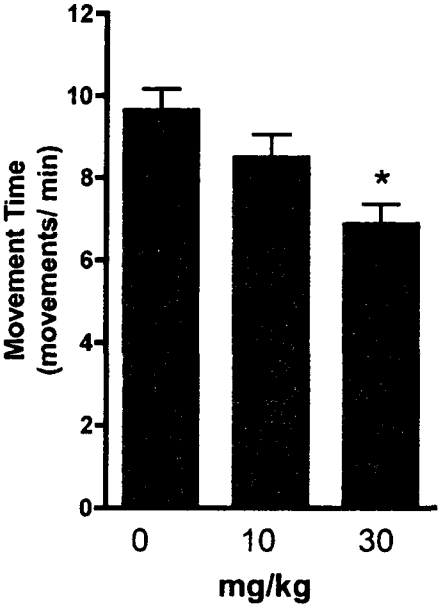
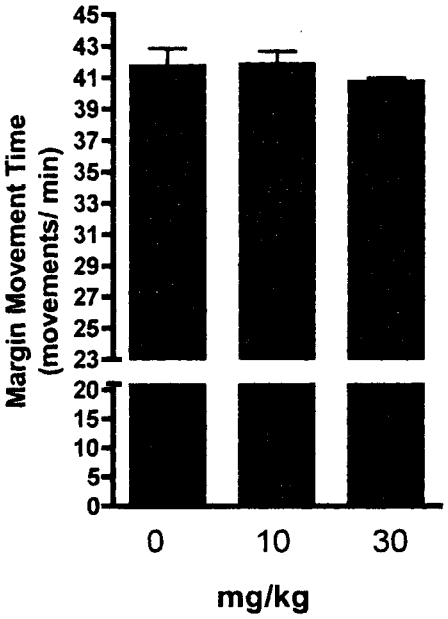


Figure 22. Pre-exposure to Mn as a juvenile induce a vulnerability in neurobehavior if exposed again as an adult. Total movement number recorded in open-field activity chambers was determine for each animal post exposure to Mn. (A) Female and (B) juvenile mice exposed to 10 and 30 mg/kg $MnCl_2$ had no significant change. (C) Female and (D) male mice exposed only as adults were not vulnerable to neurobehavioral changes with exposure. (E) Female mice pre-exposed to Mn as juvenile and then again as an adult had no change while males (F) pre-exposed did have a significant decrease in movement at 30mg/kg $MnCl_2$ compared to control. Significance is denoted by * $p>0.05$.

FIGURE 23- Time spent in the Margin

A. Juvenile exposure- Female



B. Juvenile exposure- Male

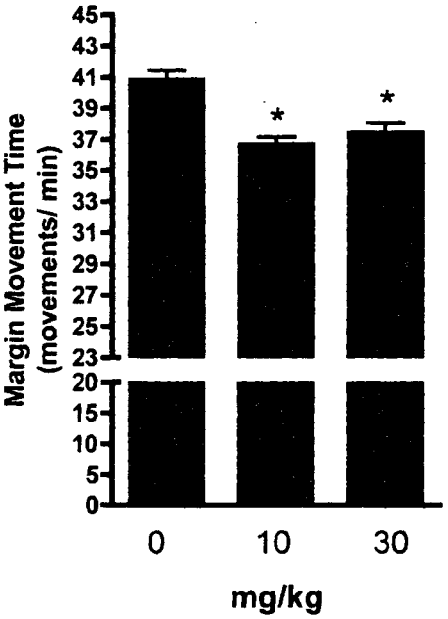
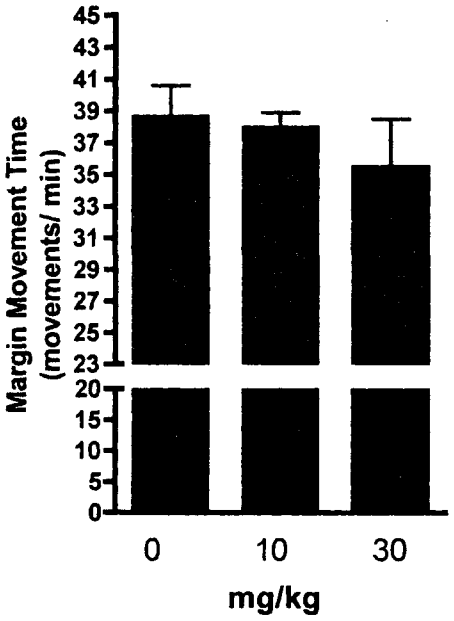


FIGURE 23- Time spent in the Margin

C. Adult exposure- Female



D. Adult exposure- Male

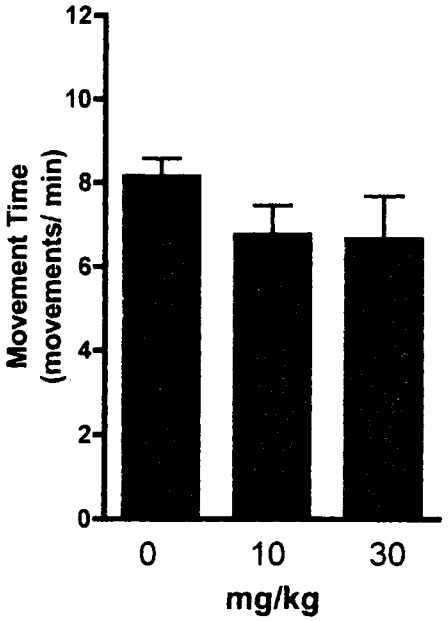
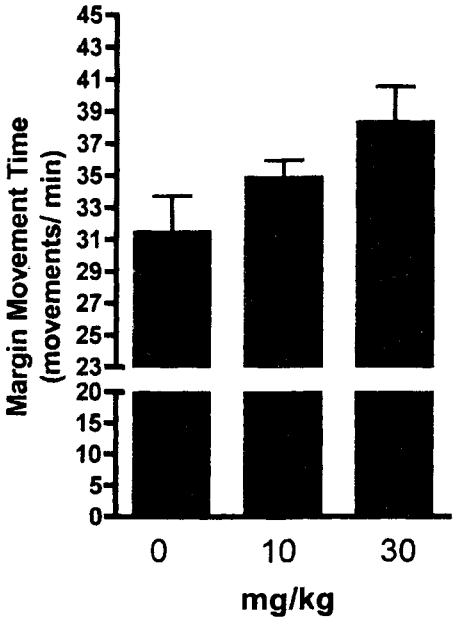


FIGURE 23- Time spent in the Margin

E. Juvenile + Adult exposure- Female



F. Juvenile + Adult exposure- Male

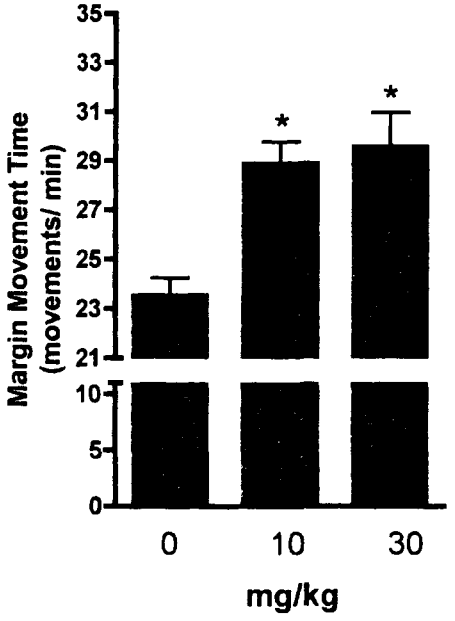


Figure 23. Young male mice exposed to Mn and adult male mice pre-exposed to Mn as a juvenile causes vulnerability in neurobehavior. Time spent in the margin was recorded in open-field activity chambers and determined for each animal post exposure to Mn. Male juvenile mice exposed to 10 and 30 mg/kg MnCl2 had a significant decrease in time spent in the margin (B) while female juvenile mice had no significant change. (C) Female and (D) male mice exposed only as adults were not vulnerable to neurobehavioral changes with exposure. (E) Female mice pre-exposed to Mn as juvenile and then again as an adult had no change while males (F) pre-exposed did have a significant increase in time spent in the margin with both the high and low dose of $MnCl_2$, 10 and 30mg/kg, as compared to control. Significance is denoted by * $p>0.05$.

FIGURE 24- Dopamine

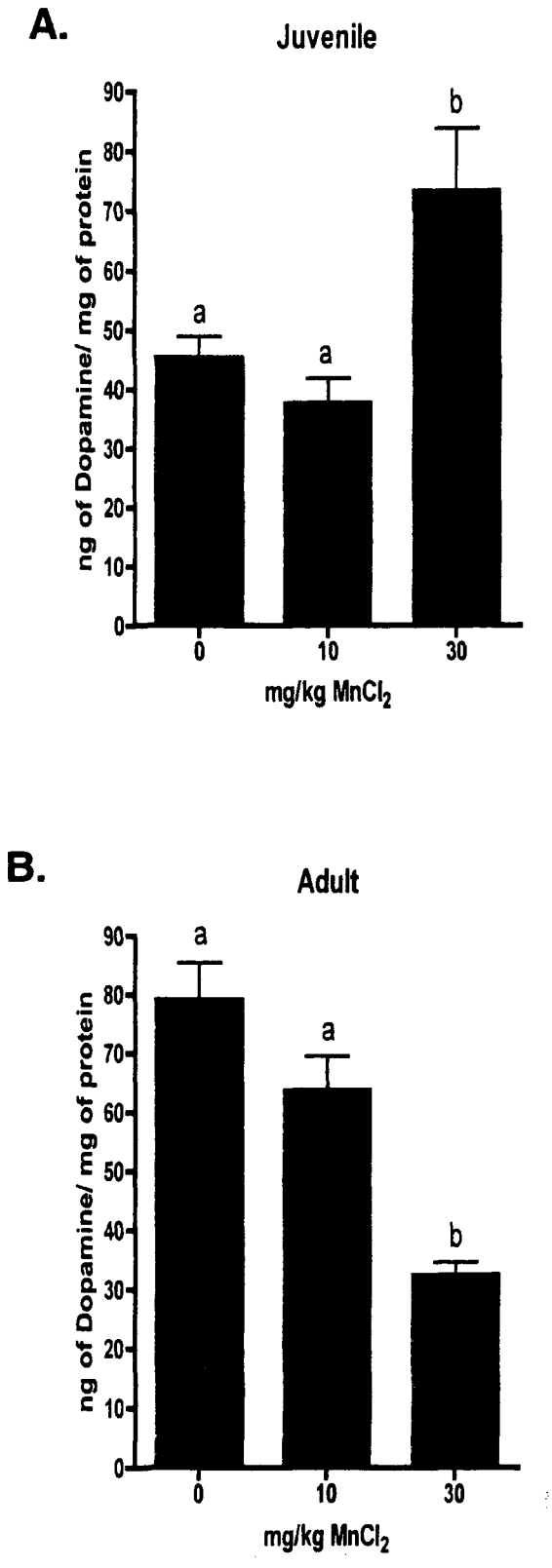


FIGURE 24- DOPAMINE

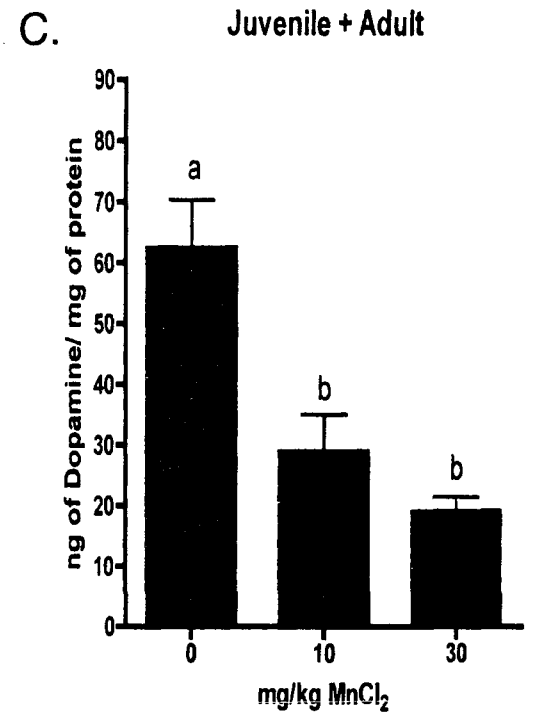


Figure 24. Mn toxicity leads to modulation of dopamine synthesis in the striatum. Dopamine is the key neurotransmitter involved in extrapyrimidal movement and was analyzed through HPLC. Mice exposed to 10 and 30 mg/kg $MnCl_2$ had a significant increase in striatal dopamine production (A) while mice exposed only as adults had no significant change (B). Pre-exposure to Mn as a juvenile resulted in a decrease in dopamine production at both Mn doses when exposed again as an adult (C). Significance is denoted by differing letters $p>0.05$.

FIGURE 25- DOPAC

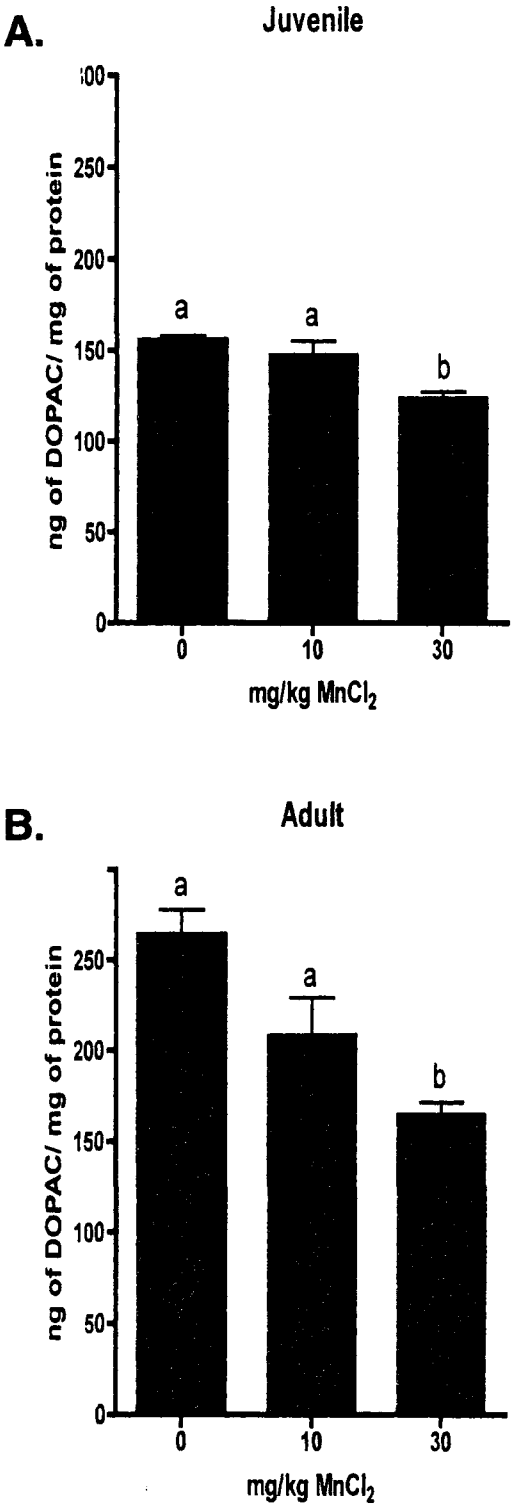


FIGURE 25- DOPAC

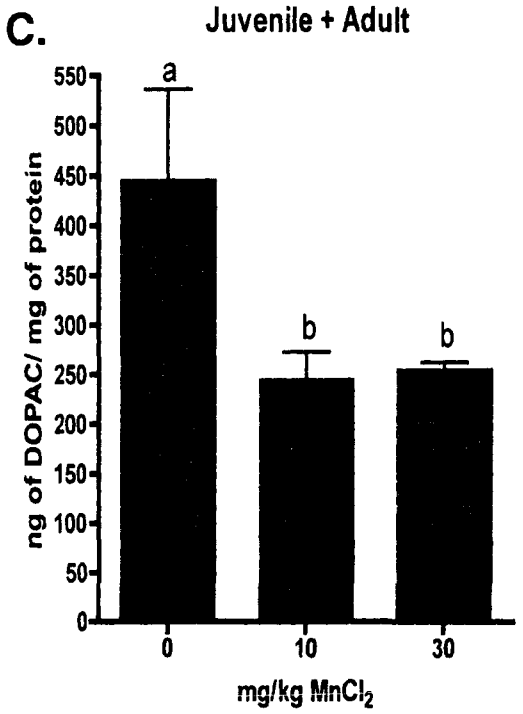


Figure 25. Production of a dopamine metabolite, DOPAC, is modulated by Mn exposure in the striatum. DOPAC is a dopamine metabolite also involved in extrapyrimidal movement. Juvenile (A) and adults (B) mice exposed to 30 mg/kg $MnCl_2$ had a significant decrease in striatal DOPAC production. Mice exposed to Mn as a juvenile and then again as an adult had significant decrease in DOPAC production at both doses (C). Significance is denoted by differing letters $p>0.05$.

