DISSERTATION

# NEUROINFLAMMATION IN MANGANESE NEUROTOXICITY

Submitted by:

Karin Marie Streifel

Department of Environmental and Radiological Health Sciences

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Doctoral Committee:

Advisor: Ronald Tjalkens

Scott Earley
William Hanneman
Marie Legare

ABSTRACT

NEUROINFLAMMATION IN MANGANESE NEUROTOXICITY

Excessive environmental or dietary exposure to the essential nutrient manganese (Mn) causes neuroinflammation, particularly in the striatial-pallidum and substantia nigra *pars reticulata* of the CNS, concominant in the loss of striatal dopamine and motor features resembling, but distinct from, Parkinson's disease (Newland et al., 1989, Calne et al., 1994, Perl and Olanow, 2007). Previous work in our laboratory and others has identified reactive gliosis associated with an increase expression of inducible Nitric Oxide Synthase (NOS2) inflammatory, and important inflammatory gene. Increases in NOS2 leads to a selective increase in reactive oxygen and nitrogen species that is associated with basal ganglia neuronal injury during the progression of the disease. Recent epidemiological studies have associated childhood Mn exposure with neurological dysfunction, indicating that juveniles may be a susceptible population to Mn neurotoxicity. Furthermore, studies of Mn exposure have generally focused on adult exposures and there is much less information regarding the effects of Mn exposure during juvenile development. Therefore in this research we investigated the role of NOS2 in Mn-induced neuroinflammation in juveniles. Using a NOS2 deficient mouse model we demonstrated that gene deletion of NOS2 attenuates peroxynitrite adduct formation in the

striatal-pallidum and substantia nigra *pars reticulata* protects against changes in neurobehavioral parameters. These findings indicate that Mn-induced production of NO by activicated glial cells contributes to nitrosative and neurobehavioral dsyfunction. In addition, I conducted *in vitro* studies to investigate the effect of acute treatment of basal ganglia neurotoxins on agonist-induced intracellular calcium transients in primary striatal astrocytes. These findings indicate that endogenous and exogenous cationic neurotoxins inhibit physiological calcium signaling in astrocytes through transient receptor potential channel proteins. This suggests a novel mechanism by which Mn and other cationic toxicants of the basal ganglia may inhibit critical trophic functions in astrocytes that depend on calcium signaling, such as metabolism and regulation of cerebral blood flow, that could promote neuronal injury. The overarching objective of this research was to investigate the role of astrocytes in the progression of injury in neurodegenerative disease models, thereby increasing our understanding of how dysfunction within glial cells contributes to pathophysiology in the CNS.

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# LITERATURE REVIEW

## Introduction

Manganese (Mn) is the 12th most abundant element on Earth. Manganese is widely distributed in rocks and soil, where leaching processes provide a route into the water and food supply. It is the fourth most commonly mined metal due to it importance in iron and steel manufacturing. Industrial wastes from manufacturing, combustion, and improper disposal can add to the background levels of Mn in the environment. Animals and humans are naturally exposed to Mn on a daily basis through diet and it is physiologically essential that Mn is biologically available. There are many routes of environmental and occupational exposure that can lead to excessive concentrations of Mn in animals, which can be detrimental to biological functions.

### 1.1 **Manganese**

A. Routes of exposure

The National Research Council recommends for safe and adequate daily intake of Mn not to exceed an estimated 0.6 mg/day for infants and approximately 5 mg/day for adults and adolescents 12 years of age or older (ATSDR, 2000) whereas the Environmental Protection Agency's (EPA) appropriate reference dose for Mn is 10 mg/day (IRIS, 2011). One of the primary routes for Mn for the general population is through nutritional sources. Consumption of foods rich in Mn such as whole grain, legumes, nuts and tea (i.e. heavy tea consumption of >1L/day range from 2-12 mg/day alone (Hope et al., 2006)) can elevate the Mn levels from the recommended adequate daily intake. Foods rich in Mn generally have high levels of phytic acid, that contain 6 negatively charged phosphate groups chelating divalent cations, such as Mn (Lonnerdal,

1994). Dietary supplements may further increase daily Mn intake with approximately 2.3 mg/daily supplement (EPA, 2004) amongst other food sources.

Soy-based infant formulas have substantially higher Mn concentrations compared to human and cows milk, whereas soy contains approximately 200 times more Mn than the latter (200-300ug/L; 1.8-27.5ug/L, respectively (Lonnerdal, 1994)). Juveniles ranging from ages 1-12 have considerably lower upper limits for Mn intake set by the Food and Nutrition board of the Institute of Medicine due to body size. Extrapolation from adult studies found 2 mg/day for ages 1-3 to up to 6 mg/day ages 9-12, however there are great uncertainties with these set levels due to lack of human data (Karin Ljung, 2007, Santamaria and Sulsky, 2010). The main source of Mn exposure to these children are from food and water sources. In the literature, animal studies in this post weanling age group has been understudied in respect to Mn exposure, yet most epidemiological data find behavioral and cognitive dysfunction in these ages when exposed to elevated Mn in drinking water (See 1.2A).

Depending on geologic composition and anthropogenic influence, concentrations of Mn in freshwater typically range from .001 to 3 mg/L and up to 1.3-9.6 mg/L in groundwater, dependent upon the pH and anaerobic conditions (WHO, 2004, Santamaria and Sulsky, 2010). The median level of Mn in groundwater is .005 mg/L whereas the EPA's secondary maximum contaminant level for drinking water is .05 mg/L primarily based on aesthetic concerns. If the median level of Mn in public drinking water is .01 mg/L and adults consuming an average of 2 L/day the ingested levels would be .02 mg/L of Mn from drinking water alone (WHO, 2004). Municipal drinking water is strictly

regulated and monitored for Mn, and levels are typically lower than the secondary maximum contaminant level and not of concern for public health.

Rural areas with water access through private and public supply wells are of greater concern due to higher levels of Mn in aquifers. In a study conducted by the U.S. Geological Survey, Mn was detected above any other element in the glacial aquifer system in the northern U.S., whereas 10% of public use wells sampled exceed 300 ug/L (Groschen G, 2008). It is estimated that 6% of domestic wells in the entire US have high levels of Mn at approximately 300 μg/L (Wasserman et al., 2006). Additionally, the EPA has established a drinking health advisory for Mn with a lifetime level at 300 μg/L, whereas these levels are associated with adverse neurological effects. Although in the general population the majority are not affected or exposed to enough excess Mn to cause toxicity, there are susceptible populations such as juveniles that when exposed to elevated concentrations of Mn in the water supply adverse health effects pose a significant risk.

In addition to ingestion of Mn, inhalation has been causative route of exposure in cases of neurotoxicity. Eight million tons of Mn are extracted every year whereas Mn can be found in and used for potassium permanganate having disinfectant properties, manganese dioxide is used for dry batteries, glass and fireworks, fertilizers and fungicides for agricultural purposes and some countries use methylcyclopentadienyl manganese tricarbonyl (MMT) as a antiknock agent in gasoline (Calne et al., 1994, WHO, 2004). Without the presence of point source pollution Mn levels in the atmosphere range from .01 to .07 $\mu g/m^3$ whereas pollution from mining, manufacturing, agricultural and MMT automobile emissions results in elevated Mn levels ranging from

0.22 to .3 $\mu g/m^3$ (Barceloux, 1999). Baseline atmospheric levels even with point source pollution are considerably lower than the lowest observed adverse effect level (LOAEL) at 50 $\mu g/m^3$ (IRIS, 1995). Since first described in 1837 by Couper of Glasglow, occupational exposures such as welding and mining have been associated with Mn toxicity (Couper, 1837). At concentrations of >1000 $\mu g/m^3$ neurological behavioral impairment has been linked to Mn toxicity (IRIS, 2011), therefore occupational health agencies suggest exposure limits for general industry ranging from 200 to 1000 $\mu g/m^3$ for an eight hour work day (Curran et al., 2009). Furthermore, due to contemporary personal protective equipment and workplace compliance with U.S. agencies, currently workers are rarely exposed to detrimental levels of inhaled Mn.

The greatest exposure to Mn for the general population is through dietary consumption of food and water. Although inhalation is a significant route of occupational exposure leading to neurological dysfunction, a large proportion of the body of Mn literature over the last half century has concentrated upon inhalation studies due to occupational exposure concerns. This has left many unknowns about the implications of Mn ingestion and possible age dependent susceptibilities. Therefore, exposure to Mn though dietary water is the primary emphasis ofthis literature review and research discussion.

B. Physiological Requirements

1-5% of ingested Mn is absorbed from the gastrointestinal tract, with slighter greater bioavailability of Mn from water than from food (Johnson et al., 1991, Davis et al., 1993, IRIS, 2011). Although the complete mechanism of absorption is unknown, it is

evident that divalent Mn transports across the enterocytes to the portal blood where 80% forms complex protein units to primarily beta-globulin and albumin, as well as a small proportion of trivalent Mn bound to transferrin which predominantly binds iron (Aschner et al., 2005). Distribution of Mn tends to deposit in mitochondrial rich tissues such as the liver and brain, in addition to the pancreas and bone (Aschner and Aschner, 2005, Aschner et al., 2005). In the liver, Mn is disassociated from the protein transporters and Mn is primarily excreted through the biliary system. Under physiological conditions only a small portion is eliminated in the urine (WHO, 2004) and therefore increased urine concentration is a biomarker for excess exposure. In order to maintain Mn homeostasis, absorption is strictly regulated when Mn intake is high in adult mammals, resulting in reduced gastrointestinal absorption, increase hepatic metabolism and enhanced biliary excretion and elimination (Aschner and Aschner, 2005). However, developmental stages as well as other trace minerals such as iron affect the absorption and excretion of Mn (see 1.2A).