FIGURE 26- ratio DOPAC:Dopamine

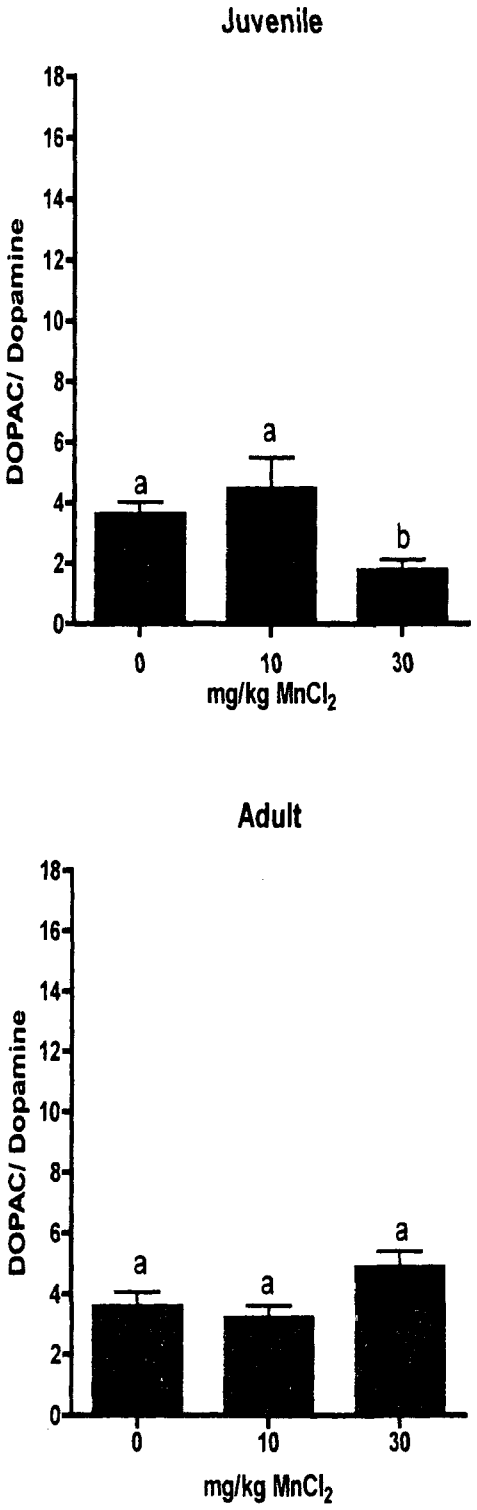


FIGURE 26- ratio DOPAC:Dopamine

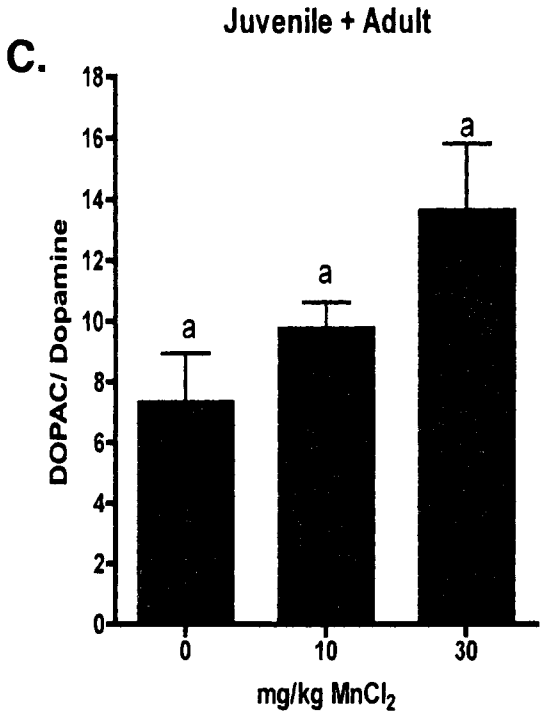


Figure 26. The turnover of dopamine is only affected in the mice only exposed to 30mg/kg $MnCl_2$ as juveniles. The turnover of dopamine is assessed by examining the ratio of DOPAC to dopamine production in the striatum. Juvenile mice exposed to high dose of Mn had a significant decrease in dopac/dopamine (A) while groups exposed to Mn as adults and juveniles + adults had no significant change dopamine turnover (B-C). Significance is denoted by differing letters $p>0.05$.

FIGURE 27- 5-HIAA

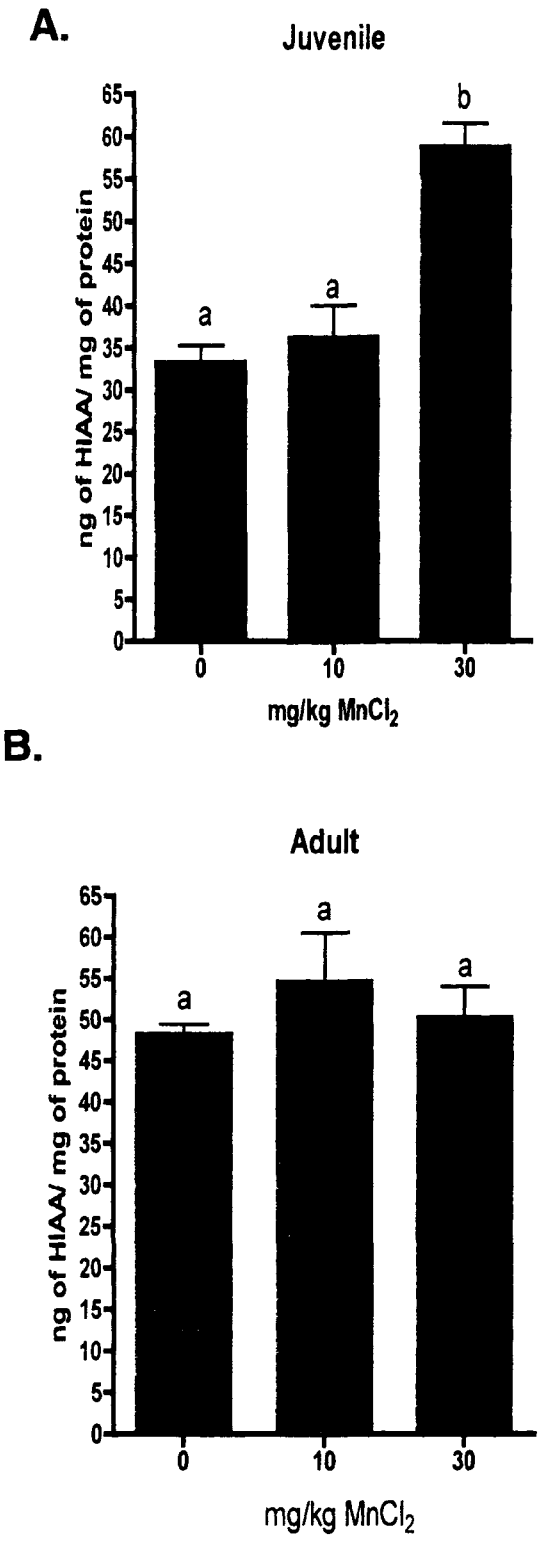


FIGURE 27- 5-HIAA

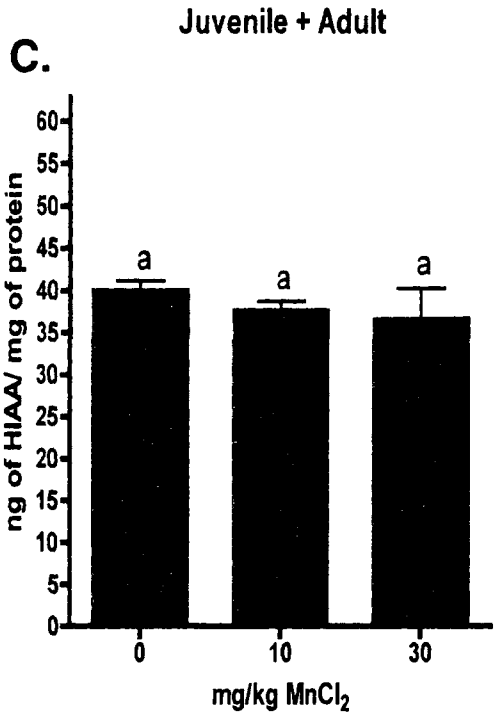


Figure 27. 5-HIAA the serotonin metabolite is modulated by Mn exposure in juvenile exposure group. The production of the serotonin metabolite, 5-HIAA gives insight into serotonin synthesis, the neurotransmitter involved in emotional stability. Juvenile mice exposed to the high dose of Mn had a significant decrease in 5-HIAA (A) while groups exposed to Mn as adults or junveiles + adults had no significant change in 5-HIAA synthesis (B-C). Significance is denoted by differing letters $p>0.05$.

FIGURE 28- 5-HT

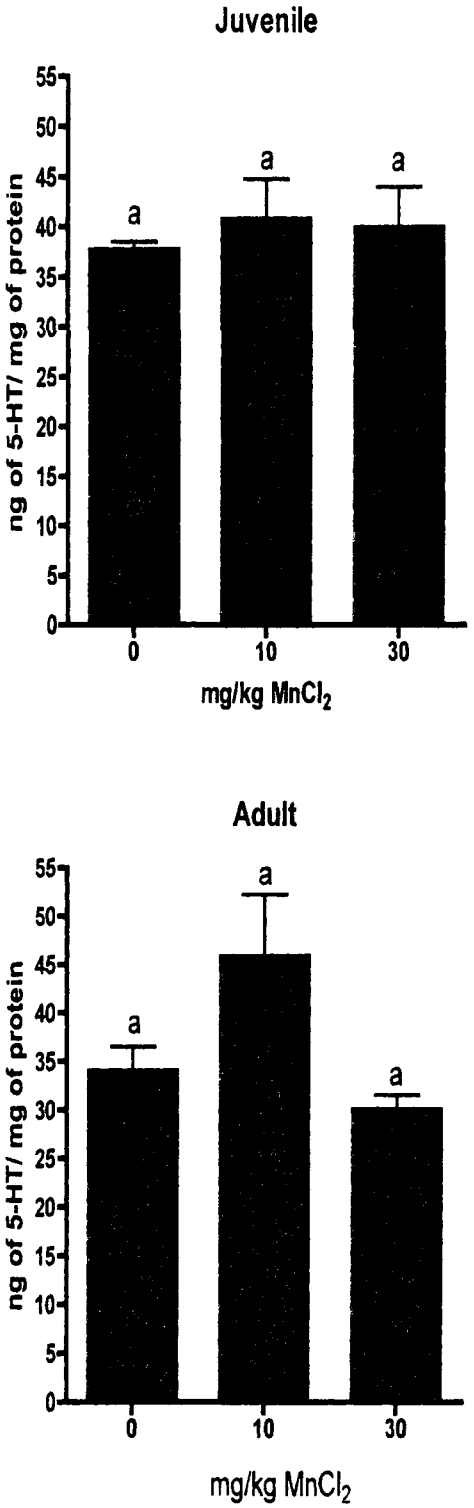


FIGURE 28- 5-HT

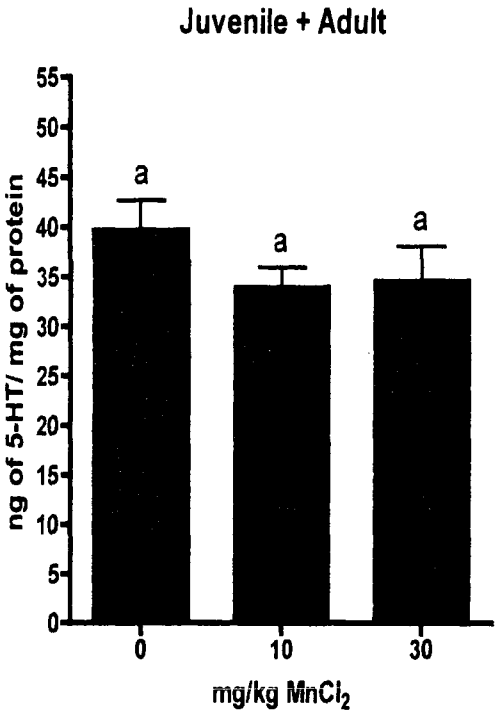


Figure 28. Serotonin production is not modulated by Mn toxicity in the striatum brain region. Serotonin is the key neurotransmitter involved in emotional stability. (A) Juvenile, (B) adult only and (C) juvenile + adult exposure groups had no significant change in serotonin synthesis. Significance is denoted by differing letters $p>0.05$.

CHAPTER 5

DEVELOPMENTAL NEUROTOXICITY OF MANGANESE: MODULATION BY ESTROGEN

ABSTRACT

Manganese (Mn) is an essential element, however, at high exposure levels, Mn causes dopamine deficiencies and Parkinsonian-like symptoms. Interestingly, neurodegenerative diseases have shown a higher incidence in males than females indicating a sex-dependent susceptibility possibly linked to the presence of estrogen. Moreover, young male mice exposed to Mn are vulnerable to neurobehavior changes. Pathological features of Mn neurodegeneration include glial inflammation and increases in NOS2 expression, specifically in regions of the basal ganglia. Previous research in our laboratory has shown NF-κB activation to be the primary pathway in which NOS2 is induced when exposed to Mn. However, it is unknown if estrogen modulates this pathway by altering glial inflammation and the factors promoting transcriptional activation of NOS2 with Mn exposure. Therefore, we postulate that with supplemented estrogen in male mice there will be an attenuation of astrogliosis and NOS2 expression with low dose Mn exposure. Juvenile transgenic mice expressing an NF-κB-GFP reporter were separated into groups composed of females, males, and males surgically implanted with Silastic capsules containing E_2, or a control implant containing corn oil. Mice were treated with 0, 50mg/kg, or 100mg/kg $MnCl_2$ by gastric gavage from postnatal day 21-34. Mice were sacrificed and brain samples were stained for protein expression or utilized for metabolite analysis. Glial NF-κB and NOS2 staining increased with treatment of Mn and were reduced back to control levels with estrogen treatment. Dopamine levels increased with exposure to Mn and reduced to control levels with supplemented

estrogen while DOPAC and the ratio of DOPAC to dopamine levels diminished with Mn exposure and were returned back to control levels in males given estrogen. These data indicate that a sex difference exists in developmental vulnerability to Mn and that estrogen acts as a neuroprotectant to reverse the vulnerability of male mice to Mn neurotoxicity. This appears to be, in part, through the capacity of E2 to prevent neuroinflammation by suppressing activation of microglia and astrocytes, with a concomitant decrease in NF-kB-dependent expression of NOS2 in glia and a parallel decrease in neuronal nitrosative stress.

INTRODUCTION

Manganese (Mn), an essential dietary element with healthy daily human intake being 2-3 mg/day (WHO, 1973) but levels above this intake can result in toxicity in many human populations. Mn environmental and dietary distribution has become a concern due to clinical similarity to Parkinson's disease when exposed at excess. The increased exposure to Mn through both dietary and inhalation routes leads to neurotoxicity with targeted damage to the extrapyramidal motor nuclei of the basal ganglia. In the past decade, Mn exposure to young children has been documented in varying countries indicating cognitive deficits and hyperactivity in children exposed to moderate levels of Mn in drinking water (Bouchard et al., 2007; Wasserman et al., 2006; Woolf et al., 2002). Dietary intake studies have shown soy-based infant formula has approximately 200-fold more Mn than breast milk (Krachler et al., 2000). Futhermore, brain Mn levels are elevated by chronic iron deficiency, a major world health problem (Garcia et al., 2007), which has also been shown to associated with attention-deficit/hyperactivity disorder (ADHD) in children (Konofal et al., 2004).