Mn is an essential element, because it functions as a cofactor in enzymatic reactions in structural, metabolic, antioxidant and CNS homeostatic properties. Mn is vital for skeletal development as it assists glycosyltransferases biosynthesis of mucopolysaccarides to form bone matrix. In rare cases, Mn deficiency can impair skeletal growth and cause deformities due to inadequate glycosyltransferase activity (Erikson et al., 2007). In the CNS, Mn is required for pyruvate carboxylase, specifically in the astrocyte, for metabolic reactions as well as replenishment of glutamate and GABA (Shank et al., 1985). Also in the astrocyte, Mn bound glutamine synthetase converts glutamate and ammonia to glutamine preventing excitotoxicity in the synaptic region

(Takeda, 2003). Mitochondrial enzyme, Mn superoxide dismutase (MnSOD), is a combatant of superoxide toxicity and critical for survival (Li et al., 1995). Finally, the enzyme arginase, acting in the urea cycle, functions with Mn to excrete ammonia. Additionally, Mn is necessary for cholesterol synthesis, blood clotting, hormone production, regulation of blood sugar and calcium absorption (Santamaria and Sulsky, 2010).

Mn is abundant in our environment and an essential element for living organisms. Deprivation of Mn is atypical in humans and non-experimental cases have not been reported (Aschner and Aschner, 2005), yet overexposure is far more common. High concentrations are known to lead to neurological disease and low level chronic exposure has more recently been of concern. The severity of health affects are dependent upon factors such as route, age, sex, health and nutritional status.

### 1.2 **Manganism**

Excessive exposure of Mn leads to a progressive neurological disorder termed manganism that primarily affects the forebrain and basal ganglia and presents with neurological symptoms resembling the clinical features of Parkinson's Disease (PD). The first record of the disease was in 1837, Couper of Glasgow described the "shaking palsy" disorder in occupational Mn ore-crushers and in 1941 the first environmental cases were reported due to water contamination. The onset of manganism occurs in phases, the initial phase gradually starts with subjective psychological symptoms, leading into more apparent symptoms and finally, between 1-2 years permanent extrapyramidal dysfunction induces motor deficiencies (Rodier, 1955). Whereas many of these symptoms are similar

to Parkinson's disease, manganism is distinct in particular symptoms and pathological features in the CNS (see 1.2B)(Calne et al., 1994).

A. Epidemiology and Susceptibility

The first reported case of contaminated well water appeared in 1941, where buried dry cell batteries leached into the water supply consumed by 25 people. Of the 25, 15 were systematic exposed to levels as high as 28 mg Mn/L (Kawamuka et al., 1941). In these cases, the elderly where more susceptible that the young and the Mn levels are uncertain at the time of ingestion, however they are estimated to be 30 times the daily dietary levels (EPA, 2004). In Greece, a study was conducted in three areas with high, medium and low concentrations of Mn (3.6-14.6 µg/L, 81.6-252.6 µg/L, 1800-2300 µg/L respectively) correlated to the prevalence of Mn toxicity in men and women above 50 years of age (Konkdakis et al.,1989). Later studies revealed that sewage irrigation in China increased Mn concentration in drinking water to 241-346 µg/L, compared to control area at 30-40 µg Mn /L. Pre-adolescents (ages 11-13) in these areas were found to have lower performance for memory and reactions tests correlating with hair Mn concentrations and location of water consumption (He et al., 1994). In 2002, a 10 year old male consumed high levels of Mn in drinking water (1.21 ppm) for 5 years and was declining in attentiveness in the classroom as well as verbal and visual memory (Woolf et al., 2002). A more thorough investigation in children's intellectual function was found to be negatively associated with Mn concentrations in well water of Bangladesh in a dose dependent fashion (Wasserman et al., 2006). A study conducted in Quebec, Canada containing naturally high levels of Mn in the drinking water (160-610 µg/L) linked

school aged children (6-15) with high hair Mn concentrations to a significant demonstration of attention deficit hyperactivity disorder (Bouchard et al., 2007, Bouchard et al., 2011). Although Mn toxicity is generally well documented affecting adult populations from occupational exposures there are few studies of well conducted environmental exposures in juveniles. Suggestions of this susceptible population, which is dependent upon age and developmental exposure time, future studies are warranted.

Infants and children have altered Mn pharmacokinetics in their developing stages of the GI and greater dietary consumption per volume than that of adults. In the neonatal period absorption and retention of Mn is higher compared to adults, and the biliary excretion of infants is poorly developed (Aschner and Aschner, 2005). Formula feed infants commonly consume 200-300 μg/L of Mn daily which is greater than 100-fold difference from human milk at levels of 3-8 μg/L (Lonnerdal, 1994). Infant intake is approximately 0.78 L/day of breast milk or formula with consumption of approximately 4 μg Mn/L or 195 μg Mn/L, receptively (EPA 2004). Animal studies have indicated that Mn can cross the blood brain barrier in young animals 4 times greater than adults, predisposing infants to Mn-induced neurotoxicity (Mena, 1974). Formula fed infants retain 20% of the Mn (EPA, 2004) and infants have been reported to have elevated hair Mn levels compared to breast feed infants (Collipp et al., 1983). Additionally, the study found that learning disabled children had significantly increased hair Mn concentration. These pharmacokinetic differences are regulated after approximately a year however children may be more sensitive to the effects of Mn at these times and exposure should be regulated.

Total Parenteral Nutrition (TPN) in patients can infrequently cause cholestasis and hepatic dysfunction through obstruction of the bile that results in the accumulation of Mn due to lack of excretion (Fell et al., 1996). Dietary absorption of Mn is approximately 115 μg/day for a healthy adult whereas intravenous doses of TPN range from 100-800 μg/day and neurological deficits have been found in patients greater than 500 μg/d (Santamaria and Sulsky, 2010). T1-weighted MRI scans have indicated that there is an accumulation of Mn within the basal ganglia particularly evident in children receiving long-term TPN (Fell et al., 1996). Withdrawal from TPN significantly decreases these accumulations over time without longterm neurological damage (Erikson et al., 2007) as well as improved liver function (Aschner and Aschner, 2005).

Iron deficiency is the most common nutritional deficiency, with 30% of the world's population in developing and industrialized countries lacking iron (WHO 2010, website). This deficiency is prevalent among women and more so amongst children. Children are especially susceptible to iron deficiency as there is a larger demand for iron during development. Furthermore, Mn and iron are interdependent upon transferrin mediated endocytosis (see 2.1.3) into the brain where there is an inverse relationship in brain Mn concentrations and iron absorption. Iron deficiency studies have shown that there is an increase of Mn absorption in the basal ganglia coinciding with increased expression of transferrin receptors and DMT-1 (Barbeau, 1984, Erikson et al., 2005b). In fact the regions in which manganese neurotoxicity is most impacted, the basal ganglia, are also iron-rich brain regions, implying the interdependence of Mn and iron transport (Erikson et al., 2005b).

B. Clinical symptoms and treatment

The initial stage of chronic Mn exposure involves sometimes subtle and gradual neurophysiological symptoms such as headache, irritability, fatigue, aggression and alterations in libido (Calne et al., 1994). These psychiatric symptoms have been referred to as "manganese madness" or "locura manganica" (Mena et al., 1967). These stages of the disease are said to be reversible with treatment or removal of exposure. In the latter stages of the disease, neuromotor symptoms become apparent, some of which resemble Parkinson's disease. Symptoms include gait dysfunction; specifically bradykinesia, rigidity, dystonia and postural instability, however distinct from Parkinson's disease, there is a lack of resting tremor, a hypertonic "cock walk" and a greater propensity to fall backwards (Calne et al., 1994).

Continual exposure of Mn results in a chronic, progressive neurodegenerative disorder. At this stage there are dissimilarities in treatment of the disorders where manganism responds to L-Dopa treatment very infrequently and in a transient manor (Calne et al., 1994). Chelation therapy for Mn neurotoxicity with ethylene-diamine-tetraacetic acid (EDTA) has been shown to increase the rate of elimination, although clinical symptoms are not completely alleviated after long duration exposures. Para-aminosalicylic acid has been shown to reduce the body burden of Mn yet the mechanism of action is unknown (Zheng et al., 2009). In the advanced stages of the disease, chelation therapy does not lead to the reversal of the disease although there can be a reduction of the symptoms.

C. Pathology

As previously stated, Mn affects regions of the basal ganglia, which is comprised a network of nuclei associated with the regulation of movement and executive functions. These nuclei include the striatum (caudate and putamen), globus pallidus (internal and external), substantia nigra (pars reticulata and par compacta) and subthalamic nucleus. The striatum, globus pallidus and substantia nigra pars reticulata are the primary targets for Mn neurotoxicity but not the only; the prefrontal cortex, subthalamic nucleus, pituitary gland and hypothalamus have also been shown to be affected by Mn (Yamada et al., 1986, Newland et al., 1989, Perl and Olanow, 2007, Aschner et al., 2009). In Parkinson's disease, degeneration in primarily in the substantia nigra pars compacta, whereas these neurons are spared in cases of manganism. Furthermore, Parkinson's disease is characterized by the presence of protein aggregates called Lewy bodies which (Perl and Olanow, 2007) that are rarely found in cases of Mn neurotoxicity (Hazell, 2002). Previous research in our laboratory has shown that the enkephalin positive interneurons in the striatal-pallidum are sensitive to Mn neurotoxicity, as a result there is dysfunction in the indirect pathway (D2) via increased inhibition of corticothalamic projection neurons additionally hindering movement (Liu et al., 2006). Furthermore, cholinergic and neuronal NOS positive interneurons had increased TUNEL staining in the striatal-pallidum which are associated with the direct pathway (D1) and initiation of behavior (Saka et al., 2002). Therefore loss of these interneurons facilitate inhibition through the D2 pathway and diminished activation of the D1 pathway all resulting in the clinically relevant Mn-induced motor hypokinesia.

Dopaminergic, glutamatergic, and GABAergic synaptic activity have been shown to be modulated by Mn neurotoxicity. Dopamine loss with Mn toxicity has been well documented in other models of Mn toxicity further indicating the dopaminergic sensitivity (Liu et al., 2006, Moreno et al., 2009b). However, an age-dependent susceptibility with environmentally relevant levels Mn has been observed indicating that juvenile exposure leads to an increase in dopamine tissue levels and correlated locomotor activity (Dorman et al., 2000, Moreno et al., 2009b, Kern et al., 2010). Dopamine transporter (DAT) studies utilizing knock out mice have indicated that accumulation of striatal Mn is dependent the DAT but not exclusive (Erikson et al., 2005a). Pre-weanling mice exposed to Mn have deprecations in dopaminergic expression of D1 receptors in addition to DAT in the striatum, which correlate to behavioral disinhibition and a suggestive increase in dopamine (Kern et al., 2010). Furthermore, alteration of DAT are observed in Mn intoxicated patients although they suggest to not be the main target of Mn-induced dopaminergic toxicity (Huang et al., 2003). These data indicate Mn as being a target for Mn neurotransmission, however exact mechanisms are unknown. Due to the complexity of basal ganglia circuity there is a close correlation with the affects of the dopaminergic activity and other modulating and interconnected neurotransmitters.

The primary inhibitory neurotransmitter in the brain is γ-Aminobutyric acid (GABA), which is at the highest concentration in the globus pallidus and substantia nigra pars reticulata. GABAergic neurons project from the striatum to the globus pallidus and substantia nigra modulating glutamatergic and dopaminergic output, respectively (Anderson et al., 2008). Mn exposure through drinking water has been shown to increase extracellular GABA concentration, as a result of decreased receptor and transporter

expression in weanling rodents (Anderson et al., 2008). Mn-induced locomotor changes with increases in GABA concentrations have also been observed with low doses through intraperitoneal injections although not affecting dopamine neurotransmission (Gwiazda et al., 2002). These data demonstrated that striatal GABA progressively increases dysfunction with Mn toxicity prior to adverse affects upon striatal dopamine levels. *In vitro* and *in vivo* analysis has demonstrated that glutamate decarboxylase, which converts glutamate to GABA, decreases with Mn toxicity therefore decreasing the concentrations of extracellular GABA (Stanwood et al., 2009). Furthermore, more *in vivo* results have found no changes in GABA levels (Liu et al., 2006, Burton and Guilarte, 2009), leading researcher to believe that GABA affects are treatment and species dependent (Aschner et al., 2009) due to the conflicting data that exists.

In the CNS, glutamate is the most abundant excitatory neurotransmitter, which as previously mentioned is the precursor molecule for GABA, and is thought to be a indirect participant of Mn neuronal degeneration. Mn may potentiate activation of postsynaptic NMDA receptors in a dose dependent fashion (Brouillet et al., 1993). Increased release of glutamate can occur in energy deprived cells (Erikson and Aschner, 2003) that can manifest with Mn exposure (see 1.3) further perpetuating metabolic and homeostatic dysfunction. Astrocytes play critical role in the regulation of extracellular glutamate and Mn intoxication reduces the astrocytes ability to maintain physiological function (see 1.4A). All of these perturbations with Mn on the neurotransmitters regulating the basal ganglia leads to a diverse array of interactions and modifications to motor function and neuropathological characteristics.

Excessive Mn exposure affects the glia in the CNS, specifically the astrocytes in nuclei of the basal ganglia. This has been shown by a robust increase in GFAP staining, hypertrophy of the processes representing Alzheimer type II astrocytosis and selective increases in Mn concentration in specifically the astrocytes (Yamada et al., 1986, Aschner et al., 1992, Olanow et al., 1996, Henriksson and Tjalve, 2000). Under these conditions, certain physiological functions of the astrocytes are affected such as homeostatic and metabolic support of the neurons. Microglia have also been shown to be activated and migrate in the striatal pallidal and substantia nigra region in Mn exposure models (Moreno et al., 2009a). These midbrain regions are rich in microglia which could be a factor in the progressive inflammatory reaction seen with exposure cases of Mn (Kim et al., 2000). Morphological changes in the resident immune cells are observed in proximity to dopaminergic neurons, leading to the production of pro-inflammatory cytokines such as TNF-alpha, NO and ROS modulating the microenvironment of the neurons and surrounding astrocytes (Verina et al., 2011).

The homeostatic dysfunction in the Mn exposed astrocytes are characterized by a decrease in glutamate uptake in the extracellular environment and decreased expression of the glutamate transporter, GLAST, or the human homologs EAAT1 on the astrocyte (Erikson and Aschner, 2002). The increased concentration of glutamate in the synaptic cleft can lead to excitotoxicity in adjacent neurons resulting in cell death. As previously mentioned, Mn has been shown to interact with NMDA receptors leading to the production of excitotoxic lesions and neuronal degeneration, which can be attenuated by the NMDA antagonist MK-801 (Brouillet et al., 1993). These studies further support the

critical homeostatic and metabolic roles of astrocytes and how Mn-induced excitotoxicity through glial modification can progress neuronal degeneration.

### 1.3 **Cellular transport of Mn and gene activation**

Mn enters the brain through the capillaries of the cerebral cortex or the choroid plexus into the cerebral spinal fluid. Excessive Mn exposure levels can rise 2 to 3 folds from physiological levels ranging from 2 to 8 uM (Aschner et al., 2007, Aschner et al., 2009, Moreno et al., 2009b). At the cellular level, uptake of Mn across the blood brain barrier (BBB) into the astrocytes have been suggested to be transported through several mechanisms. The active, divalent metal transporter 1 (DMT-1) is associated amongst other transporters with Mn delivery into the CNS, in addition to iron regulation (Aschner et al., 2007). Mn transport by DMT-1 was indicated by radioactively labeled Mn in a defective allele model for DMT-1, that showed a lesser degree of Mn uptake into the CNS (Chua and Morgan, 1997). Another transporter is ZIP8 that is involved in divalent metal transport primarily in plants, however there is expression of a subfamily in mammalian cells and specifically in the capillaries of the BBB (Erikson et al., 2007, Girijashanker et al., 2008). The primary iron carrying protein, transferrin, binds to trivalent Mn and can mediate transport across the cerebral endothelial cells through endocytosis of the transferrin receptor complex and Mn is further released into the extracellular fluid (Aschner et al., 2007). Interestingly, transferrin receptor density is in high abundance in brain regions susceptible to Mn accumulation and toxicity (Aschner et al., 2007).

Mitochondria are an important organelle target once Mn has entered the cell. Intracellular Mn is sequestered in the mitochondria and transported into the matrix by the calcium uniporter (divalent cation transporter) (Gavin et al., 1990). 60%–70% of the accumulated manganese is sequestered in mitochondria whereas the rest is localized to the cytosol (Wedler et al., 1989). This accumulation leads to disruption of calcium homeostasis (Allshire et al., 1985), inhibiting energy production by decreasing activity of complex 1 in the electron transport chain and potentiating the formation of reactive oxygen species (Zhang et al., 2004). Mn accumulation in the mitochondria results in calcium dysfunction and ATP deprecation, directly affecting cellular integrity and energy failure in the CNS (Gunter et al., 2006).

Low dose Mn and cytokine treatment has been shown to activate the immediate-early transcription factor, nuclear factor kappaB (NF-kB), that coordinately regulates expression of multiple neuroinflammatory genes in astrocytes, including inducible nitric oxide synthase (NOS2) (Xie et al., 1993, Moreno et al., 2008). Upstream activators in this mechanism have been elucidated to activate MAP kinase pathways and cyclic guanosine monophosphate (cGMP)-dependent extracellular response kinase (ERK) activation resulting in translocation of NF-kB into the nucleus that enhances expression of NOS2 (Moreno et al., 2008). In the presence of Mn there is an enhanced activation of soluble guanylate cyclase (sGC), a heterodimeric enzyme that converts guanosine triphosphate to cGMP, further potentiating ERK-dependent activation of NF-kB (Winger and Marletta, 2005, Moreno et al., 2008). Extracellular stress stimuli activate the innate immune response through this pathway among others to induce NOS2, which produces the pro-inflammatory cytokine nitric oxide (NO). NO can also activate sGC increasing

the cGMP levels and potentate the production of NO (Moreno et al., 2008), these disruptions in signaling cascades are associated with alterations striatal neurotransmission and basal ganglia pathophysiology (West and Tseng, 2011). Glial-derived NO has been implicated in Mn neurotoxicity among a multitude of other neurodegenerative disease due to an abundance of NO producing neuroinflammation and demise for neuronal integrity (Hirsch et al., 1998, Spranger et al., 1998, Liberatore et al., 1999).

## 1.4 **Neuroinflammation and Nitric Oxide**

Oxidative stress and excitotoxicity are critical mechanisms that can lead to neuronal degeneration. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, prion disease, multiple sclerosis, AIDS dementia, and manganism are associated with activation of astrocytes and microglia, known as gliosis. Mn-activated astrocytes and microglia produce reactive oxygen and nitrogen species (ROS and RNS, respectively) that injure neurons.

### A. Physiological roles of glia

Neurons have a high demand for energy and rely on astrocytes for metabolic support in an activity-dependent manner. When neurons are actively releasing glutamate, astrocytes have been shown to produce pyruvate though glycolysis further converting the pyruvate into lactate and releasing it into the extracellular space for neuronal uptake (Pellerin et al., 2007). During intense neuronal activity and depletion of glucose, catabolism of glycogen stores in astrocytes are observed and increases in lactate shuttling to neurons for energy supplement (Brown and Ransom, 2007). Mn exposure *in vitro* has not been shown to decrease astrocytic metabolism and the shuttling of neuronal energy

substrates but alteration of glutamate processing has contributed to neuronal toxicity (Zwingmann et al., 2003). Subsequently, Mn can alter the efficiency of mitochondrial energy production which further impairs the neuronal environment (see 1.3). Such alterations in metabolic coupling between astrocytes and neurons maybe a significant factor in the mechanism of Mn neurotoxicity.