Moreover, occupational exposure to toxic levels of Mn has been recognized in factory workers, smelters, and more recently, welders (Bowler et al., 2006b). Environmental human exposure to Mn has increased since the addition of methylcyclopentadienyl Mn tricarbonyl (MMT) an anti-knock agent in gasoline in both United States and Canada (Aschner and Allen, 2000; Mergler et

al., 1999). All of these studies indicate collectively that with elevated daily Mn exposure through ingestion or inhalation the basal ganglia is targeted for neurotoxicity.

It is well known through various epidemiology studies that PD, among other degenerative diseases, have been shown to have a sex-dependent susceptibility with a higher incidence in males than females (Kenchappa et al., 2004). The ovarian steroid hormone, 17-β-estradiol (E_2), holds many functions both reproductive and non-reproductive. E_2 has been implicated in regulation of synaptic plasticity and involved as a protectant in various neurodegenerative diseases and ischemic events (Morale et al., 2006; Stirone et al., 2005). A decrease in striatial dopamine and substantia nigra pars compacta (SNpc) dopaminergic neurons is characteristic of animals exposed to 1-methyl-4-phenyl-3,6-dihydro-2H-pyridine (MPTP) and 6-hydroxydopamine (6-OHDA) but in the presence of E_2 the loss of the functional dopaminergic system was recovered (Callier et al., 2001; Murray et al., 2003; Tripanichkul et al., 2007) indicating a protective role for E_2. Recent evidence has shown a decrease in the amount of astro- and micro-gliosis normally acquired with MPTP exposure in rodent males exposed to E_2 (Tripanichkul et al., 2006) but the underlying protective signaling mechanism acting through the glia cells is unknown.

The binding of E_2 to estrogen receptors (ERs) is known to modulate transcription of factors such as NF-κB (Dodel et al., 1999; Vegeto et al., 2003). NF-κB, a Rel protein family member (Xie et al., 1993) is involved in inflammation, cell division, apoptosis, and immune responses (Karin and Ben-Neriah, 2000;

Perkins, 2000) and the principal transcription factor for inducible nitric oxide synthase (NOS2) expression. NF-κB is activated following phosphorylation of the inhibitory subunit (IκBα) by the IκB kinase (IKK) complex, targeting it for degradation by 26S proteosome and allowing the p65-RelA/p50 subunits of NF-κB to translocate into the nucleus (Nakano et al., 1998; Vermeulen et al., 2002). MAP kinases, such as extracellular signal-responsive kinase (ERK) have been shown in prior studies to be activated in astrocytes upon exposure to Mn and cytokines, leading to activation of NF-κB and expression of NOS2 (Moreno et al., 2008a). ERK has been shown to be activated upon E_2 exposure as well in neuronal cultures (Mannella and Brinton, 2006) but it is simply unknown if the same is true in astrocytic cultures. The identification of the mechanism that E_2 modulates the transcriptional activation of NOS2 following Mn exposure is essential to understanding the key elements of NO overproduction in Mn neurotoxicity.

Collectively, these data raise concern that elevations in brain Mn may predispose males to dysfunction of the basal ganglia early in life. Although the increased environmental and dietary exposures to Mn are recognized, the fundamental mechanisms leading to damage of the basal ganglia are simply unknown. Studies from our laboratory have indicated a sex-dependent susceptibility to Mn *in vivo* (Chapters 3 & 4) as well has demonstrated Mn role in induction of inflammatory signaling pathways such as NOS2 expression through soluble guanlyl cyclase (sGC) promoting ERK-dependent enhancement of NF-kB signaling (Moreno et al., 2008a), but the role of E_2 in the signaling pathway(s)

activating NF-κB is unclear. It is hypothesized that E_2 will modulate catecholamine neurotransmitters and NOS2 protein expression in glial cells through NF-κB signaling in the basal ganglia. In order to address this hypothesis we exposed transgenic NF-κB-green fluorescent protein (GFP) mice to $MnCl_2$ for two weeks during development with and without estrogen exposure. The results indicate that E_2 modulates basal ganglia neurotransmitters and decrease NOS2 expression in glial cells through NF-κB signaling.

MATERIALS AND METHODS

Reagents. All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Primary antibodies for glial fibrillary acidic protein (GFAP) were from Dako Cytomation (Denmark) and Sigma (St. Louis, MO). Antibodies for ionizing Ca^{2+} binding adaptor molecule-1 (IBA-1) and inducible nitric oxide synthase (NOS2) were from Wako Chemicals Inc. (Osaka, Japan), and BD Biosciences (San Jose, CA), respectively. Primary antibodies to 3-nitrotyrosine (3-NTyr) were from Upstate, (Charlottesville, VA), and Major Microtuble Association Protein 2 (MAP2) from Abcam (Cambridge, MA). Horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine reagents were part of the Vectastain ABC kit from Vector Labs (Burlingame, CA). AlexaFluor-555 and 647–labeled secondary antibodies were from Invitrogen (Eugene, OR).

Animal exposure model. Male and female NF-κB-GFP mice were housed in microisolator cages and kept on 12-h light/ dark cycles with access to laboratory chow and water *ad libitum*. Littermates were utilized throughout the study. Mice were split up into three groups; female and one group of males had Silastic capsules surgerically inserted subcutaneously at day 19 with safflower oil as a control while the other group received 25ug of 17-β-estradiol benzoate suspended in safflower oil Silastic capsules, throughout the study as indicated in Handa's paper (Kudwa et. al., 2008). All mice received 0.9% normal saline, 50mg/Kg $MnCl_2$, or 100mg/Kg $MnCl_2$ by gastric gavage daily from day 21-34

postnatal. Animals were weighed prior to each gavage and the amount of Mn delivered was adjusted accordingly. Additionally, the amount of Mn delivered was adjusted for the molar concentration in the tetrahydrate form to achieve a precise dose of 50 or 100 mg/Kg. All procedures were performed under the supervision of the Animal Care and Use Committee at the Colorado State University and were approved prior to onset of the studies.

Immunofluorescence. Mice were anesthetized by inhalation of isofluorane and perfused intracardially with 4% paraformaldehyde in 20mM cacodylate in 1X PBS buffer (pH 7.4). Subsequently brains were collected and kept in 4% paraformaldehyde for 2 hours then transferred to 15% sucrose in 20mM cacodylate in 1X PBS overnight followed by overnight exposure to 30% sucrose in 20mM cacodylate in 1X PBS until frozen in OCT embedding medium. Brains were kept frozen at -80°C until coronal 10mm serial sections were sliced using a Microm HM 500 cyrostat. Co-immnunofluorescence was utilized to examine protein expression in SNpr, globus pallidus (Gp), and striatum (St). Primary antibodies to anti-GFAP (1:250) and anti-IBA-1 (1:100) combined with anti-NOS2 (1:100) to examine gliosis. In order to examine the signaling pathways inducing NOS2 expression co-immunofluorescence was performed using primary antibodies to anti-GFAP (1:250) in combination with either anti-ERK (1:100) or anti-cGMP (1:100). Anti-MAP-2 (1:500) antibody was utilized in combination with anti-3-nitrotyrosine (1:100) to levels of protein nitration in neurons. Specific protein epitopes were visualized with secondary antibodies labeled with AlexaFluor- 555 or -647 and slides were mounted in media containing DAPI to

identify cell nuclei. Images were acquired using either a Zeiss 20X or 40X air PlanApochromat objective and 6 – 8 microscopic fields were examined per treatment group in 2 – 4 animals per group.

Determination of catecholamines and monoamine neurotransmitters. The following method and equipment has been modified from Champney et. al., in order to assess levels of five metabolites from brain samples exposed to MPTP; Dopamine, 2-(3,4-dihydroxyphenyl)acetic acid (DOPAC), Homovanillic acid (HVA), Serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA)(Champney et al., 1992). The Schimadzu LC-10AS high performance liquid chromotograph was utilized along with Schimadzu LC-10A pump that circulated the 7% methanol mobile phase isocratically at a 1.0 mL/min flow rate. The LED-6A electrochemical detector working electrode was set at +0.67 V, recorder output set at 8nA, response output at STD or 0.5 seconds, negative polarity for the output and polarity of electrode set at reduction. Experiments were performed through a Microsorb-MV C-18 reverse phase column with a pore size of 100A, 5mm size and 25cm in length (Varian; Walnut Creek, CA). The mobile phase consisted of 0.946g Na_2HPO_4, 2.8g of citric acid, 18.6mg of EDTA and 20mg of sodium ocytl sulfate in 930 of Milli-Q water maintained at a pH of 4.6. The buffer was filtered through 0.45mM inorganic sterile filter followed by the addition of 70 mL of HPLC grade methanol was added to the buffer followed by gentle mixing of the buffer.

Brain samples were snap frozen with liquid nitrogen after extraction from the brain and stored in -80°C for up to two years. The samples were then

removed and kept on ice, weighed, and 300ul of 0.2M perchloric acid (PCA) containing 0.5 mg/ml of Deoxyephinephrine (EPN) was applied per 10mg of wet weight brain. Tissue samples were then disrupted by 2sec sonication and placed directly onto ice. 20ul of sample was saved for total protein concentration via BCA protein assay. The following sample was then centrifuged at 14,000 x g for 10 min at 4°C. Aliquots of the sample were loaded into glass HPLC vials and ran within eight hours of sonication. Standards for all 5 compounds were processed in the same manner as the samples and 1.0 mg/mL, 0.6 mg/mL, 0.3 mg/mL and 0.1 mg/mL concentrations of standards concluded the runs for each day. The internal standard EPN was included in all runs in order to account for minute changes in detector sensitivity. The sample peaks areas were compared to the standard peaks area and values are expressed as ng of neurotransmitter/ mg of total protein.

Levels of Mn, Cu and Fe in brain tissue. Brain regions were collected and stored at -80°C until sample preparation. Brain samples were weighed and transferred to acid-cleaned polypropylene tubes, 100µl of Nitric Acid was applied to each sample and cooked for 2 hours at 95°C to digest the tissue. Lipids were then digested with 50µl of 30% Hydrogen Peroxide for 15 min. at 80°C followed by 50µl of Hydrochloric Acid to complex the metals for 15 min. at 80°C. Samples were then brought up in 1mL of Milli-Q water. Analysis of the samples was performed on a Perkin-Elmer Elan DRC-II ICP-MS and throughout the digestion standards and blank tubes underwent the same digestion procedures as the

samples. Concentration of metals analyzed in brain samples are expressed a ppm of metal.

Statistical Analysis. Comparison of two means was performed by Student's *t*-test. Comparisons three or more means was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison *post-hoc* test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). For all experiments, $p < 0.05$ was considered significant.

RESULTS

Young NF-κB-GFP transgenic mice were utilized in this study to enable the assessment of the role of Mn toxicity on activation of NF-κB during the development period that were found to be vulnerable in Chapters 3&4. Following the two weeks of Mn exposure serum samples were taken and analyzed for estrogen (E_2) levels. It was determined that the females exposed to saline (control) and 50mg/kg $MnCl_2$ had no change in levels of E_2 but interestingly the female mice exposed to the high dose of Mn (100mg/kg $MnCl_2$) did experience a decrease in E_2 levels (Fig. 29). The males implanted with a control capsule had relatively low E_2 levels found in the serum independent of Mn exposure while the males implanted with E_2 did have a significant increase in levels of E_2 in the serum compared to both males without E_2 capsules and females (Fig. 29).

In order to identify neurochemical dysfunction due to Mn treatment striatal brain tissues were analyzed by HPLC to determine alterations in the basal ganglia. The neurochemical analysis revealed that the male mice exposed to 100mg/kg $MnCl_2$ alone did have an increase in dopamine levels compared to controls and the low dose treatment (Fig. 30A). No significant change was detected with either females or males with E_2 exposure (Fig. 30B-C). Upon observation of the dopamine metabolite, DOPAC, it was found that 100mg/kg of $MnCl_2$ caused a significant decrease in the amount of DOPAC produced in the striatum (Fig. 31A) and no significant change in DOPAC occurred in the females or the males with E_2. The assessment of dopamine turnover by examining the

ratio of DOPAC to dopamine showed a significant decrease in male mice with 100mg/kg $MnCl_2$ while the females and males with E_2 had no change in the ratio with exposure to Mn (Fig. 32A-C). Finally, serotonergic neurotransmitters, 5-HT and 5-HIAA, were assessed and no change was seen with treatment in any of the groups of mice (Fig. 33-34).

The transgenic mouse allowed for the assessment of the role of Mn toxicity on activation of NF-κB in addition to examination of cell-specific transcription of NOS2 occurring via a NF-κB dependent mechanism as seen in previous *in vitro* studies done in the laboratory. Through the use of microglia specific antibody, IBA-1, we were able to visualize microglia, along with NF-κB activation and NOS2 protein expression simultaneously in the substantia nigra pars reticulata (SNpr) and identified the percent of microglia expressing the above mentioned inflammatory markers. Upon doing this we ascertained that an increase NF-κB activation was observed in activated microglia within the male mice exposed to 100mg/kg $MnCl_2$ (Fig. 35A) while no change was found with E_2 supplemented male mice (Fig. 35B). The same finding was observed with NOS2 expression increasing with high dose exposure in microglia of male mice (Fig. 36A) furthermore, with no change in male mice with E_2 was detected (Fig. 36B-C). More importantly analysis also found that microglia had an increase of co-localization of both NF-κB activation and NOS2 expression in microglia at the low-dose of Mn, 50mg/kg $MnCl_2$ (Fig. 37A) while the mice with E_2 supplementation had no change (Fig. 37B).

Next, we determined if NF-κB activation and NOS2 expression was occurring in astrocytes located in the SNpr, in order to do this we used a specific astrocyte antibody, GFAP and identified the percent of positive GFAP cells that either had NF-κB activation, NOS2 expression or co-localized with both. A significant increase was observed in astrocytes having NF-κB activation within males exposed to both 50 and 100 mg/kg $MnCl_2$ compared to control (Fig. 38A) while the E_2 exposed male mice did not change in astrocytes undergoing NF-κB activation (Fig. 38B). NOS2 expression in astrocytes also increased significantly with both 50 and 100 mg/kg $MnCl_2$ (Fig. 39A) while again the male mice supplemented with E_2 had no change in astrocytic NOS2 expression (Fig. 39B). Finallly, co- localization of both NF-κB activity and NOS2 expression in astrocytes did increase significantly for 100 mg/kg $MnCl_2$ and 50 mg/kg $MnCl_2$ signifies a trend of an increase but it was not significant from the controls (Fig. 40A). Lastly, the male mice with E_2 containing capsules did not experience a co-localization of both NF-κB and NOS2 in astrocytes located within the basal ganglia specifically the SNpr (Fig. 40B).

DISCUSSION

Neurodegenerative diseases are known to affect more men than women and in models of Parkinson's disease (PD) specifically, a neuroprotective effect is observed with exposure to physiological levels of E_2. Previous findings in our laboratory observed Mn-induced hyperactivity in young developing male mice. Along with the knowledge of the neuroprotective effects of E_2 we wanted to investigate if the same was true in Mn neurotoxicity. The inflammatory signaling mechanism identified *in vitro* has given some insight into how Mn induces NO signaling in astrocytes but this specific mechanism has not been identified *in vivo*. In order to examine the pathway we will utilize the NF-κB-GFP transgenic mouse allowing visual determination of NF-κB activation and subsequently involved in NOS2 simultaneously expression in both microglia and astrocytes in the mouse brain.

Neurochemical analysis of the key neuronal systems involved in Mn toxicity, dopamine and serotonin, were determined by HPLC for the striatum, which is the nuclei of the brain that is damaged by Mn toxicity. Increases in dopamine were observed in the high dose Mn group within the male mice but with supplementation of E_2 the dopamine levels recovered to control indicating a neuroprotective role of E_2 (Fig. 30). A metabolite of dopamine, DOPAC was decreased in male mice exposed to 100 mg/kg of Mn but with the exposure of E_2 there was no decrease emphasizing the protective role of E_2 (Fig. 31). An increase in dopamine and DOPAC has been observed in other rat models of Mn toxicity (Cotzias et al., 1976; Dorman et al., 2000; Shukla et al., 1980), which is

thought to be attributed to an increase in catecholamine production. Interestingly, the turnover of dopamine to DOPAC is decreased with both 50 and 100 mg/kg $MnCl_2$ which can be explained because dopamine is not being catabolized to DOPAC causing a decrease in levels found in the striatum. In a MPP+ model of PD, mesencephalic tissue had a decrease in dopaminergic neuron death with exposure to E_2 (Bains et al., 2007) and 6-OHDA induced dopaminergic lesions of the striatum are protected by E_2 bringing levels of dopamine back to controls (Murray et al., 2003). No changes of the sertoninergic system were observed, only the dopaminergic system had detectable changes in this study revealing that exposure to Mn during development leads to a dopaminergic dependent dysfunction.