In addition to metabolic and homeostatic functions that support neurons, astrocytes are essential for regulation of cerebral blood flow. Neuronal activity and release of neurotransmitters, such as glutamate, activates adjacent astrocytes inducing a calcium propagation throughout the astrocytic network. When the rise of intracellular calcium reaches the perivascular end foot of the astrocyte, complex signaling mechanism yet to be elucidated, induce vasomodulation of the cerebral vasculature increasing the metabolic support to the active neurons. Impairment of astrocytic signaling could deprive active, metabolically challenged neurons rendering them susceptible to oxidative stress.

Microglia are the resident immune cells in the central nervous system. These monocyte-derived cells have phagocytic capacity and monitor the CNS to rapidly respond to chemical or metabolic stress, infection, and neuronal injury. Activated microglia undergo a morphologic change from a resting or ramified state into the phagocytic and amoeboid cells expressing inflammatory genes and producing an array of cytokines (Verina et al., 2011). These phenotypic changes alter their immune functions and chronic exposure to infectious agents leading to activation of these cells could predispose the CNS to detrimental damage. The cellular and molecular signals between microglia and astrocytes are not elucidated, and these cellular communications could

potentiate the neurodestructive microenvironments observed in neurodegenerative disease (Saijo et al., 2009). Therefore the molecular and cellular signaling pathways between microglia and astrocytes would be beneficial to understand the processes of inflammation in the CNS.

B. Nitric Oxide in Mn toxicity

The innate immune response in the CNS leads to activation of microglia and astrocytes and a secretion of potent cytokines are apparent, such as IL-1B, TNFα and IFNγ. These cytokines lead to the propagating activation of the inactive glia leading to the sensitization of dopaminergic neurons to these pro-inflammatory cytokines (Hirsch and Hunot, 2009). In the substantia nigra of PD patients, increased amounts of these cytokines have been attributed to glial activation and furthermore the increased expression of COX-2 and NOS2 (Hirsch and Hunot, 2009). *In vitro* Mn exposure in the presence of these pro-inflammatory cytokines, such as TNFα, initiates NF-kB signaling in astrocytes, potentiating activation of NOS2 (Moreno et al., 2008). *In vivo* Mn exposure has also indicated increased activation of NF-kB and NOS2 (Moreno et al., 2009a, Moreno et al., 2011). This inflammatory gene activation observed with Mn exposure is potentially due to the increased concentration of pro-inflammatory cytokines from the glia, specifically IL-1B and TNFα, that leads to neuronal injury (Liu et al., 2009).

NO is biosynthesized from NADPH-dependent conversion of L-arginine by three isoforms of nitric oxide synthase; neuronal, endothelial, and inducible. NO production through neuronal and endothelial isoforms is necessary for physiological cell communication. In addition, these constitutively expresses cytosolic isoforms are

calcium/calmodulin dependent and activated by calcium influx in neurons or endothelial cells (Bredt and Snyder, 1994). However, the inducible form of NOS, NOS2, is expressed *de novo* as an inflammatory response to cellular stress stimuli in the CNS and is independent of calcium/calmodulin. This isoform is exclusively expressed in the astrocytes and microglia and is induced by exposure to Mn and inflammatory cytokines such as TNFα and IFNγ (Moreno et al., 2009a). The activation of NOS2 results in the excessive accumulation of NO that in the presence of reactive oxygen species, primarily superoxide, produces the highly reactive anion, peroxynitrite. This electrophile forms adducts with tyrosine residues at position 3 on the aromatic ring, yielding 3-nitrotyrosine (3NT) during the progress of inflammatory disease (van der Veen et al., 1997). Widespread protein nitration during stress and injury in the CNS leads to nitrosative stress and neuronal dysfunction (Ischiropoulos, 2003). This pathological feature has been observed in multiple neurological disorders, including ischemia, Alzheimer's disease, Parkinson's disease, and manganese neurotoxicity. As previously mentioned, studies in our laboratory have supported the glial inflammatory hypothesis demonstrating *in vitro* Mn treatment of astrocytes potentiates cytokine-induced expression of NOS2 and production of NO through activation of Nf-kB leading to apoptosis in neuronal cells (Moreno et al., 2008, Moreno et al., 2011). *In vivo* Mn exposure induced glial activation, up-regulation of NOS2 and increased peroxynitrite adducts on neurons with in the basal ganglia concurrently with neurobehavioral changes (Liu et al., 2005, Moreno et al., 2009a, Moreno et al., 2009b, Moreno et al., 2011). What is left unclear is if glial NOS2 activation is responsible for the pathologic features observed in manganese neurotoxicity and if deletion of NOS2 is neuroprotective (see Chapter 3).

## 1.5 **Astrocytic Signaling and disease**

Parkinson's disease (PD) is a common neurological disorder that is chronic, progressive, multifactorial and currently incurable in 1.5 million Americans (National Parkinson Foundation). In 1992 alone, it was estimated that the total annual cost of PD was 6 billion dollars (National Institute of Neurological Disorders and Stroke). Currently, PD affects 1% of the population over the age of 60 (Maragakis and Rothstein, 2006), but it is predicted that the number of PD patients will increase corresponding to the rise in the elderly population. Microvascular pathology has been observed in PD, and recent imaging data confirms altered regional cerebral blood flow (rCBF) in the basal ganglia of PD patients (Hsu et al., 2007, Borghammer et al., 2010). The mechanisms responsible for regulating rCBF is mediated by astrocyte activation from metabolically needy neurons through rises in intracellular calcium where at the perivasular end foot release of vasoactive signals increase blood flow, termed neuro-vascular coupling (NVC) (Filosa et al., 2006, Metea and Newman, 2006). Yet to be deciphered is the endogenous affecter prompting the dysfunction in rCBF and potentially altered signaling in the astrocytes leads to modification in the vasoactive response. Therefore, elucidating the role of the astrocyte signaling and exogenous agents that impede calcium signals could further explicate the progressive dysfunction observed in rCBF.

A. Neurovascular coupling (NVC)

Depletion of rCBF has been associated with the progression of PD (Borghammer et al., 2010), yet the causation of this hypoperfusion is unknown. Decreases of blood flow in the brain will hinder oxygen and glucose delivery to remaining metabolically active neurons, leading to cellular dysfunction and neuronal death (Iadecola and Nedergaard, 2007). This component of PD is a possible explanation of the progressive nature of PD and neuronal demise, and may explain the progression of other neurodegenerative diseases.

Astrocytes are the primary support cell in the brain, responsible for metabolic maintenance of neurons, transmitter uptake and release, regulation of the blood brain barrier and homeostasis of extracellular ionic concentrations. Astrocytes are in close proximity to pre- and post-synaptic nerve terminals, where neurotransmitters activate bidirectional mechanisms between the active neurons and astrocytes influencing neurotransmission (Pascual et al., 2005, Fellin et al., 2006). Signal transduction pathways activated in adjacent astrocytes travel to their end feet that surround the cerebral arterioles which regulate rCBF (Simard et al., 2003, Fellin et al., 2006). Astrocytes are non-excitatory cells that communicate through calcium waves, which propagate through the syncytium (Koehler et al., 2006). Calcium waves travel through two different mechanisms, gap junctions between neighboring cells and paracrine release of gliotransmitters that activate membrane bound receptors on the surrounding cells (Blomstrand et al., 1999, Guthrie et al., 1999). Long range communication in astrocytes has been shown to primarily occur though paracrine signaling due to the modest change in calcium wave propagation and purinergic receptor expression upon knockout of a

major gap junction protein, connexin 43 in mice (Scemes et al., 2003). This intercellular signaling mechanism between astrocytes is the primary focus of the research described in Chapter 4.

Gliotransmitters released by astrocytes include glutamate and ATP (Parpura et al., 1994, Guthrie et al., 1999), subsequently activating glutamate and purinergic receptors on neighboring cells. Activation of these receptors increases intracellular calcium concentrations, which stimulate exocytosis of gliotransmitters through via the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) (Pascual et al., 2005, Striedinger et al., 2007). SNARE proteins are critical in gliotransmission; alterations in astrocytic SNARE proteins results in modification of calcium oscillations and in wave propagation (Striedinger et al., 2007). Thus receptor activation and subsequent exocytosis of gliotransmitters resulting in a propagation of an intercellular calcium wave propagates up to 300 μm through the astrocytes.

ATP is an essential product of the electron transport chain and is the principal cellular energy source in eukaryotes. ATP was first identified as a modulator of neurotransmission and vasomodulation in dorsal root ganglion nerve terminals, where application of ATP caused a dilatory response in adjacent capillaries (Holton and Holton, 1953). More than a century ago it was suggested by Ramon y Cajal that astrocytes regulate vascular tone in the CNS (Cajal, 1911). Recently, a large body of literature has indicated that ATP is a primary extracellular gliotransmitter that activates purinergic receptors on the plasma membrane of astrocytes (Guthrie et al., 1999, Koehler et al., 2006). There are two categories of ATP-dependent purinergic receptors: P2X receptors, which are ligand gated ion channels, including the low affinity $P2X_{1-7}$ subtypes (James

and Butt, 2002); and P2Y receptors, which are G-protein coupled receptors, including many high affinity subtypes $P2Y_{1,2,4,6,11,12,13,14}$(Burnstock, 2007). ATP stimulation of P2Y receptors activates the phospholipase C (PLC)-inositol triphosphate (IP3) pathway, increasing intracellular calcium from the endoplasmic reticulum and inducing release of gliotransmitters through calcium-dependent SNARE proteins. This signaling transduction pathway is critical to initiate an increase in intracellular calcium in astrocytes and exocytosis of ATP to adjacent astrocytes in order to activate P2 receptors (Newman, 2001, Koehler et al., 2006).

The P2Y pathway functions as the physiologic signaling pathway at namomolar to low micromolar concentrations of ATP (James and Butt, 2002). $P2Y_{1,2,4,6,11}$ are specific subtypes expressed on astrocytes that utilize the $G_{q/11}$ transduction, which is associated with IP3 -dependent calcium release (Burnstock, 2007). IP3 mediates release of calcium from internal stores and in the astrocytic end foot this induces a vasodilatory response in adjacent cerebral arterioles (Straub et al., 2006, Straub and Nelson, 2007). Furthermore, subtypes $P2Y_2$ and $P2Y_4$ are highly expressed at the astrocytic end feet associated with the vasculature (Simard et al., 2003). Due to the proximity and concentration of these receptors at the glio-vascular interface, activation of receptors by purines participates in the glia-vascular signaling and regulates functional hyperemia (Fields and Burnstock, 2006).

Release of IP3 generates an intracellular calcium increase in astrocytes that leads to a rapid vasodilatation or vasoconstriction of the adjacent arteriole (Straub et al., 2006). Elevations of calcium at the perivascular end foot cause secretion of vasoactive substances, such as arachidonic acid (AA) metabolites, nitric oxide (NO), and potassium

ions (Koehler et al., 2006, Attwell et al., 2010), all of which effect vascular tone, yet the precise role of each particular substance in regulating vasodilation and neurovascular coupling is still in dispute. Calcium-dependent potassium conductance, notably through BK channels on the end foot processes in astrocytes, has been shown to be a critical regulator of vascular tone (Girouard et al., 2010). Astrocytes have elevated potassium permeability relative to neurons and the majority of the conductance occurs at the end foot (Newman et al., 1984). When calcium-sensitive BK channels at astrocytic end foot surrounding the vasculature are inhibited, vasoactive responses significantly decline without affecting intracellular calcium increases in astrocytes (Price et al., 2002, Filosa et al., 2006). These studies support the role of potassium as a potent vasoactive substance in neurovascular coupling.

An alternate hypothesis regulating cerebrovascular is through the production of arachidonic acid metabolites. The astrocytic rises of intracellular calcium activates phospholipase $A_2$ leading to the production of arachidonic acid, arachidonic acid is metabolized to prostaglandins and epoxyeicosatrienoic acids (ETTs) (Metea and Newman, 2006). Prostaglandin production, most likely $PGE_2$, results in vasodilation which additionally has been associated with EETs concentrations and consequently potassium channel activation (Metea and Newman, 2006, Attwell et al., 2010). For instance, BK channels are modulated indirectly by EETs activation of calcium on astrocytes as well as on smooth muscles cells (Earley et al., 2005, Koehler et al., 2006). On the other hand arachidonic acid can be metabolized to 20-HETE by cytochrome P450, CYP4A, leading to vasoconstriction of the cerebrovasculature (Metea and Newman, 2006). Through either the direct mechanism, increasing intracellular calcium to elicit

potassium release, or the indirect production of arachidonic acid metabolites subsequently affecting BK channels, a rapid vasoregulation from potassium ions and arachidonic acid metabolites lead to a contraction or relaxation of the vascular smooth muscle cells at the glia-vascular interface regulating rCBF (Price et al., 2002).

NO is a potent vasodilator in the vascular smooth muscle cells of the PNS through transcription of eNOS in endothelial cells. However, in the CNS, NO is both a vasoconstrictor and vasodilator dependent upon concentration (Metea and Newman, 2006). At low concentrations of NO, vasodilation is more likely do occur. This has been suggested to be through activation of sGC and subsequent production of cGMP or through inhibition of the enzyme producing 20-HETE (Sun et al., 2000, Metea and Newman, 2006). High concentrations of NO favors constriction of the vasculature, where NO is postulated to inhibit the enzyme producing EETs decreasing vasodilator activators (Metea and Newman, 2006).

Astrogliosis may affect NVC mechanisms of the astrocytes through direct and indirect pathways, subsequently enhancing the progression of neurodegeneration (Hirsch et al., 2005). This has been capitulated through the up-regulation of iNOS in the diabetic retina. In a model of diabetic retinopathy, expression of NOS2 (iNOS) and overproduction of NO by retinal glial cells inhibited production of arachidonic acid-derived PGE2 and enhanced vasoconstriction (Mishra and Newman, 2010). Notably, the loss of rCBF and neurovascular coupling in the retina was recovered with use of iNOS inhibitors, indicating that excess NO directly inhibited functional hyperemia. These mechanisms of astrocytic signaling disruption in functional hyperemia are still unknown, however, the association with astrogliosis is known in this dysfunction.

B. Divalent Cation Channels

Transient receptor potential (TRP) proteins are a family of non-selective cation channels that are expressed on astrocytes, in specific abundance are the canonical TRPC3 channels (Grimaldi et al., 2003), which are physically linked with the $G_{q/11}$ transduction pathway (Montell et al., 2002). TRPC3 has been shown to be activated by the PLC-dependent release of diacylglycerol and specifically associated with P2 receptor activation (Hofmann et al., 1999, Reading et al., 2005). Capacitative calcium influx provides extracellular calcium that allows refilling of the endoplasmic reticulum to maintain calcium homeostasis although TRPC3 channels have evidence distinct from this mechanism (Grimaldi et al., 2003). TRPC3 plays an important role in the calcium oscillations observed in astrocytes. These oscillations have been attributed to physiological functions such as transmitter secretion as well as pathophysiological implications such as mediating astrogliosis (Berridge, 1990, Tashiro et al., 2002). Although the exact role of TRP channels remains to be elucidated, evidence for TRP expression on astrocytes and the role of calcium influx remains essential for physiological functions in the astrocyte.

There is a small amount of evidence suggesting that modulation of intracellular calcium can be regulated by extracellular cation channels on the plasma membrane of the astrocyte. Mn has been shown to disrupt the propagation of calcium in mechanically stimulated waves, which was similarly abolished by the non-selective P2 receptor inhibitor (Tjalkens et al., 2006). The Mn exposures in this study were low dose $MnCl_2$ for 24 hours, notably that allows time for changes in gene and protein expression.

However, the sub-acute treatments evidently indicate that Mn interferes with calcium homeostasis and mitochondria sequestration that disrupts ATP-regulated calcium waves.

Other metals have been observed like the inhibitory actions of zinc ($Zn^{2+}$), which is another transition metal and divalent cation larger than Mn. Agonist activation of the metabotropic $P2Y_4$ receptor in the presence of $Zn^{2+}$ interrupts calcium responses in a concentration dependent manner, which were reversed with chelation of $Zn^{2+}$ in addition to washout (Wildman et al., 2003). These data suggested an extracellular site of action in which $Zn^{2+}$ interacts to modulate calcium. Another divalent cation which has been shown to disrupt purinergic signaling is magnesium ($Mg^{2+}$) ions. $Mg^{2+}$ is questioned to compete for calcium entry through non-selective cation channels and or the possible blockage of calcium influx channels (Ikonomidou and Turski, 1996, Ko et al., 2004, Kwak and Weiss, 2006, Deshpande et al., 2008). Although these studies observe a range of channels involved with the essential cation, all have observed modification calcium influx (Lee et al., 2011).

Finally, in the presence of pyridinium ions, a positively charged molecule similar in structure to MPP+ and PQ (see 1.5C) suggest that TRPC channels modulate calcium signaling in astrocytes (Li et al., 2009). Although it is believed that these permeant pyridinium dyes, enter in to the astrocyte through channels in a questionable density dependent manner. All of this evidence of cationic molecules suggest the association with calcium influx and affinity for extracellular cation channels, specifically TRP channels, have the potential to lead to signaling dysfunction in the presence of these molecules a dose dependent manner.

C. Basal ganglia neurotoxins

The basal ganglia is a sensitive group of brain nuclei that are susceptible to a multitude of neurotoxins inducing a neuroinflammtory response. For instance, 6-hydroxydopamine (6-OHDA) which in endogenously produced yet has been used as a model for PD. The acute responses on astrocytes and calcium signaling in the presence of these chemicals are unknown, however there chronic exposures have been extensively studied. Systematic administration of 6-OHDA does not cross the blood–brain barrier (BBB), therefore it is injected into the striatum or substantia nigra pars compacta to induce rapid dopaminergic neurotoxicity (Jackson-Lewis V., 2011). This compound is formed through the oxidation of dopamine, by the hydroxyl radical attack on the catechol ring of the neurotransmitter. Cell death in dopamine neurons is mediated through ROS production and inhibition of complex I and IV in the electron transport chain (Jackson-Lewis V., 2011).

The dopaminergic-selective neurotoxicant, MPTP, is lipophilic and uncharged at physiological pH and therefore crosses the BBB. MPTP in the astrocyte is converted to its active metabolite, MPP+, by monoamine oxidase B and exits the cell via the organic cation transporter (Cui et al., 2009). Once released into the extracellular space, neurons expressing dopamine transporters take up the toxin and MPP+ has the ability to inhibit complex I of the electron transport chain, producing ROS and depleting ATP. Furthermore, MPP+ has affinity for the vesicular monoamine transporter 2 in neurons which packages dopamine and other neurotransmitters (Liu et al., 1992), releasing the toxin back into the extracellular space propagating the toxic effects of MPP+.

The herbicide paraquat (1,1'-dimethyl-4,4'-bipyridilium dichloride) is banned in the US and Europe but has gained much interest due to the chemical similarity to the dopaminergic neurotoxic MPP+ (Drechsel and Patel, 2008). Epidemiological evidence has associated chronic exposure to paraquat with PD-like neurodegeneration and observed in the exposed brains of rodents that paraquat induces oxidative and nitrosative stress mechanisms (Jackson-Lewis V, 2011). Paraquat toxicity is primarily through redox-cycling concomitant increases in superoxide concentration. The crossing of the BBB by paraquat is controversial due to its charge and polar chemical structure, but is postulated to cross though an amino acid transporter (Drechsel and Patel, 2008), which is how the adverse health effects are observed.