In order to validate previous *in vitro* data that NF-κB is involved in the transcription of NOS2 expression in glial cells we assessed the amount of NF-κB activation and NOS2 expression in both microglia and astrocytes. It was determined that NF-κB activation increased with 100 mg/kg of $MnCl_2$ in both microglia and astroglial cells in SNpr brain region of male mice (Fig 35A & 38A). Supplementation of E_2 *in vivo* decreased NF-κB activation to control levels in both glial cell subtypes indicating E_2 is modulating the functional activation of NF-κB and NOS2 activation (Fig. 35B & 38B). The observation that E_2 inhibits p65 to bind to κB response element has been documented in a variety of diseases (Guzeloglu-Kayisli et al., 2008) and specifically in cases of neurodegeneration.

The expression of NOS2 is known to occur both *in vivo and in vitro* in Mn toxicity rodent models (Liu et al., 2006; Moreno et al., 2008a) but until this study

it has not been identified that E_2 will modulate the expression of NOS2 halting NO production and subsequent neuronal injury with Mn exposure. Young male mice did experience a significant increase in NOS2 expression with the high dose of Mn in both microglia and astrocytes (Fig. 36A & 39A). Upon exposure to E_2 in both glial cell subtypes minute NOS2 expression is seen with Mn exposure leading to the conclusion that E_2 can modulate NOS2 expression in the glia (Fig. 36B & 39B). E_2 has been shown to inhibit NOS2 expression in an ischemic model by not allowing the binding of p65 to the κB response elements on the NOS2 gene (Shih et al., 2006), a possible mechanism that is occurring in these young male mice exposed to Mn. Co-expression of both NF-κB and NOS2 was identified in both microglia and astrocytes exposed to Mn but more importantly this co-localization of both NF-κB and NOS2 in glial cells was inhibited by E_2 (Fig. 37 & 40), emphasizing that E_2 is modulating NF-κB transcriptional activation of NOS2.

Interestingly, the astrocytes also seem to be more vulnerable to Mn induced inflammatory signaling than the microglia, due to the increase in both NF-κB activation and NOS2 expression at the low dose of Mn only in the astrocytes. Astrocytic sensitivity could be due to the ability of astrocytes to uptake Mn at high concentrations (Maurizi et al., 1987) leading to increased activation of signaling pathways promoting inflammatory signaling dependent on NF-κB transcription of NOS2. In conclusion, the data from this study thus far indicate a protective role of E_2 on Mn induced NF-κB activation and NOS2 expression in both microglia and astrocytes. Understanding how estrogen

modulates specific molecular pathways underlying the production of inflammatory mediators in activated glial will provide valuable molecular insight into the pathways influencing susceptibility to environmental Mn during development.

CHAPTER 5

FIGUR ES

FIGURE 1
E_2 Serum Levels

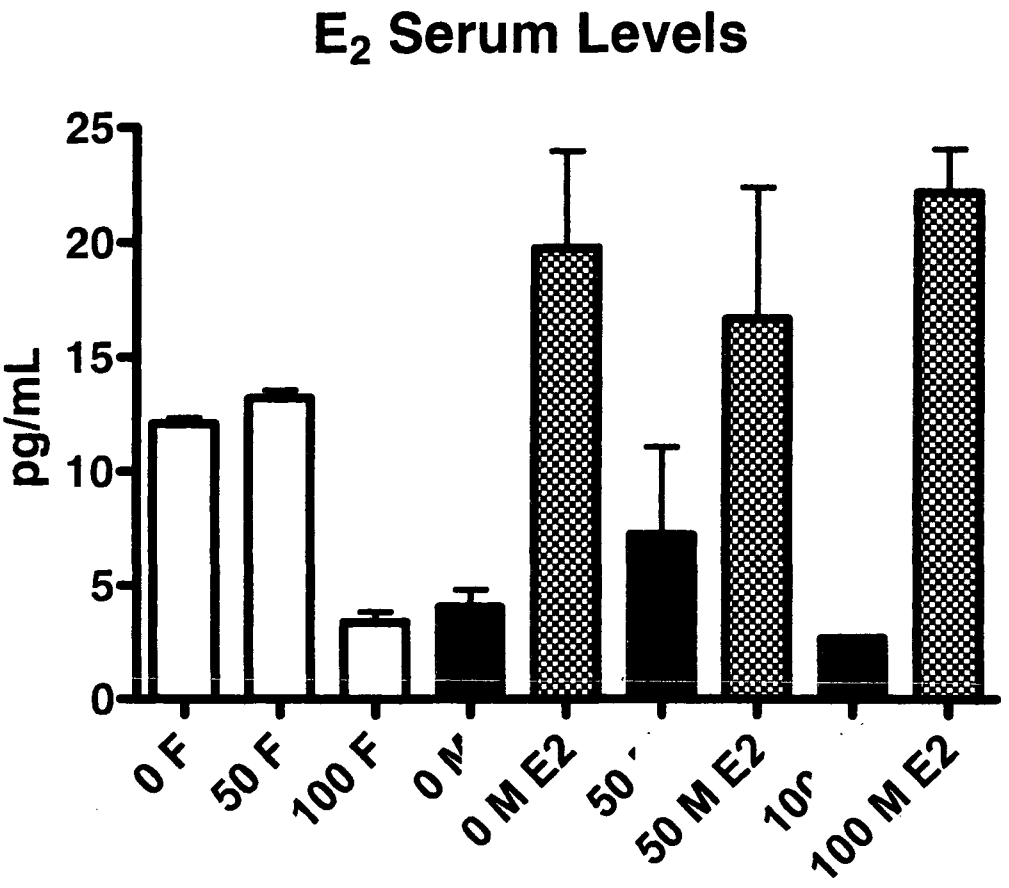
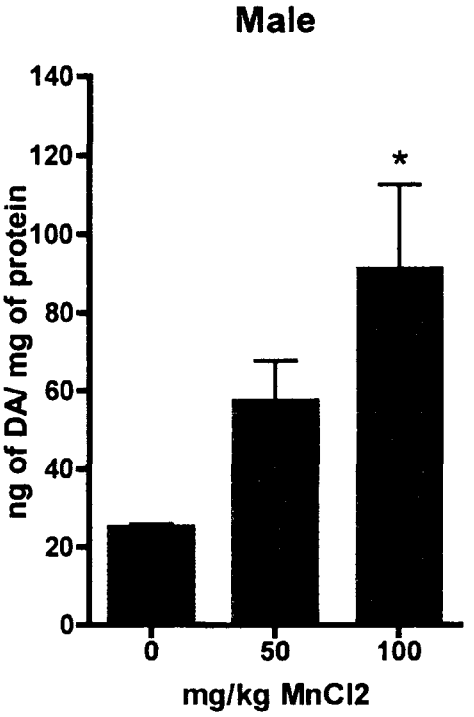


Figure 29. Estrogen (E_2) serum levels were tested in all mice to examine if the males received E_2 during the study. Male mice were exposed to 25ug of 17-β-estradiol through Silastic capsules inserted subcutaneously into each animal. Female and control male mice received capsules with safflower oil (vehicle control) while a group of male mice received E_2. Female control and 50mg/kg Mn had no significant change while females exposed to 100mg/kg MnCl had a significant decrease in E_2 serum levels. Male mice exposed to control capsules had low levels of E_2 while males exposed to E_2 had a significant increase.

FIGURE 2
Dopamine

A.



B.

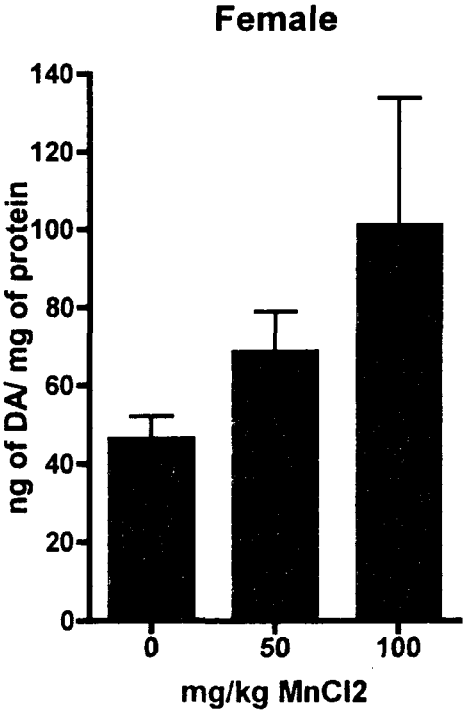


FIGURE 30
Dopamine

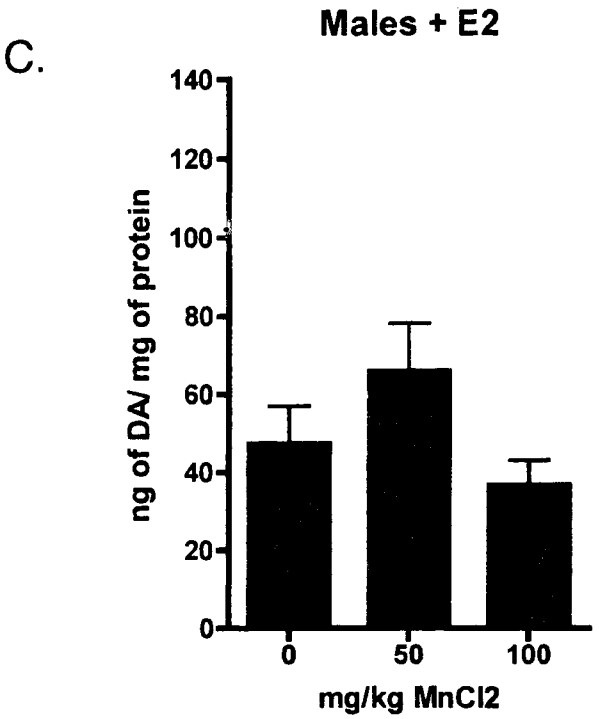
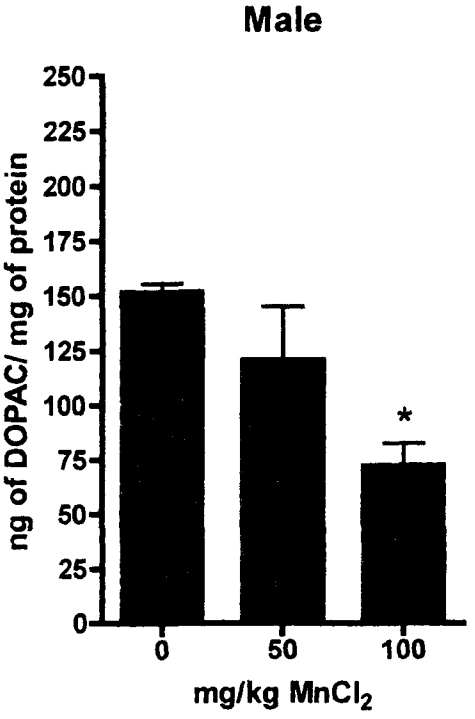


Figure 30. Mn- induced increases in dopamine synthesis is recovered by E_2. Dopamine is the key neurotransmitter involved in extrapyrimidal movement in the basal ganglia. Young male mice had a significant increase in dopamine levels in the striatum (A) while female mice had no significant change (B). Dopamine concentrations were sustained at control levels in male mice exposed to Mn and 25μg of E_2 over a period of 14 days (C). Significance is denoted by *. $p<0.05$.

FIGURE 31
DOPAC

A.



B.

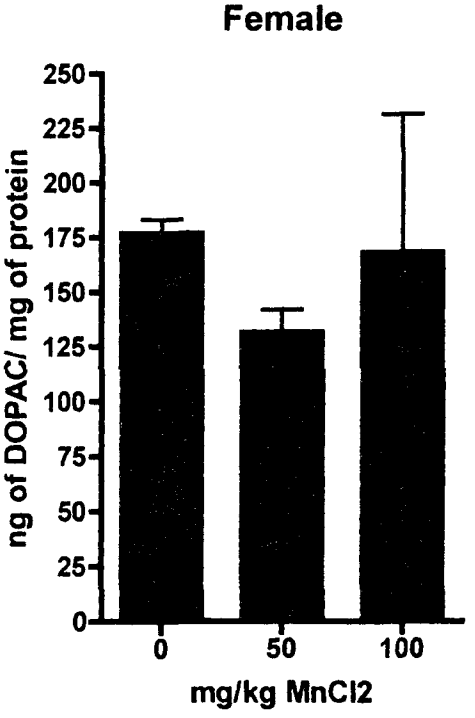


FIGURE 31
DOPAC

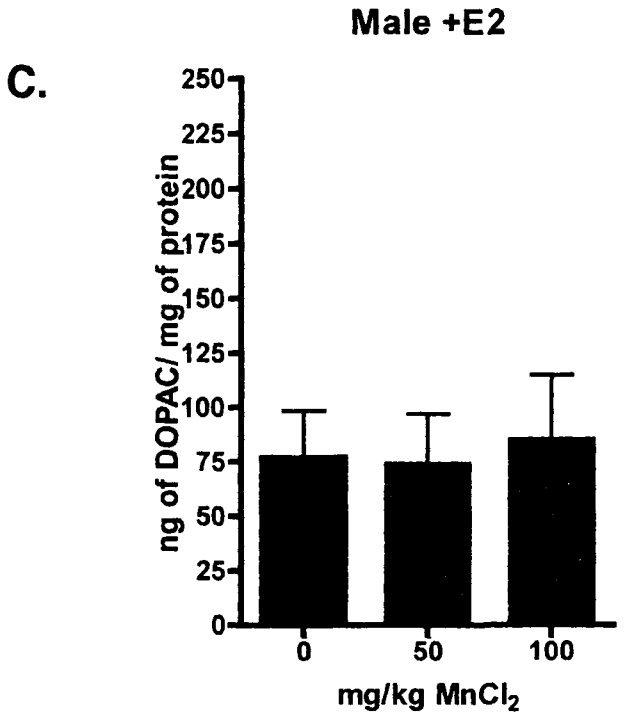
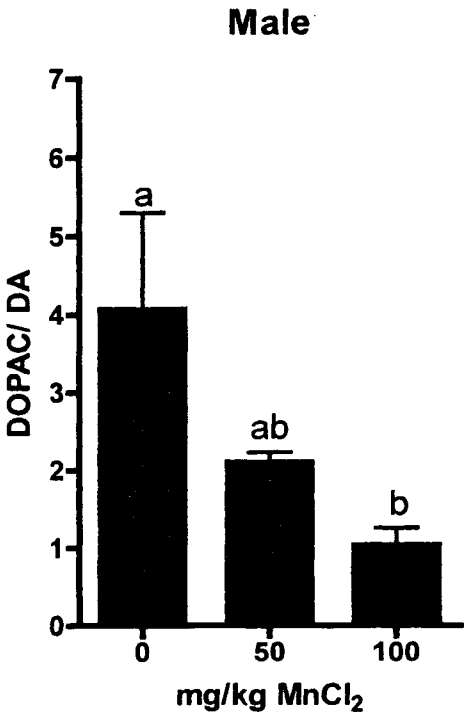


Figure 31. E_2 allows for recovery of DOPAC production in the striatum with Mn toxicity. DOPAC is metabolized from dopamine and is essential for normal dopaminergic system functioning. Young male mice had a significant decrease in DOPAC levels in the striatum (A) while female mice had no significant change (B). Dopamine concentrations were sustained at control levels in male mice exposed to Mn and 25μg of E_2 (C). Significance is denoted by *. $p<0.05$.

FIGURE 32
ratio DOPAC/ Dopamine

A.



B.

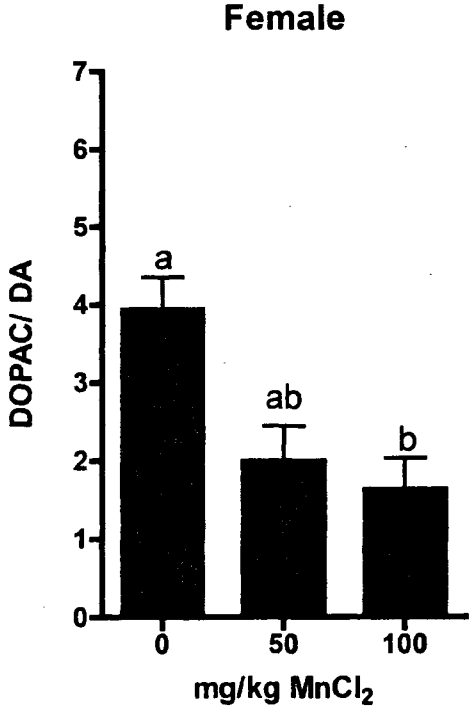


FIGURE 32
ratio DOPAC/ Dopamine

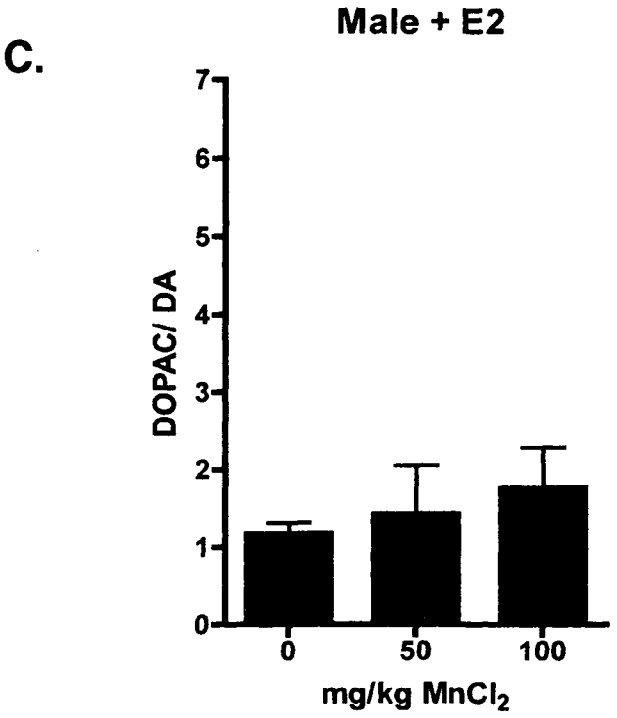
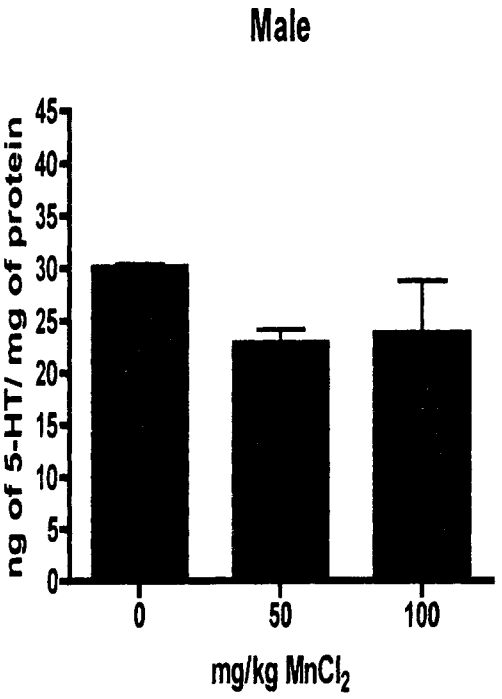


Figure 32. Decrease in turnover of dopamine occurs with exposure to Mn but is modulated by E_2. The turnover of dopamine is assessed by examining the ratio of DOPAC to dopamine production in the striatum. Exposure to 100 mg/kg $MnCl_2$ significantly decreases dopamine turnover in males (A) and females (B) juvenile mice while 50mg/kg $MnCl_2$ shows a trend to decrease turnover as well. E_2 exposure causes no significant changes in the ratio of DOPAC to dopamine. Significance is denoted by differing letters $p>0.05$.

FIGURE 33
5-HT

A.



B.

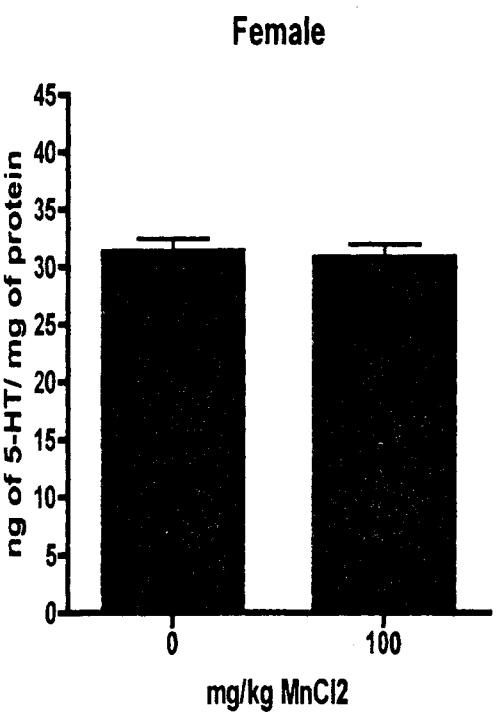


FIGURE 33
5-HT

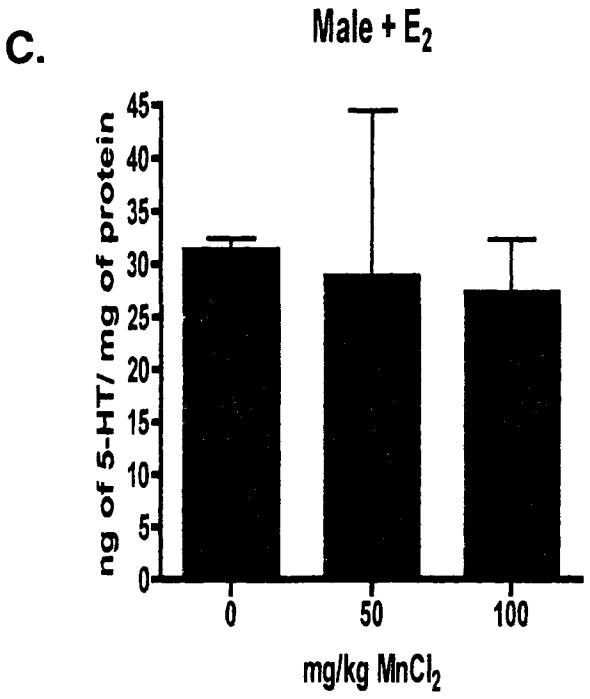
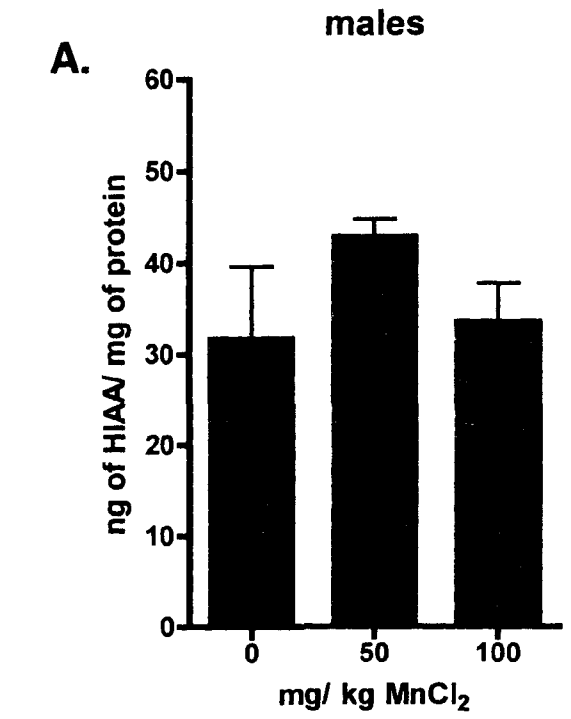


Figure 33. There is no modulation of serotonin levels with relatively low dose Mn or E_2 exposure. Serotonin is the key neurotransmitter involved in emotional stability. (A) Male, (B) Females and (C) Males with E_2 exposure groups had no significant change in serotonin synthesis. Significance is denoted by differing letters $p>0.05$

FIGURE 34
5-HIAA



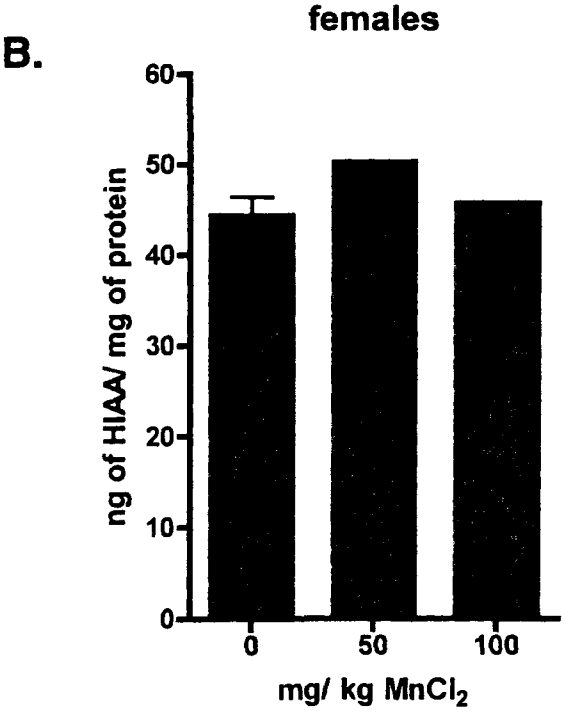


FIGURE 34
5-HIAA

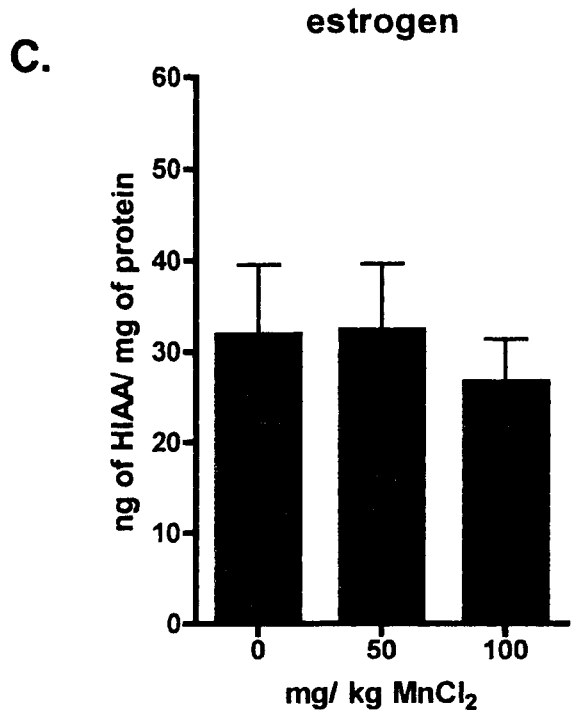


Figure 34. Striatal 5-HIAA levels are not changed by Mn or E_2 exposure. The production of the serotonin metabolite, 5-HIAA gives insight into serotonin synthesis the neurotransmitter involved in emotional stability. No change of 5-HIAA concentrations in the striatum was observed in (A) males (B) females or (C) males with E_2. Significance is denoted by differing letters $p>0.05$.