These chemicals, along with Mn (see 1.3), chronic effects have been well documented in the literature, however, their acute interactions with physiological signaling functions in the CNS are unknown. The cationic charge and structures of these chemicals were postulated to interfere with calcium influx in astrocytes due to previous observations in our laboratory (Tjalkens et al., 2006). Implications of the inhibitions in calcium influx would prevent sufficient increases in intracellular calcium depleting the propagation of astrocytic signals to the vasculature. In order to observe this calcium dysfunction, acute application of these chemicals were applied to primary striatal astrocytes and imaged by real time fluorescent microscopy. Alterations in calcium homeostasis have concerns for uncoupling NVC, therefore understanding if endogenous and exogenous chemicals modulate these mechanism could help to explain the deprivations in rCBF observed with the progression neurodegeneration (see Chapter 4).

**Summary**

The progression of neurodegeneration is associated with a robust astrogliosis as well as deprecations in rCBF. However, it is not known if these post mortem and clinical observations are due to early dysfunction in NVC mechanism. This dysfunction in signaling could lead to the progression of the disease state further implicating activation neuroinflammatory gene expression leading to adverse pathology. The data generated from these studies will help 1) demonstrate that Mn neurotoxicity is age and sex dependent, and that early exposure predispose the basal ganglia to greater neuroinflammatory susceptibility upon subsequent re-exposure during aging, 2) demonstrate that juvenile mice lacking functional NOS2 gene are protected from Mn induced locomotor dysfunction and glial-mediated nitrosative stress, indicating that Mn-induced production of NO contributes to neurotoxicity and 3) demonstrate that ATP-induced cation currents in primary astrocytes exhibit inhibition of TRPC3 in the presence of cationic exogenous and endogenous neurotoxins. Overall, these studies indicate that astrocytes have a direct role in neuroinflammatory injury and nitrosative stress following Mn exposure and the microenvironment of the astrocytes can indirectly influence the trophic support for neurons, implicating the progression of neurodegenerative disease state.

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# CHAPTER 2

# GLIAL INFLAMMATION: AGE AND SEX SUSCEPTIBILITY

## Introduction

Manganese (Mn) is a well documented neurotoxin in occupational exposure settings, such as welding and steel manufacturing. However, Mn exposure from environmental and dietary sources are the primary routes of exposure for the majority of the population. Little is known about childhood Mn exposure and the susceptibilities on the developing nervous system, in addition to if secondary exposure upon aging renders the pre-exposed nervous system more vulnerable to further neuroinflammation. Epidemiological evidence of children exposed to Mn in drinking water are associated with cognitive and behavioral deficits (Menezes-Filho et al., 2009). Additionally, early studies linked hyperactivity to children exposed to high Mn levels with high concentrations of Mn hair follicles (Collipp et al., 1983); which is a prevalent childhood disorder and public health concern. Children have an increased risk of Mn exposure due to the immature development of the regulating pharmacokinetics compared to adults, which warrant age-dependent susceptibility studies. This collection of publications, demonstrate age-dependent neuroinflammatory and neurobehavioral susceptibility, including sex-dependent vulnerability to Mn neurotoxicity. This chapter is composed of the principle topics written in brevity from the published manuscripts; for full depth details and discussion of the work discussed here access the Journal of Toxicological Science.

A. Developmental exposure to manganese increases adult susceptibility to inflammatory activation of glia and neuronal protein nitration. Moreno JA, Streifel KM, Sullivan KA, Legare ME, Tjalkens RB. Toxicol Sci. 2009 Dec;112(2):405-15.

Chronic manganese (Mn) exposure leads to the activation of glia and increased expression of inflammatory genes such as inducible nitric oxide synthase (NOS2) (Liu et al., 2006). Current literature has extensively researched cases of adult Mn exposure, where early stages of the disorder exhibit cognitive and physiological alterations. As the disease progresses, irreversible neurodegenerative symptoms are pronounced such that resemble, but are distinct from Parkinson's disease (Calne et al., 1994). However, little is know about the effects of Mn exposure on the developing CNS and furthermore if early childhood exposure pre-disposes for greater neurological injury upon aging.

Recent epidemiological evidence has indicated that childhood exposure to elevated levels of Mn in drinking water is correlated with neurobehavioral and neurocognitive effects (He et al., 1994, Woolf et al., 2002, Wasserman et al., 2006, Bouchard et al., 2011). Because infants and children have immaturely functioning pharmacokinetics in their developing stages, these age periods have increased absorption and retention of Mn compared to adults, and poorly developed biliary excretion prolonging the half-life of Mn exposure (Aschner and Aschner, 2005). Concerns due to increased Mn content in soy-based infant formula have been raised; however, with the post weanling age of development studied in this research, contaminated drinking water of greater relevance.

It is known that astrocytes accumulate 50 times more Mn than neurons (Maurizi et al., 1987), and in cases of excess Mn are a target for physiological dysfunction. Mechanisms underlying Mn-induced activation of the microglia and astrocytes are still

inconclusive. However, observations of the glia undergoing proliferation and morphological changes have been implicated in manganism among other neurodegenerative diseases (Verity, 1999). Glial activation results in an up-regulation of inflammatory genes increasing pro-inflammatory cytokines, in example NOS2 elevating levels of nitric oxide (NO). Astrocytic Mn exposure increases NO through activation of Nf-kB in an ERK-dependent manner (Moreno et al., 2008); however, much less is understood about microglia NO production. Excessive NO accumulation from the glia can lead to the protein nitration of tyrosine adducts by peroxynitrite on the neurons, progressing neuronal injury. Therefore, this study focused on the increased activation of NOS2 in the glia and the concomitant nitrosative stress of the neurons found in the basal ganglia.

The study included three treatments of Mn by oral gavage in juveniles, adults, and a group treated as juvenile then again as adults. Furthermore, the dosing paradigm chosen were low dose exposures to test the potentiation and susceptibility upon re-exposure to Mn with aging. It was hypothesized that low dose exposure to Mn as juveniles would distinctly activate microglia and astrocytes, that upon subsequent re-exposure to Mn, would result in greater glial NOS2 expression and induction of nitrosative stress in the basal ganglia.

Results of pathological immunohistochemical analysis of astrogliosis in juveniles revealed high basal levels of GFAP in the substantia nigra pars reticulata (SN), which was attributed to the proliferative state of the developing region (Rice and Barone, 2000). Upon Mn treatment in juveniles, a significant suppression in intensity of staining was observed, indicating a possible dysfunction in the development of the SN (Figure 1;

Table 1). In the adult treatment group as well as re-exposed adults, an increase in astrogliosis was detected in the striatal-pallidum and SN. This supports previous Mn exposure models that renders the striatal-pallidum and SN susceptible to Mn and the role in which gliosis plays in neuronal toxicity.

Pathological immunohistochemical analysis of microgliosis in Mn-treated juveniles resulted in enhanced morphological phenotypes of the resident immune cells (Figure 2; Table 2). However, this was only observed in the juvenile mice and not in the adult and re-exposed adult groups. This suggests that microglia activation is predominant in early stages of exposure. Furthermore, with astrocytic activation prevalent in the adult and re-exposure treatment groups these data support the hypothesis that microglia promotes the activation of astrocytes with Mn exposure and other brain injury models (Liberatore et al., 1999, Norton, 1999, Filipov et al., 2005, Saijo et al., 2009).

Increased activation of the glia can be associated with an inflammatory reaction to Mn exposure. Therefore, NOS2 expression was accessed via co-immunofluorescene in astrocytes and microglia using specific immunobodies. In Mn-treated juveniles all regions of the basal ganglia had a significant increase in NOS2 and astrocyte co-localization; however, Mn-treated adults only had significant increases in NOS2 expression in the globus pallidus (GP) and SN, but a suggestive trend in the ST (Figures 3 and 4). The re-exposed adults had significant increases in NOS2 throughout the basal ganglia, furthermore, the increase in quantitive measures were much more robust suggesting an enhanced neuroinflammatory response.

Juvenile microglial co-localization with NOS2 was significantly increased with Mn exposure, yet in adults only the SN had microglia and NOS2 expression significantly

increased with Mn (Figures 5 and 6). In the re-exposed group there was a significant co-localization in all brain regions accessed. These data are superficially correlated with pathological scoring of microgliosis considering the differences in re-exposed adult activation, but do indicate that there is a robust neuroinflammatory reaction upon subsequent re-exposure in the microglia cells.

Observations in the NOS2 production of NO as 3-nitrotyrosine are utilized using co-immunofluorescene. 3-nitrotyrosine adduct levels indicated that the GP and SN of juveniles were most susceptible to nitrosative stress (Figures 7 and 8). Adults had increased adduct formation in the GP whereas re-exposed adults had a significant increase in the SN. These data indicate that juvenile mice are significantly susceptible to neuronal injury. Furthermore, medium spiny projection neurons expressing D1 receptors in the striatum (DARPP-32), were selectively vulnerable upon subsequent re-exposure to Mn (Figure 9).

These data demonstrate that early activation of microglia may enhance the sensitivity of the activation of astrocytes (Figures 1 and 2), potentiating the neuroinflammatory response (Figures 7 and 8). Juvenile Mn exposure enhances the injury of SN and DARPP-32 neurons upon re-exposure to Mn (Figures 9). Moreover, juvenile exposure to Mn appears to be a critical window of sensitivity which can promote susceptibility to secondary exposures and neurological disease during aging.

B. Age-dependent susceptibility to manganese-induced neurological dysfunction. Moreno JA, Yeomans EC, Streifel KM, Brattin BL, Taylor RJ, Tjalkens RB. Toxicol Sci. 2009 Dec;112(2):394-404.