FIGURE 35
Microglia co-expressing NOS2 and NF-κB

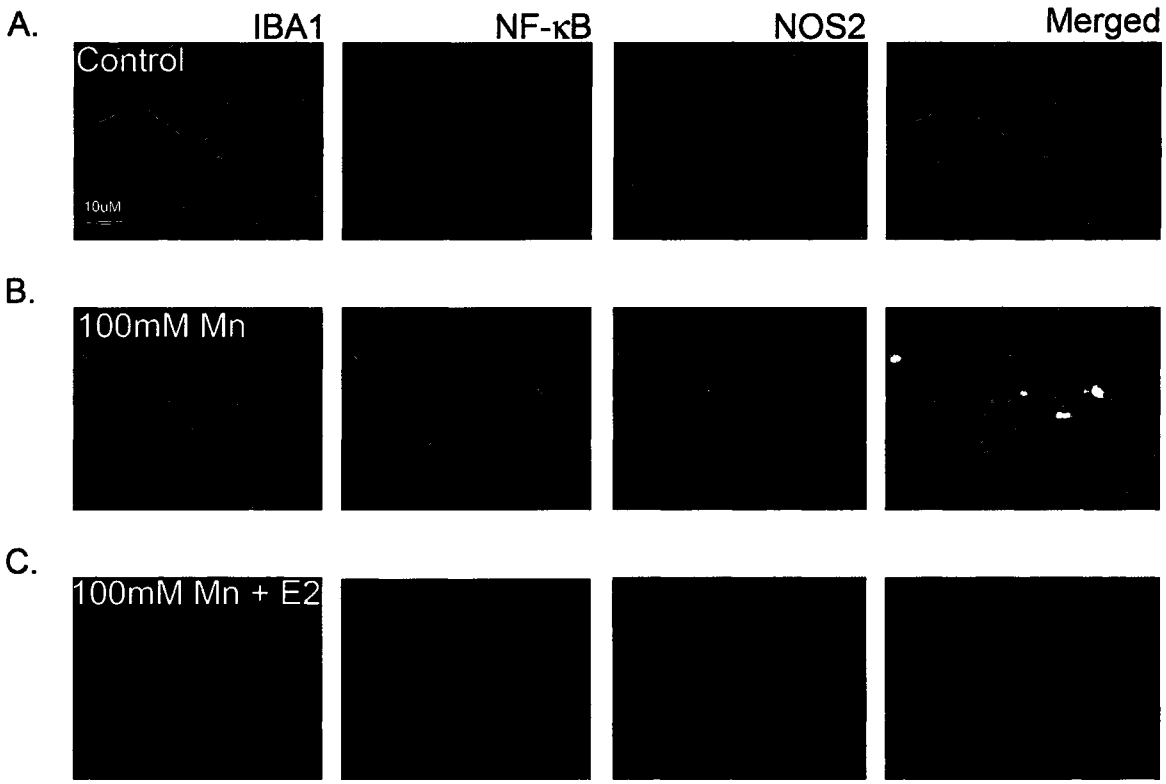


Figure 36
Microglia co-expressing NF-κB

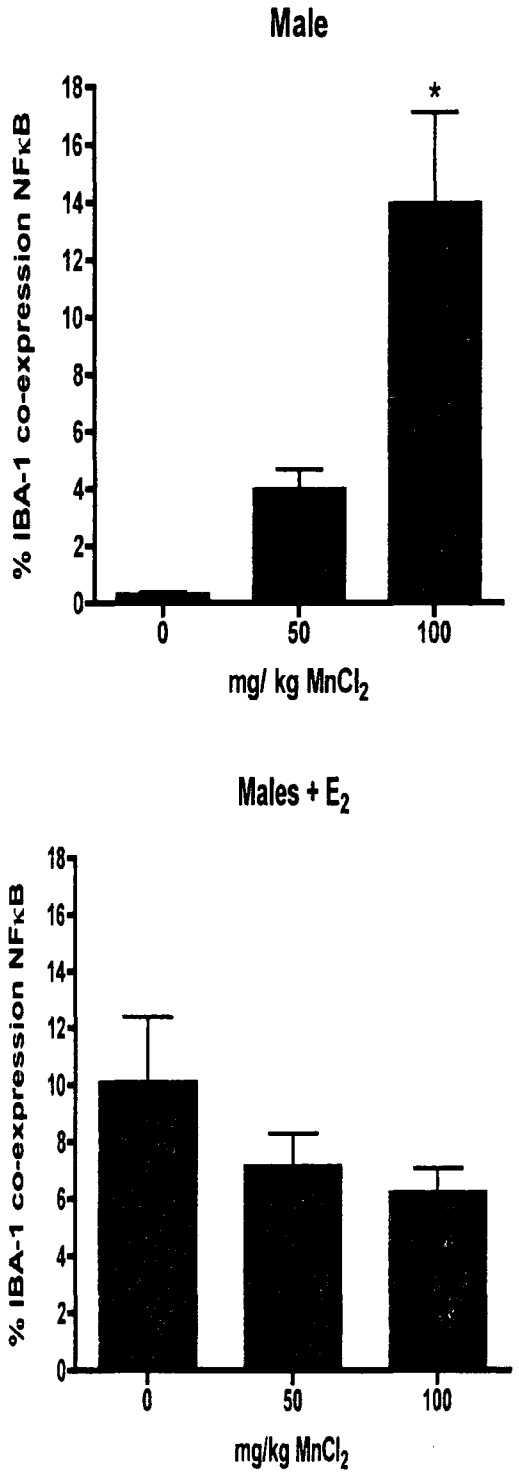


FIGURE 37
Microglia co-expressing NOS2

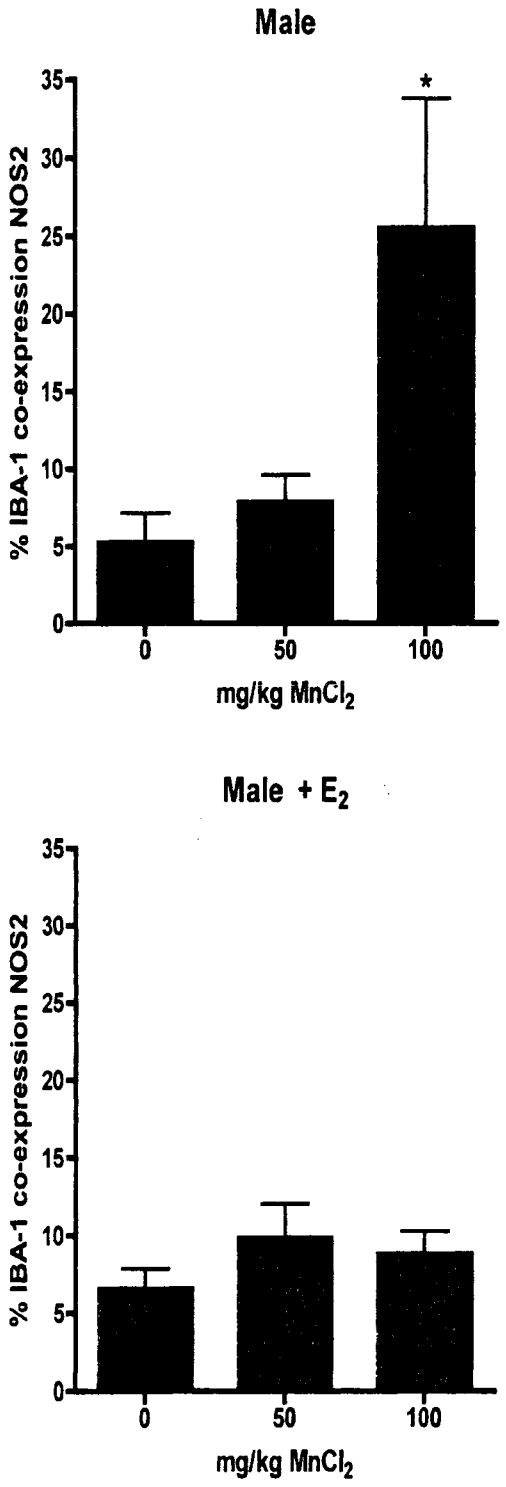
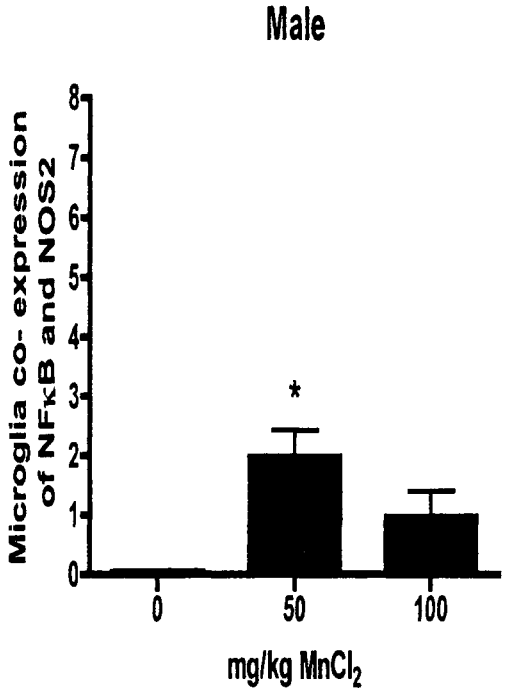


FIGURE 38
Microglia co-expressing NF-κB and NOS2



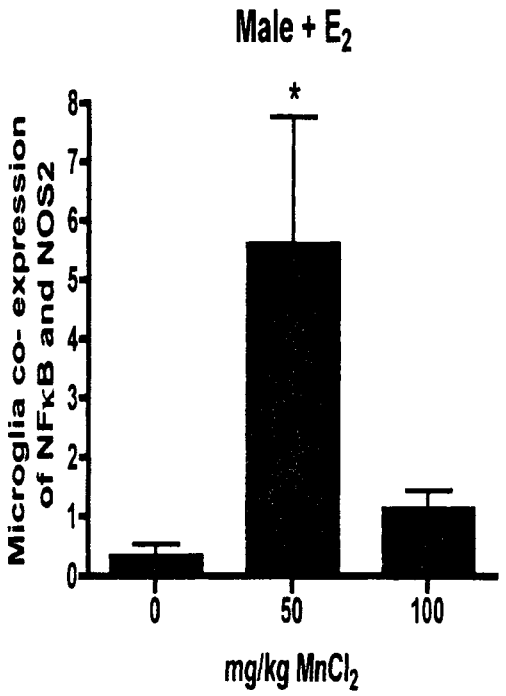


Figure 35. E_2 modulates activated microglial cells in NF-κB-GFP mice express NOS2 and NF-κB following differential exposure to Mn as juveniles. Microglial activation of NF-κB and expression of NOS2 was assessed via co-immunofluorescence in multiple regions of the basal ganglia from mice exposed to Mn. Representative images of the SNpr are presented from control mice and those treated with 100 mg/kg $MnCl_2$ as control treated animals (A) 100mg/kg $MnCl_2$ animals (B) or 100mg/kg $MnCl_2$ with E_2(C). Images of Iba-1, NF-κB activation and NOS2 expression are shown in purple, green and red channels, respectively, and cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Merged images indicate co-localization of NF-κB and NOS2 expression in Iba-1-positive microglia in mice exposed to Mn. Scale bar = 10 μm

Figure 36. Mn exposure causes activation of NF-kB in microglia found in the substantia nigra pars reticulata (SNpr) and was modulated by E_2. In order to assess the mechanism of Mn induced inflammatory signaling NF-kB transgenic mice were utilized to be able to quantitatively assess activation of the transcription factor. Male exposed to 100mg/kg $MnCl_2$ had a significant increase in NF-kB activation compared to control and 50mg/kg $MnCl_2$ (A). E_2 subsided the activation of NF-kB observed in the male mice without E_2. Significance is denoted by *. $p< 0.05$

Figure 37. NOS2 expression increases in microglia located in the SNpr of mice exposed to Mn. NOS2 expression in glial cells is a marker of inflammatory signaling and is well characterized in Mn toxicity. Using immmunofluoresence co-localization of the protein in microglia were quantitated. Male mice exposed to 100mg/kg $MnCl_2$ had a significant increase in NOS2 expression compared to control and 50mg/kg $MnCl_2$ (A). The male mice exposed to E_2 had no change in NOS2 expression indicating a protective effect. Significance is denoted by *. $p< 0.05$.

Figure 38. Quantitative analysis of co-expression in microglia was attained for NF-κB activation and NOS2 expression with an increase observed with Mn exposure subsided by E_2. Co-expression of NF-κB and NOS2 will give insight into the inflammatory signaling in microglia with Mn toxicity. Male mice had a significant increase of microglial NF-κB activation and NOS2 expression with 50mg/kg $MnCl_2$ while 100mg/kg had a trend of increasing inflammation but was not significant compared to control (A). The E_2 exposure did not allow significant changes of co-expression in microglia located in the SNpr (B). Significance is denoted by *. $p< 0.05$.

Figure 39
Astrocytes co-expressing NF-κB and NOS2

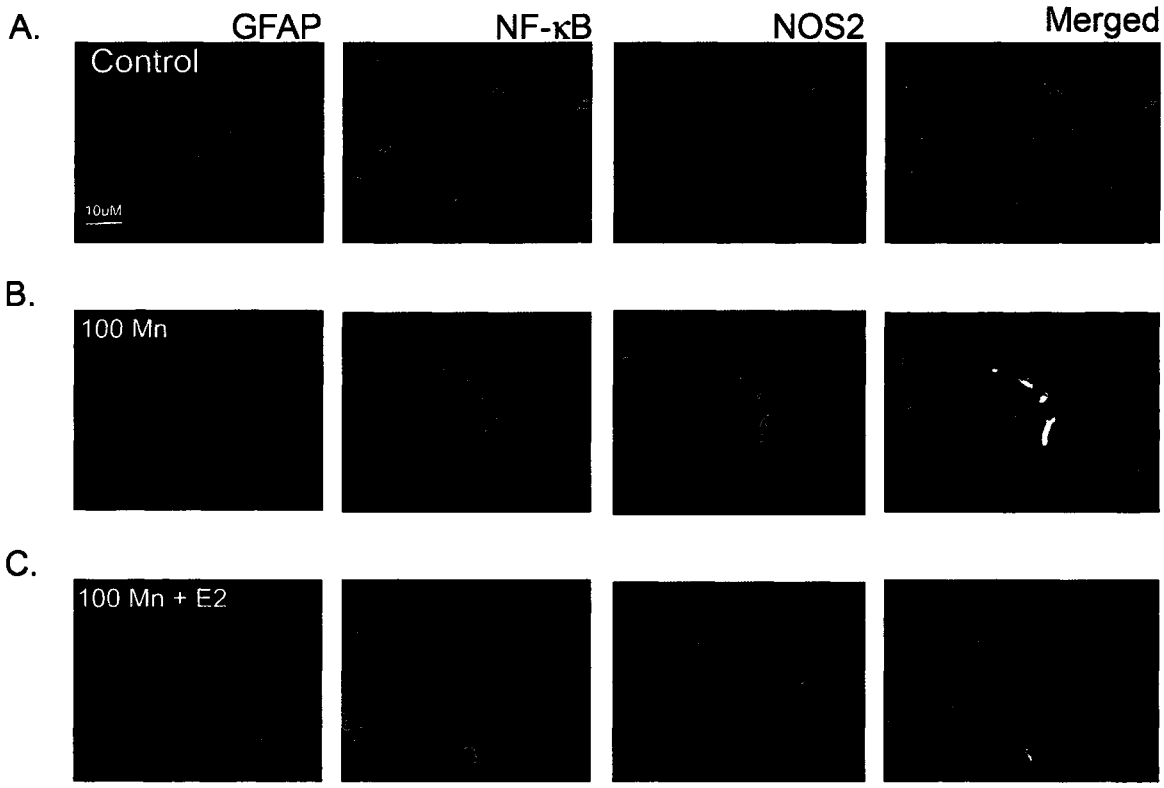


Figure 40
Astrocytes co-expressing NF-κB

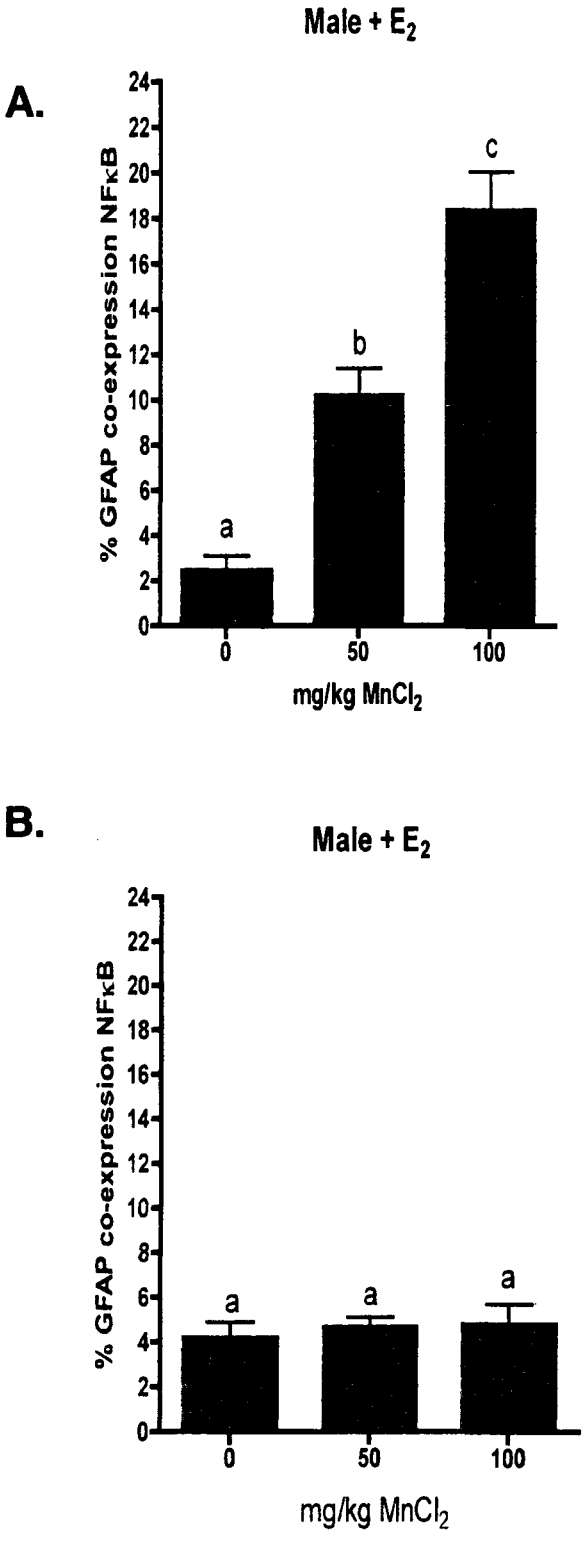
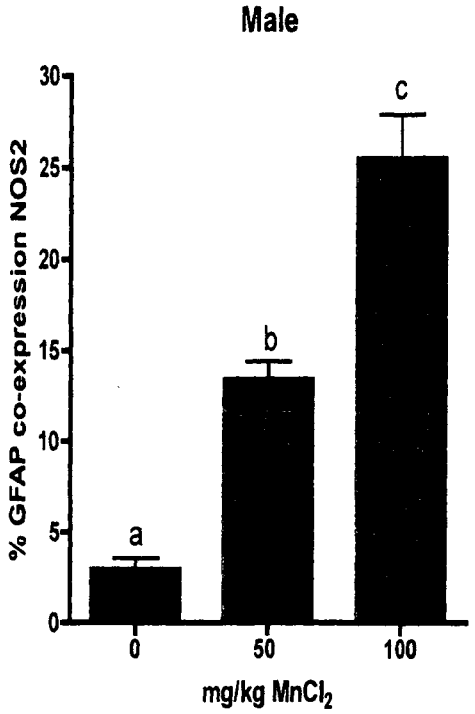


Figure 41
Astrocytes co-expressing NOS2

A.



B.