Excess exposure to manganese (Mn) leads to a progressive neurodegenerative disorder of the basal ganglia, termed manganism. Motor deficits appear with chronic exposure characterized by hypokinesia, dystonia, rigidity and a propensity to fall backwards (Calne et al., 1994). These motor symptoms induced by Mn correspond to changes in catecholamine levels in the striatum, which receives input from motor neurons (Saka et al., 2002), primarily seen as a loss of dopamine and therefore loss of γ-Aminobutyric acid (GABA) output to the pallidum (Perl and Olanow, 2007). Previous studies in Mn neurotoxicity have extensively researched the affects on adult models and extrapolate to developing animals; however, these extrapolations could be greatly miscalculated due to the immaturity of the developing system. Studies of Mn exposure in juvenile are lacking and are important to address the concerns of Mn neurotoxicity in the developing brain.

Dopaminergic, glutamatergic, and GABAergic synaptic activity have been shown to be modulated by Mn neurotoxicity. As previously mentioned, dopamine loss with Mn toxicity has been well documented in the adult model indicating the dopaminergic sensitivity (Liu et al., 2006, Guilarte et al., 2008a). GABAergic neurons project from the striatum to the globus pallidus (GP) and substantia nigra (SN) modulating glutamatergic and dopaminergic output, respectively (Anderson et al., 2008). Due to the complexity of basal ganglia circuity, there are close correlations with alterations in neurotransmitters that affect the locomotor activity observed with Mn toxicity. Furthermore, epidemiological evidence has demonstrated an age correlation with Mn exposure and

locomotor activity changes; whereas adult exposure resembles the hypokinesia previous discussed and juvenile exposure demonstrates attention deficits and behavioral disinhibition (Menezes-Filho et al., 2009). The current research using a mouse model addresses whether there is an age dependent susceptibility with environmentally relevant levels Mn that leads to altered levels in dopamine, which is postulate to be correlated with adverse locomotor activity.

The study conducted had three treatments of Mn by oral gavage in juveniles, adults, and a group treated as juvenile then again as adults. Furthermore, the dosing paradigm chosen were low dose exposures to test the potentiation and susceptibility upon re-exposure to Mn with aging. It was hypothesized that low dose exposure to Mn as juveniles would distinctly alter neurochemical and neurobehavioral assays compared to adults, and that subsequent re-exposure would further impact the neurological changes observed with manganism.

Locomotor assays accessed the time spent in the margin, thigmotaxis, which is an indicator of anxiety and lack of novelty seeking behavior. Results found Mn treatment in males decreased margin time in juveniles, with inverse effects in male adults that were again exposed as juveniles (Figure 1). Interestingly, female mice were refractory to the increases in Mn-induced juvenile hyperactivity. Furthermore, male mice that were re-exposed to Mn as adults had a significant decrease in movement (Figure 2). These data reaffirm that low dose Mn exposure differentially affects locomotor behavior dependent upon age, sex, and previous exposures.

Dopamine neurotransmission has roles in regulating behavior, voluntary movement, cognition, and reward pathways, among other functionalities. Therefore,

accessing neurochemical levels in the striatum as an indicator of the observed neurobehavioral differences correlated with Mn toxicity. Mn-treated juveniles had a significant increase in dopamine with the highest dosing of Mn whereas adult and re-exposed adults have decreased levels of dopamine compared to control (Figure 3). Re-exposed adults were more susceptible because even the low dose Mn group had suppression of dopamine levels. These dopamine levels were associated with behavioral observations and further demonstrated that the developmental exposure pre-disposed the dopaminergic integrity upon secondary exposure.

Dopamine is metabolized by monoamine oxidase to 3,4-dihydroxyphenyl acetic acid (DOPAC). In all treatment groups the highest level of Mn decreased DOPAC levels; however, only in the low dose group in the re-exposed adults was striatal DOPAC levels significantly decreased supporting the secondary exposure vulnerability (Figure 4).

Calculation of the DOPAC to dopamine ratio helps understand the catabolism and turnover of the neurotransmitter. The Mn-treated juvenile mice had a significant decrease in this ratio, implying a greater susceptibility to catabolic activity of dopamine and a decrease in dopamine turnover (Figure 5). However, there were no change in turnover in either of the Mn-treated adult groups. Furthermore, serotonergic neurotransmission was assessed because previous studies have found a decrease in serotonin with high dietary Mn exposure (Kimura et al., 1978). There were no measurable changes in serotonin levels (Figure 6); however, the metabolite 5-HIAA was increased in juveniles (Figure 7). This demonstrates that Mn also affects serotonergic pathways, and that the increases in 5-HIAA levels could have implications in the observable hyperactivity in juveniles as alterations in these levels have been observed in abnormal behavior (Miczek et al., 2002).

Accumulation of brain Mn was also analyzed to account of pharmacokinetic variability between age and treatment groups. Mn deposition in the juvenile mice occurred in the greatest extent in ST and the SN, additionally in the cortex at the highest Mn treatment (Table 1). However, in adult and re-exposed adult mice, these low dose concentrations did not result in a robust increase in accumulation. Iron and copper were also accessed (Table 2 and 3), indicated an increase in these transition metals in the SN of juveniles. This provides further evidence that juveniles developing systems are more susceptible to altered pharmacokinetics of transition metals, as adult mice did not have accumulation of these metals in the basal ganglia.

These results indicated that low dose Mn exposure induces differential neurobehavioral effects according to age and sex, and the neurochemical levels correspond to changes in locomotor activity. Along with the neuroinflammatory results from the companion paper, these studies equate enhanced activation of the glia and neuronal injury with the neurobehavioral and chemical modifications. Furthermore, these studies further authenticate that juvenile Mn exposure constitutes a critical window of sensitivity, which can promote susceptibility to secondary exposures and neurological disease during aging.

C. Manganese-induced NF-kappaB activation and nitrosative stress is decreased by estrogen in juvenile mice. Moreno JA, Streifel KM, Sullivan KA, Hanneman WH, Tjalkens RB. Toxicol Sci. 2011 Jul;122(1):121-33.

Manganese neurotoxicity in children is of concern considering the fragile disposition of the developing system, which have enhanced absorption and retention with decreased hepatic excretion of Mn (Aschner et al., 2005). Associated with this pharmacokinetic vulnerability are several epidemiological reports indicating neurobehavioral and neurocognition deficits with childhood exposure to Mn in drinking water. Although Mn toxicity is generally well documented in adult populations from occupational exposures, there are much fewer studies of environmental exposures in juveniles.

Early studies in China revealed that sewage irrigation increased Mn concentration in drinking water up to approximately 350 μg/L, compared to 40 μg/L of Mn in the control area. Pre-adolescents in these areas were found to have lower performance for memory and reactions tests correlating with increased hair Mn concentrations and levels of Mn in drinking water (He et al., 1994). A more thorough investigation in children's intellectual function was found to be negatively associated in a dose dependent fashion with Mn concentrations in well water of Bangladesh (Wasserman et al., 2006). High environmental exposures to Mn in Mexico found an inverse association with intellectual function of school aged students (Riojas-Rodriguez et al., 2010). Most recently a study conducted in Quebec, Canada observed naturally high levels of Mn in the drinking water, up to 610 μg/L, associated with with high hair Mn concentrations in school aged children who significantly demonstrated attention deficit hyperactivity disorder (Bouchard et al., 2007, Bouchard et al., 2011). Additionally this study suggested a possible gender

difference, rendering males more susceptible to Mn exposure and neurological impairment (Bouchard et al., 2011). With the epidemiological evidence and previous research in our laboratory, these data collectively suggest a susceptible population to Mn dependent upon age and sex.

Epidemiological and clinical studies have indicated an association in the sex dependent incidences of neurological diseases, correlating the female hormone estrogen with neuroprotective effects. In Parkinsonian models, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have shown that estrogen (E2) attenuates glial production of pro-inflammatory cytokines *in vitro* and *in vivo* and E2 diminishes activation of the glia and subsequent loss of tyrosine hydroxylase positive neurons (Tripanichkul et al., 2006). Additionally, the 6-hydroxydopamine (6-OHDA) neurodegenerative model has associated dopaminergic integrity with the presence of E2, reducing the lesion and lessening the dopamine loss. Collectively these studies indicate the neuroprotective potential of E2, however it is not known if E2 modulates the activation of Nf-kB and NOS2 production with Mn treatment.

Therefore, the study conducted had three juvenile treatment groups; females, males and male supplemented with E2 treated with saline or 100mg/kg of Mn by oral gavage. The dosing paradigm chosen were high dose exposures to induced a robust neuroinflammatory reaction to test the suppressive neuroprotective effect with E2. It was postulated that E2 supplementation in developing male mice would inhibit Nf-kB activation in glia and prevent neuroinflammatory gene expression.

In order to validate the model, systemic E2 serum levels were analyzed (Figure 1). It was determined that exogenous E2 raised serum levels in juvenile male mice in

comparison to female mice, rendering this model relevant for studying the role of E2 in Mn neurotoxicity.

Neurochemical analysis indicated that Mn treatment in males increased levels of dopamine, had a decreasing trend in DOPAC levels and a significant decrease in the DOPAC to dopamine ratio (Figure 2). However, in males supplemented with E2, there was no change in dopamine, DOPAC or ratio levels. This signifies results similar to other neurotoxic models, that E2 suppresses the neuroinflammatory reaction that could be etiologically linked to dopaminergic activity and neurobehavioral effects.

Activation of Nf-kB and expression of NOS2 was assessed in the astrocytes and microglia, via a transgenic NF-kB-EGFP reporter mouse. In astrocytes there was no measurable activation of Nf-kB in the striatum (ST); however, in the substantia nigra pars reticulata (SN), there was a significant increase in males, which was not observed in E2 supplemented males (Figure 3). NOS2 expression had a significant increase in the ST and SN of males, which was attenuated in Mn-treated E2 males. Furthermore, in the microglia of the ST and SN of Mn-treated males there was an increase in Nf-kB and NOS2 in both the males in the presence and absence of E2 (Figure 4). However, there was a significant suppression of the percent activated and expressed between males with E2 and males without E2. These data collectively demonstrate that E2 has a modulatory mechanism on activation of the glia, suggesting that E2 interacts with the Nf-kB pathway decreasing the expression of neuroinflammatory genes.