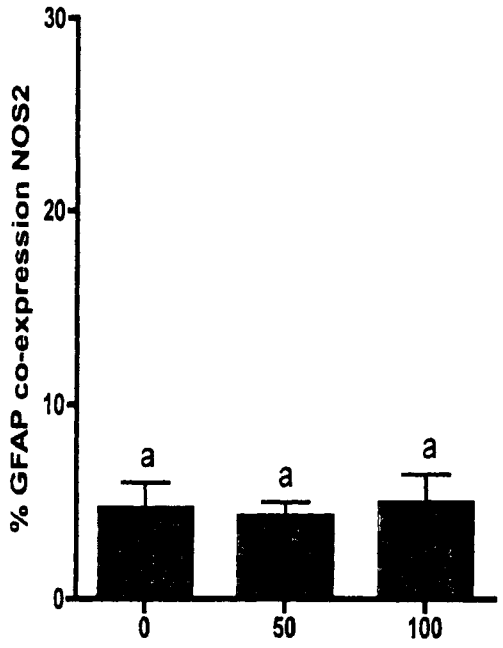


Figure 42
Astrocytes co-expressing NF-κB and NOS2

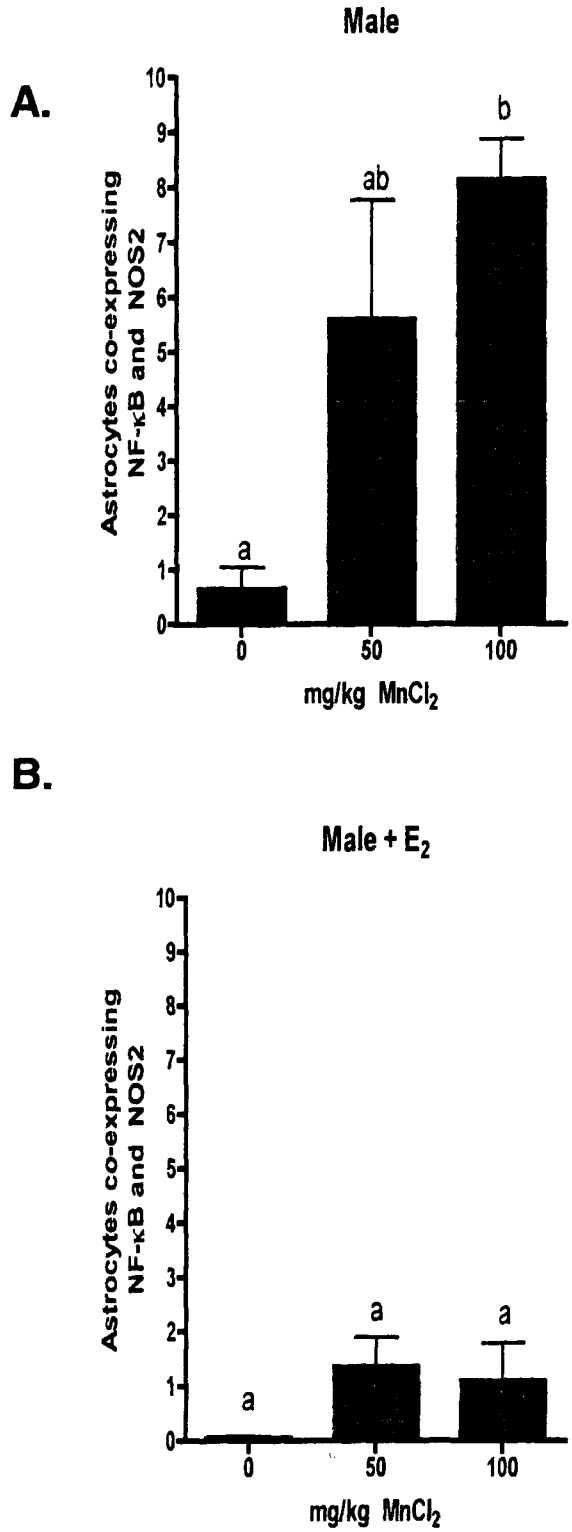


Figure 39. E_2 modulates activated astrocytes in NF-κB-GFP mice express NOS2 and NF-κB following differential exposure to Mn as juveniles. Microglial activation of NF-κB and expression of NOS2 was assessed via co-immunofluorescence in multiple regions of the basal ganglia from mice exposed to Mn. Representative images of the SNpr are presented from control mice and those treated with 100 mg/kg $MnCl_2$ as control treated animals (A) 100mg/kg $MnCl_2$ animals (B) or 100mg/kg $MnCl_2$ with E_2(C). Images of GFAP, NF-κB activation and NOS2 expression are shown in purple, green and red channels, respectively, and cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Merged images indicate co-localization of NF-κB and NOS2 expression in GFAP-positive astroglia in mice exposed to Mn. Scale bar = 10 μm

Figure 40. Mn exposure causes the activation of NF-κB in astrocytes found in the substantia nigra pars reticulata (SNpr) and modulated by E_2. In order to assess the mechanism of Mn induced inflammatory signaling NF-κB transgenic mice were utilized to be able to quantitatively assess activation of the transcription factor. Male exposed to 50 and 100mg/kg $MnCl_2$ had a significant increase in NF-κB activation compared to control (A). E_2 subsided the activation of NF-κB observed in the male mice without E_2. Significance is denoted by differing letters. $p< 0.05$

Figure 41. Astrocytes increased NOS2 expression with Mn exposure but was subsided with E_2. NOS2 expression in glial cells is a marker of inflammatory signaling and is well characterized in Mn toxicity. Co-localization of NOS2 in astrocytes located in the SNpr were quantitated. Male mice exposed to 50 and 100mg/kg $MnCl_2$ had a significant increase in NOS2 expression compared to control (A). The male mice exposed to E_2 had no change in NOS2 expression indicating a protective effect. Significance is denoted by differing letters $p< 0.05$.

Figure 42. Astrocytes co-expressing NF-κB activation and NOS2 expression in astrocytes was quantitatively analyzed with an observed increase with Mn exposure, inhibited by E_2. Co-expression of NF-κB and NOS2 will give insight into the inflammatory signaling in astrocytes with Mn toxicity. Male mice had a significant increase of microglial with NF-κB activation and NOS2 expression with 100mg/kg $MnCl_2$ while 50mg/kg had a trend of increasing inflammation but was not significant compared to control (A). The E_2 exposure did not allow significant changes of co-expression in microglia located in the SNpr (B). Significance is denoted by *. $p< 0.05$.

Figure 43
Mn induced expression of cGMP is inhibited by E_2

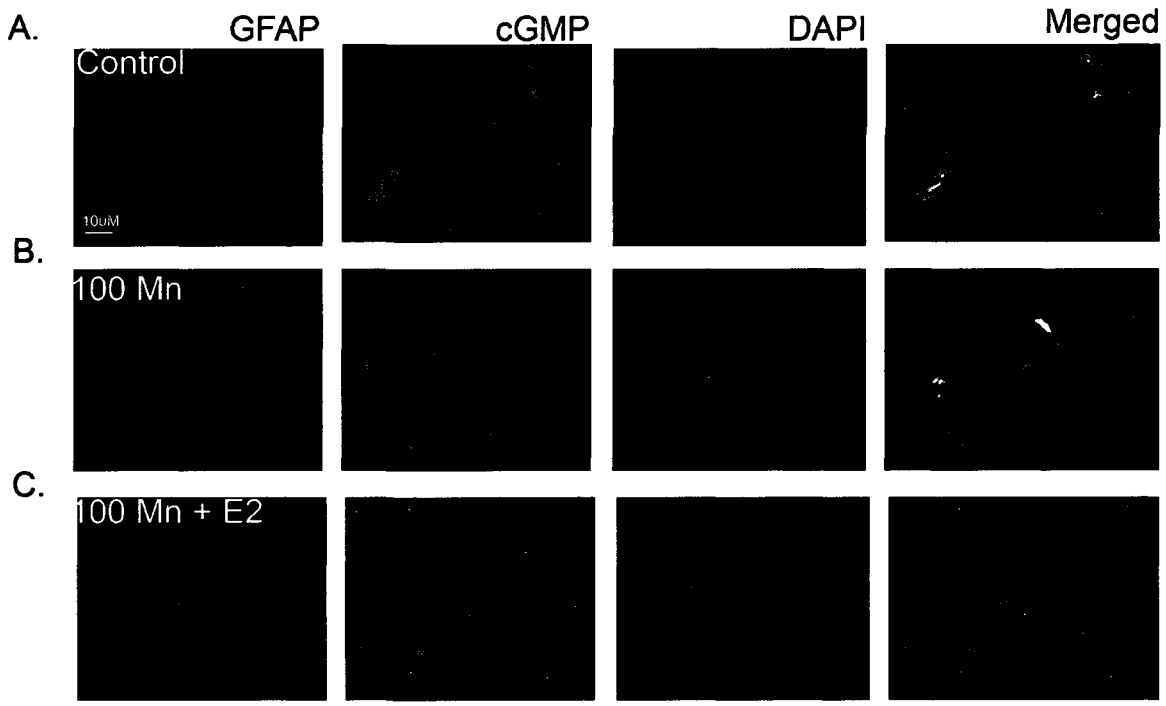


Figure 43. Mn induced activation of astrocytes increases expression of cGMP is modulated by exposure to E_2 in the SNpr. Astroglial expression of cGMP was assessed via co-immunofluorescence in multiple regions of the basal ganglia from mice exposed to Mn. Representative images of the SNpr are presented from control mice (A) and those treated with 100 mg/kg $MnCl_2$ (B) and 100mg/kg Mn with E_2 (C). Images of GFAP and cGMP expression are shown in green and red channels, respectively, and cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Merged images indicate co-localization of cGMP expression in GFAP-positive astrocytes in mice exposed to Mn. Scale bar = 10 μm.

Figure 44
3-Ntyr formation in MAP2 positive neurons decreases with E_2 exposure

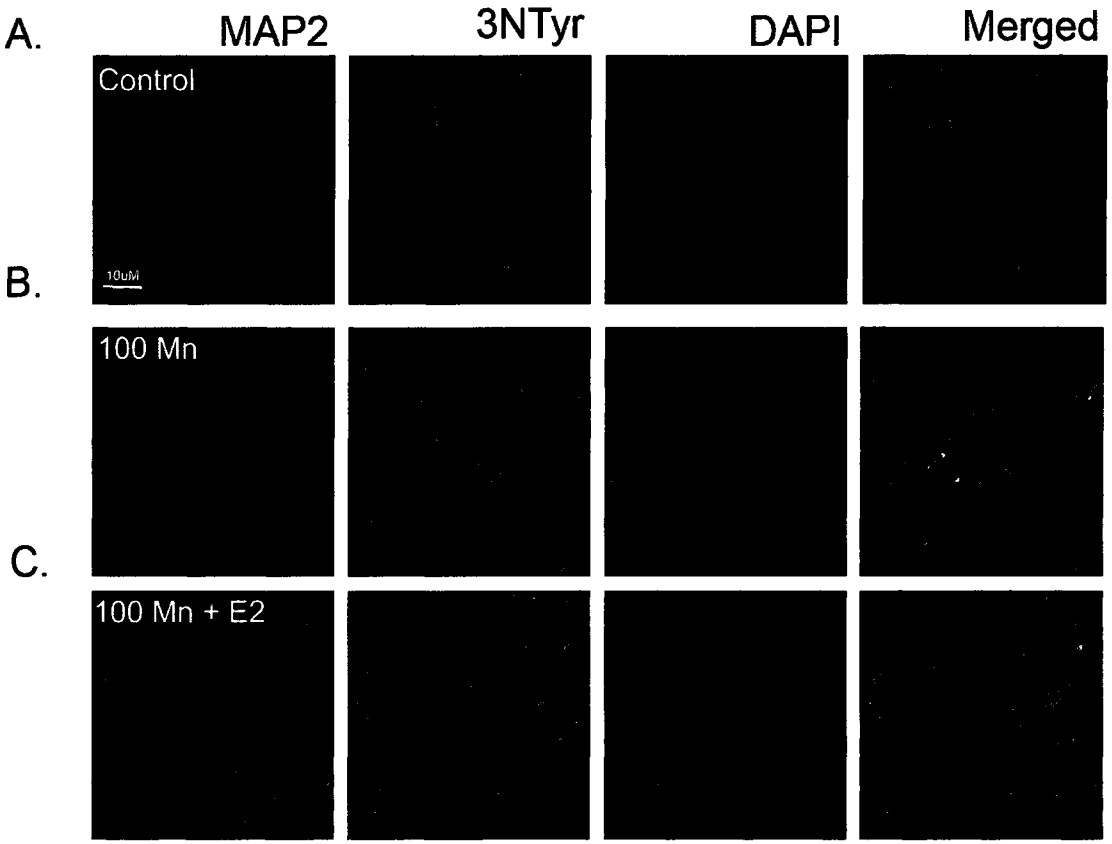


Figure 44. Manganese exposure increases levels of 3-nitrotyrosine (3-NTyr) protein adducts in basal ganglia neurons but is decreased to control levels with E_2. To detect modification of neuronal proteins by peroxynitrite ($ONOO^-$) derived from increased production of NO by activated glia, serial sections from the SNpr of juvenile mice exposed to 0 and 100 mg/Kg $MnCl_2$ from day 20 – 34 postnatal were stained with antibodies against the general neuronal marker Microtuble Association Protein 2 (MAP2; green) and 3-NTyr (red) and were counterstained with DAPI to identify cell nuclei (blue). Representative images of 3-NTyr-modified proteins indicate co-localization of 3-NTyr adducts with both neuronal soma and dendrites in the SNpr of Mn-treated juvenile mice. Scale bar = 10 μm.

CHAPTER 6

CONCLUSIONS

FINAL CONCLUSIONS

Manganism is a debilitating neurodegenerative disease affecting the basal ganglia with symptoms similar to Parkinson's disease in adults, while children exposed to Mn toxicity can suffer from cognitive deficits. The experiments discussed herein were designed to elucidate the role of gliosis-induced inflammatory signaling in both adult and young animals, to identify a temporal vulnerability of the basal ganglia to Mn neurotoxicity. Investigations into the mechanism of glial-derived nitric oxide signaling is essential in describing disease etiology and pathogenesis of Mn neurotoxicity. The role of estrogen in modulating this inflammatory pathway was assessed due to the observation that more males are diagnosed with neurodegenerative diseases than females. Furthermore, the described studies will provide valuable molecular insight into inflammatory pathways identified in many neurodegenerative diseases, thus providing new targets for therapeutic intervention.

The determination that Mn activates soluble guanylyl cyclase (sGC) and MAP kinases to potentiate NF-κB-dependent expression of inflammatory genes such as NOS2 in cortical astrocytes allows some insight into the mechanism of neuroinflammation seen in a variety of neurodegenerative diseases. More importantly, this is the first report of induction of sGC causing downstream inflammatory signaling by Mn independently of cytokines. Furthermore, these studies utilize near physiological levels, 10μM Mn, which is not only the lowest utilized in the literature but allows for better correlation between environmental

oral exposure scenarios, where individuals are being exposed to trace amounts of Mn in drinking water. Due to our identification of upstream effectors regulating NF-κB transcription of NOS2, a logical extension of this project would be to characterize transcriptional mechanisms allowing NF-κB to activate *Nos2*, thereby permitting better identification of specific protein targets that could be therapeutically modulated to mitigate neuroinflammation. It is noteworthy to state that we have identified a small part of extremely complex pathway involving many more protein kinases and molecular signals that future research could characterize as being involved in the induction of the inflammatory pathway. For example, we ascertained that p38 is also a key player in induction of NO signaling in astrocytes exposed to Mn and cytokines therefore this would be a another part of the pathway that needs to be better characterized in our Mn model. The described mechanistic studies will hopefully improve our understanding of how NOS2 and other inflammatory genes are inappropriately expressed in activated astroglia during degenerative conditions of the basal ganglia such as manganism.

Occupational exposure to Mn is well defined, and most Mn studies have attempted to understand the neurobehavior, neurochemical, and neuronal injury phenotypes observed with Mn toxicity in adults. However, much less is known regarding the effects of Mn in the developing CNS, particularly with respect to the potential risk posed by exposures early in life that may predispose to later neurological injury. In order to appreciate the sensitivity of the basal gangalia to Mn toxicity we prefomed murine *in vivo* study to determine if pre-exposure to Mn

during juvenile development would lead to increased vulnerability for glial inflammation and neuronal injury. These studies performed here revealed a striking sensitivity of the developing basal ganglia to Mn-induced activation of both microglia and astrocytes that correlated with induction of NOS2. Key basal ganglia nuclei, specifically the striatal-pallidal pathway, experienced increased nitration of neuronal proteins only in the mice exposed to Mn as a juvenile and then again as an adult, indicating that there is a increased susceptibility of neuronal injury with Mn toxicity in the developing brain. Mn exposed adult mice pre-exposed to Mn as a juvenile also had a dysfunction of the dopaminergic system with a significant decrease in dopamine and its metabolite DOPAC in the striatum. This finding emphasizes pre-exposure to Mn does render the basal ganglia vulnerable specifically the extrapyramidal system if exposed again later in life. These findings are significant because environment exposure to Mn is becoming a greater concern, especially for the young and elderly worldwide, due to iron deficiency and trace amount of Mn found in well water. Unfortunately, the combination of these effects has only begun to be investigated, and the data presented herein highlight the need for further research, as juveniles exposed to Mn during development are more responsive to re-exposure during adulthood. Therefore if damage of the basal ganglia is acquired as a child and then later in life due to occupation, liver dysfunction, environmental increases, or anemia the brain is more sensitive and can lead to increased Mn induced inflammatory pathways and neuronal injury.

Furthermore, it is also noteworthy to mention that if these studies are evidence of early exposures rendering susceptibility of the basal ganglia to neurodegeneration than not only contact with Mn later in life would be detrimental. Therotically other neurotoxins that affect the basal ganglia would be of concern as well if exposed early in life to Mn. Some concerning environmental neurotoxins such as pesticides, rotenone and paraquat would be of concern if pre-exposure to Mn occurred due to their mode of action of causing neuronal injuy in similar brain regions and neuronal systems such as dopminergic neurons. Another concern with a basal ganglia deemed sensitive early due to Mn exposure is the possibility of genetic mutations that promote parkinsonism-like neurodegeneration. For instance, the lack of the genes DJ-1, PINK1, and Parkin have been shown to lead to familial PD so if children lacking these genes were exposed to Mn then that might lead to enhanced or earlier onset of the disease.

Along with the evidence that oral exposure to Mn early in life and then again as an adult, increases injury we also observed Mn toxicity in the young male mice possibly due to modulation of normal brain development. The young mice exposed to Mn had increased hyperactivity that correlated with overstimulation of the dopaminergic system and dysfunction of the serotonergic system. Increased cognitive deficits, such as ADHD, have been characterized in children exposed to Mn in well water, lending evidence that the young mice undergoing Mn toxicity possibly are experiencing hyperactivity as seen in young humans. These studies, described in Chapters 3 and 4, suggest that juvenile development represents a critical window of sensitivity to inflammatory activation

of glia and induction of nitrosative stress in neurons and neurobehavior and chemical dysfunction. Though these studies we have found a juvenile model, weanlings to pre-pubertal, that can be utilized to test the developing brain that corresponds to elementary school-age children, a timepoint identified by epidemiology studies as being an critical time for Mn toxicity.

Upon determining degree of microgliosis and astrogliosis, an interesting finding was observed. The mice exposed as juveniles had an increase in activated microglial cells in the basal ganglia, while the degree of astrogliosis did not change. The opposite affect was observed in mice exposed to Mn as a juvenile and an adult, microgliosis did not increase with exposure while astroglisis did. Therefore, animals exposed to Mn as a juvenile and as an adult were already "primed" with microglial activation during the young age exposure, so therefore no subsequent microglia activation occurred. However, the astrocytes did become significantly activated with Mn exposure in the juvenile plus adult exposure group. It should also be noted that astrocytes were highly activated in the juvenile animals with or without treatment possibly indicating that, in the young mice, astrocytes were already in an activated state due to their age, and therefore the treatment had no effect on the inflammatory phenotype of the cells. The identification of the sequence of microglial and astroglial activation will bring a better understanding of the possible mechanism causing sustained activation of astrocytes in the brain leading, to secretion of inflammatory mediators and neurodegeneration.

Interestingly the male mice exhibited increased neurotransmittter abnormalities and behavioral changes compared to the females in the age-dependent susceptibility *in vivo* research previously mentioned. In combination of the Mn induced sex-dependent vulnerability that we identified, the concept of males were indeed more vulnerable to neuronal injury and gliosis following Mn exposure, as is well described in other neurodegenerative diseases like PD. Previous literature states that E_2 may be the primary factor in female neuroprotection. In order to evaluate the effects of E_2 in Mn toxicity, we exposed young male mice to E_2 and Mn and assessed pathological gliosis and inflammatory signaling. Our results suggest E_2 does modulate the activation of NF-κB and the subsequent transcription of NOS2 expression, a novel finding in Mn toxicity. The identification of a sex-dependent vulnerability not only gives some insight into the mechanistic pathway involved in neurodegeneration, but into the possible approach for decreasing inflammation in the brain specifically the glial cells leading to a decrease in neuronal injury.

Overall, the glial inflammatory signaling pathway in Mn toxicity has been more fully elucidated, although events at the NOS2 promoter following NF-κB binding remain unknown. Further characterization of these processes would give great insight into possible theraputic interventions of this inflammatory pathway. Future studies investigating the temporal sequence of activation of microglia and astrocytes would be beneficial in order to determine what factors are "priming the brain" for astrocyte activation, thought to be a common precursor to multiple neurodegenerative diseases. Finally, the mechanism of Mn-induced

inflammatory activation of glial cells, including variations induced by both sex and age, are more fully elucidated, which will promote a better understanding of manganism and potentially other disorders of the basal ganglia.

CHAPTER 7

LITERATURE CITED

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APPENDIX

TBALES AND PROTOCOLS

TABLE 3
Literature discussing Neurochemisty results in models on Mn neurotoxcity

Treatment	Results	Paper
90 day old male rats, 20mg/kg drinking water throughout development	MAO-A: -15% St; -13% BS, -16% cortex MAO-B: -13% hypo Insensitive to Mn trmt	Lai et. Al, 1992
Neonatal rats w/intubation; 25/50ug in water (14 or 21 days)	Monoamines did not change btw. pD14 and PD21 but 5-HT and 5-HIAA increased w/age	Kontour et. al, 1988
Rats: suppl. w/ Mn in diet (2mg/ gram of diet)	Decrease of 5-HT w/ Mn	Kimura et. al., 1978
Neonatal and Adult male rats; 25/50 mg/kg gavage	Striatal DA and DOPAC increased in neonate 50mg/kg ...no change w/adult	Dorman et. al., 2000
Rat pups: 50,250, &500ug of Mn suppl.	Negative linear relationship – decrease trend of DA	Tran et. al., 2002
Rats: maternal-fetal transfer-GD1 MnCl 1uCi a day in drinking water	No sig. effect on DA or NE in neonates	Kontur et. al, 1985
Rats: 2 or 10mg/ml Mn dissolved in drinking water of dams and the liters up to PND30	No change monoamine assay..NE,DA,DOPAC, 5-HT and 5-HIAA	Pappas et. al., 1997
Rhesus monkeys: MnSO4 aerosol	Decrease in pallidal GABA and 5-HIAA; caudate NE decreased w/ 1.5mg Mn/m^3; putamen NT's unaffected	Struve et. al., 2007
Rhesus Monkeys: chronically MnCl for 18 months	Striatal Dopamine levels normal seen w/ PET; DA normal in SNpc	Mella et. al., 1924
Rats: PN4-21 Mn via maternal milk during lactation	Increase in GABA and GABA/glutamate ratio indicating enhanced inhibition of the brain	Garcia et. al., 2006

Mn (250 or 750 ug/day) exposure PND male 1-21 then challenged w/cocaine as an adult	DA transporter binding sites decreased w/ Mn exposure (didn't have cocaine exposure)	Reichel et. al., 2006
Squirrel Monkeys: Mn expsure	Decrease in DA	Neff et. al., 1969
Rabbits: Mn Exposure	Decrease in DA	Mustafa et. al., 1971
Male rats 1, 10, 100, 1000 uM MnCl2	Striatal tissues exposed to Mn (in vitro) decrease DA formation at 100 &1000uM MnCl2	Hirata et. al., 2001
Male PD1-21 rats exposed to 50, 250, 500 ug/day gavaged	Not sig. but trend towards decrease in DA w/ increasing doses of Mn	Tran et. al., 2002
Male Rhesus monkeys: cow's milk, soy formula, or soy+Mn	5-HT and 5-HIAA decreased with age; while HVA increased	Golub et. al., 2005
4 patient worked 2yrs in Mn smelting industry in Taiwan	Normal PET scanning of DA in the patients compared to control in the striatum	Wolters et. al., 1989
4 patient worked 2yrs in Mn smelting industry in Taiwan	Same patients still no decrease of DA; but a sig. decrease in caudate/occipital ratios D2-receptors but no change in the striatal	Shinotoh et. al., 1997

TABLE 4
Literature discussing levels of metals in models on Mn neurotoxcity

Treatment	Results	Paper
90 day old male rats, 20mg/kg drinking water throughout development	Mn: +479% St ; +530% hypo Fe: +95% St; -28% cortex Cu: +43-100% in St, BS	Lai et. al, 1992
Retired breeders mice; 50/100mg/kg SC injection	Mn: increased after 1 day injection and kept increasing with more	Dodd et. al, 2005
Neonatal rats w/intubation; 25/50ug in water (14 or 21 days)	All brain regions had increase of Mn (hypo had highest) but lower at 21 days than 14 days; Liver Mn increased with expsure	Kontour et. al, 1988
Basal levels of metals-rats from 1-day to 147days old	Mn: reaches peak at 6 weeks then decreases w/age, 11wks in olfactory tubercle (OT), SN and amygdala, 2wks OT, SN; 3-6wks corpus callosum	Tarohda et. al, 2004
15ug 6-OHDA rats sacked at different days after injection	Mn: increased rapidly 3-7days Fe/Zn: 7-10days increased Mn increase caused Fe increase b/c 1.) inhibits aconitase 2.) destroys cellular Fe homeostasis 3.) then increasing Fe levels	Tarohda et. al, 2005
Rats: suppl. w/ Mn in diet (2mg/ gram of diet)	Increased Mn levels in heart, lung, kidney	Kimura et. al., 1978
Human basal metal levels in brain	Mn (irregular age patterns): highest levels in putamen and caudate nucleus; lowest levels in anterior frontal and temporal lobes in adults Fe (increase then decline): highest in putamen and GP; CN & hippo lowest in adults; Infants: lower levels of Mn and Fe than adults	Markesberry et. al, 1984
Neonatal and Adult rats; 25/50 mg/kg gavage	Mn infants: increased in St, hippo, cortex, and hindbrain at PND21 w/ 25&50 only hypo& cere w/50 Mn adults: increased in St, cere,	Dorman et. al., 2000

	brain residue	
Rat pups: 50,250, &500ug of Mn suppl.	Whole brain: increased Mn w/500 at d14; Small intestine: increased at d14 with 500; Kidney: increase at d21 with 250 &500	Tran et. al., 2002
Rats: maternal-fetal transfer-GD1 MnCl 1uCi a day in drinking water	Transfer of Mn by the placenta peaks at 15 days	Kontur et. al, 1985
Child hair samples: formula fed to 8 years old; hyperactive children	Formula-fed infants increased Mn at 6 wks but by 3 yrs. back to normal levels; children w/learning disabilities have an increase in Mn in hair	Collipp et. al., 1983
Rats: 2 or 10mg/ml Mn dissolved in drinking water of dams and the liters up to PND30	Increased cortical Mn levels w/ 10Mn group	Pappas et. al., 1997
Rats: IP of 15 mg/kg Mn on an iron def. diet	Decreased Fe leads to increased Mn	Chandra and Shukla 1976
Rhesus monkeys: MnSO4 aerosol	Mn: increase putamen and GP w/ 0.06, 0.3 & 1.5; increase caudate w/ 0.3 & 1.5;	Struve et. al., 2007
Human workers MnO2 dust- dry alkaline battery factory	Increase blood and urine in the Mn exposed workers	Roels et. al., 1992
Rats: SC injection of MnCl at 5d, 5w, 48w, 73w, 95w	Liver and brain highest Mn at 5d; needed for normal brain development; Mn high in hippo.,dentate gyrus, pons; Aging brain Mn in red, olivary nuclei and inferior collicoli	Takeda et. al., 1999
Rats: PN4-21 Mn via maternal milk during lactation	Increase in brain Mn, Chromium and Zn w/ a decrease in Fe;	Garcia et. al., 2006
Mn (250 or 750 ug/day) exposure PND 1-21 then challenged w/cocaine as an adult	Striatal Mn increased at PD14 and PD21; no change in Mn at day PD90; no change in Fe in the brain; Fe serum levels greatest in the PD21	Reichel et. al., 2006

Patients w/ long-term parenteral nutrition - children	After reduction or withdwral whole blood Mn was 615-1840 to 250-1093 nmol/L by 4 months and 79-430 by 8 months	Fell et. al., 1996
Male rats 20mg/ml MnCl2 in drinking water for 13wks	1ug/g of dry weight brain in the sensorimotor cortex and 1.5ug/g in the GP normally but w/ trmt 4-fold increase in GP, St, and cortex	Morello et. al., 2007
4 adult and 1 fetus human brain tissue	Cu and Zn found in all brains with more Zn in the fetal brain ie. Normal constituents of the human brain	Bodansky 1921
	Cu and Fe most abundant in the basal ganglia; Fe in the brain (2% of total Fe in body) eq. to liver; abundant in the astrocytes (fxn as Fe storage and regulation)	Madsen and Gitlin 2007

TABLE 5
Literature discussing glial phenotypes due to Mn toxicity

Treatment	Results	Paper
Rats: 2 or 10mg/ml Mn dissolved in drinking water of dams and the liters up to PND30	Only increase in GFAP w/ PND32 of 2 and 10Mn exposure in the dentate gyrus	Pappas et. al., 1997
Rhesus Monkeys: chronically MnCl for 18 mo	Gliosis in the medial segment of the pallidum ; Alz. Type II astrocytes seen in GP	Mella et. al., 1924
Monkeys: Mn exposure	Alz. Type II astrocytes seen in pallidum	Olanow et. al., 1996
Patients w/liver failure	Alz. Type II astrocytes seen in pallidum	Norenberg, MD 1981
Male rats 20mg/ml MnCl2 in drinking water for 13wks	No overt change in GFAP + staining	Morello et. al., 2007

TABLE 6
Literature discussing behavioral abnormalities in models of Mn toxicity

Treatment	Results	Paper
Retired breeders mice; 50/100mg/kg SC injection	Decrease in horzontal mvmt. after one injection (30.9% and 38.9%); no change in swimming, rearing, grip strength or fatigue	Dodd et. al, 2005
Neonatal and Adult male rats; 25/50 mg/kg gavage	No motor or cognitive deficits for neonates a possible decrease trend for memory	Dorman et. al., 2000
Rat pups: 50,250, &500ug of Mn suppl.	Pup w/Mn exposure took longer to roll over but not sig. ; increase in homing test w/Mn so took longer to reach goal; 3X higher # of shocks at day 32 w/ 500 ug Mn	Tran et. al., 2002
Rats: maternal-fetal transfer-GD1 MnCl 1uCi a day in drinking water	No sig. change on the development of the startle response	Kontur et. al, 1985
Rats: 2 or 10mg/ml Mn dissolved in drinking water of dams and the liters up to PND30	Sig. rearing and movement with 10Mn..no change in swim tests	Pappas et. al., 1997
Human workers MnO2 dust- dry alkaline battery factory	Decrease in visual reaction time, eye-hand coordination & hand steadiness: Lifetime exposure of 3575 total and 730ug Mn/m^3 x year respriable Mn	Roels et. al., 1992
Taiwan Cohort: 6 out of 13 workers exposed to +27mg/m^3	Gait diruption (esp. walking backwards) bradykinesia micrographia, hypophonia – no L-DOPA response	Reviewed in Olanow, CW 2004
Rhesus Monkeys: chronically MnCl for 18 months	Gait dysfunction, rigidity, bradykinesia, facial grimacing no tremor or dyskinesia or response to L-DOPA	Mella et. al., 1924
Mn (250 or 750 ug/day) exposure PND 1-21 then challenged w/cocaine	Mn didn't affect developmental landmarks (Pina detachment, incisor eruption, or eye opening); decrease travel if pre-exposed to 750 Mn	Reichel et. al., 2006

as an adult	w/cocaine	
Male PD1-21 rats exposed to 50, 250, 500 ug/day gavaged	PND 50-64 burrowing detour and passive avoidence test decrease w/Mn exposure	Tran et. al., 2002
Patients w/ long-term parenteral nutrition - children	2 pat. Had mvmt. Disorder consisting of dystonia and abnormal posturing ; one died one improved by 8mo.	Fell et. al., 1996
Male Rhesus monkeys: cow's milk, soy formula, or soy+Mn	Soy and Soy+Mn engaged in less play behavior and more clingling in social dyadic interactions , shorter wake cycles, shorter periods of daytime activity	Golub et. al., 2005
4 patient worked 2yrs in Mn smelting industry in Taiwan	Bradykinesia and rigidity w/ masked facial expression clumsiness and impaired dexterity and gait abnormalities	Wolters et. al., 1989