The increase in NOS2 leads to the excessive accumulation of NO ensuing peroxynitrite formation. In the SN of the male mice there was a robust increase in 3-nitrotyrosine adducts, which was prevented with supplementation of E2 in males (Figure

5). These results are consistent with the MPTP model finding E2 attenuates protein nitration (Morale et al., 2006), and are a collective measure to how Mn neuroinflammatory activation results in alterations in the dopaminergic neuronal integrity.

*In vitro* data in the transgenic NF-kB-EGFP reporter mouse indicated that E2 treatment attenuated the activation of Nf-kB EGFP and the expression of *Nos2* mRNA (Figure 6). Furthermore, E2 treated astrocytes mediated neuronal apoptosis (Figure 7). Additionally indicating that the activation of Nf-kB, and therefore production of NO by NOS2, is an important mediator in the integrity of neurons.

In conclusion, these data demonstrate that the activation of Nf-kB and expression of NOS2 in the glia are important mediators of neuronal injury exhibited by nitrosative stress. E2 has the capacity to modulate the neuroinflammatory activation of this Mn-activated mechanism providing further evidence for sex-dependent vulnerabilities. Collectively, these studies bring further evidence that NOS2, and moreover NO, is an important culprit in the neurobehavioral and neurochemical affects seen with Mn neurotoxicity.

**Summary**

This collection of published work demonstrates that juvenile Mn exposure leads to developmentally unique neurobehavioral and neuroinflammatory reactions. Furthermore, that upon subsequent exposure to Mn during aging, the neuroinflammatory reaction as juvenile predisposes secondary exposure to a great vulnerability to Mn. The role of sex steroids plays a role in the activation of the neuroinflammatory reaction in the glia through Nf-kB, interestingly providing evidence that estrogen in neuroprotective with Mn neurotoxicity. These data indicate Mn as a public health concern for a susceptible population, children, and environmental exposure should be monitored. Additional to the individual health effects from Mn neurotoxicity, of concern are also economic implications of intellectual deficits in developmental exposures.

# CHAPTER 2

# FIGURES

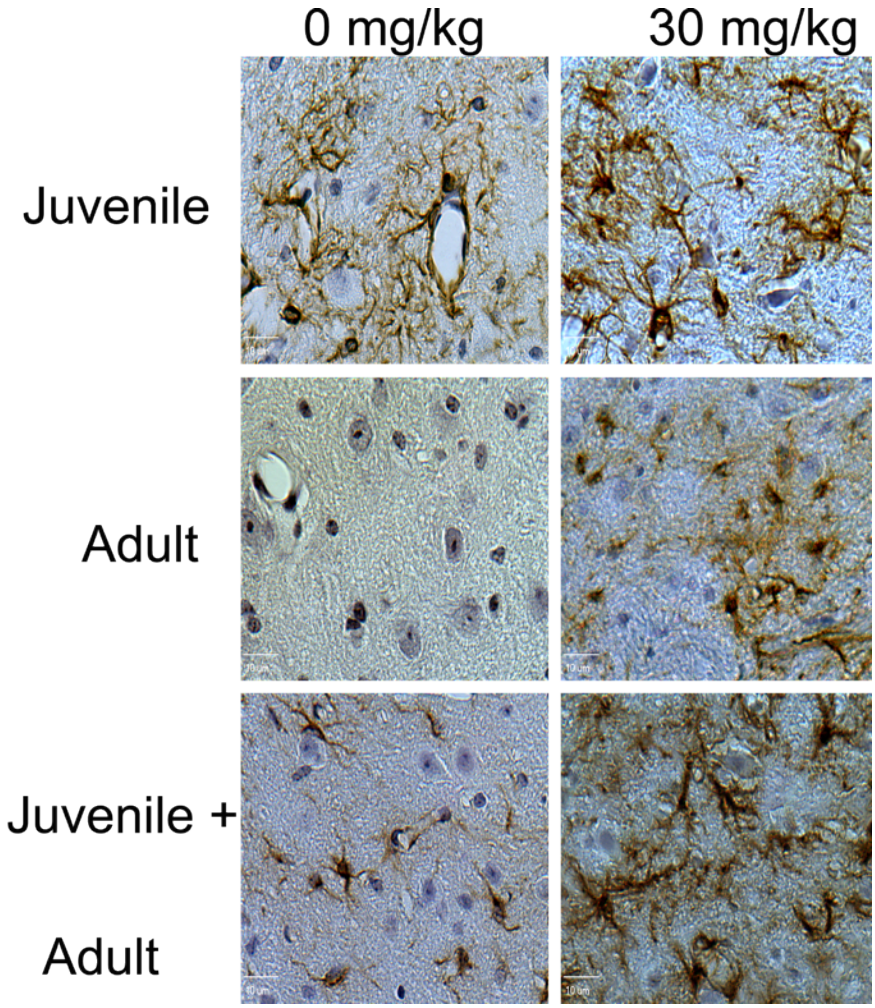


Figure 1. **Differential exposure to Mn in juvenile and adult C57Bl/6J mice induces distinct patterns of astrogliosis in the basal ganglia**. Mice were exposed to 0, 10, or 30 mg/Kg $MnCl_2$ by daily intragastric gavage from day 21 –34 postnatal (juvenile), week 12 – 20 (adult), or day 21 –34 postnatal 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.

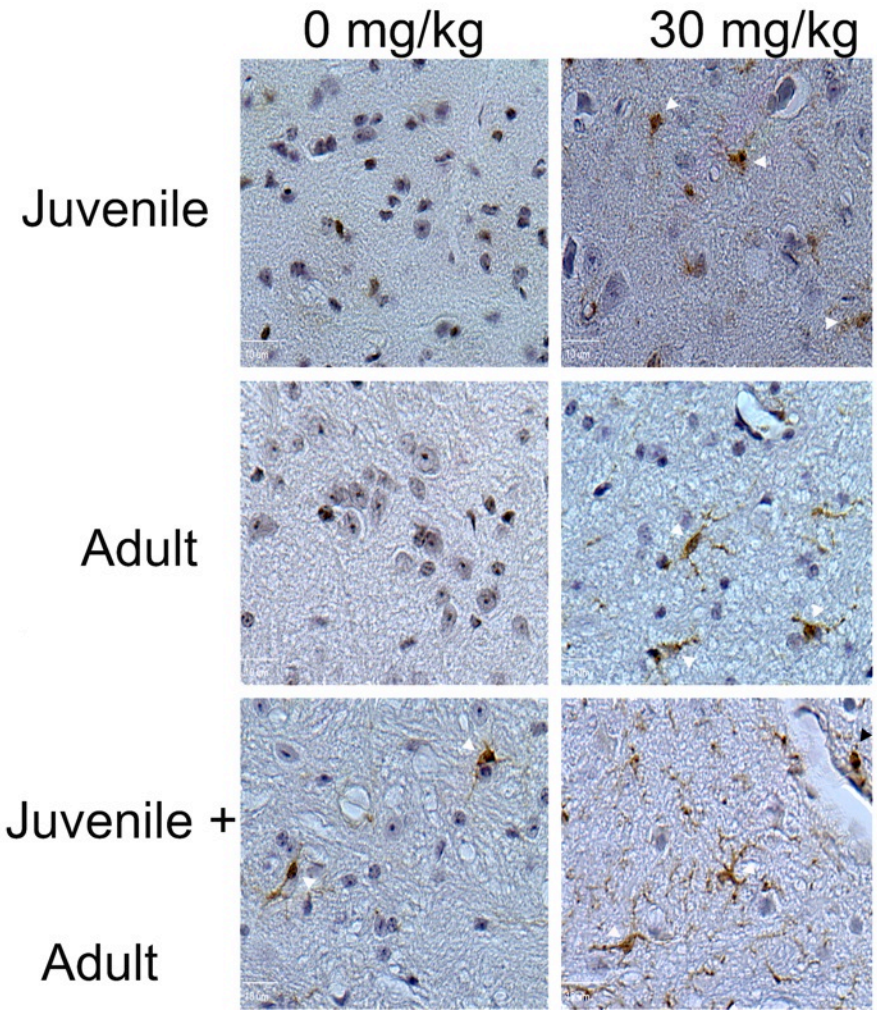


Figure 2. **Differential exposure to Mn in juvenile and adult C57Bl/6J listinct patterns of microgliosis in the basal ganglia**. Mice were exposed mg/Kg $MnCl_2$ by daily intragastric gavage from day 21 –34 postnatal k 12 – 20 (adult), or day 21 –34 postnatal plus week 12 – 20 (juvenile + e brain regions in the basal ganglia were assessed for activation of microglia ochemical staining for Iba-1 including the striatum (St), globus pallidus substantia nigra pars reticulata (SNpr). Representative images of the SNpr are ntrol mice and though exposed to 30mg/kg $MnCl_2$ as juveniles (A), adults h juveniles and adults. Scale bar = 10 μm.

Table 1. **Pathological scoring of astrogliosis in specific brain regions exposed to Mn *in vivo*.**
*Normal Astrocyte= 1 *Activated Astrocyte= 5
Data shown are means ± SEM (n ≥ 3). Significance compared to controls within a specific brain region (Gp, St, or SNpr) per exposure group is denoted by †.

| **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† |

Table 2. **Pathological scoring of microgliosis in specific brain regions exposed to Mn *in vivo*.**
*No microglia= 0 *Activated microglia= 3
Data shown are means ± SEM (n ≥ 3). Significance compared to controls within a specific brain region (GP, ST, or SNpr) per exposure group is denoted by †.

| **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 |

Table 3. **Accumulation of Mn in a variety of brain regions from mice exposed to $MnCl_2$.**
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 †.

| **Time of** | **Treatment ($MnCl_2$)** | **St** | **SNpr** | **Cortex** | **Hypothalamus** | **Serum** |
|---|---|---|---|---|---|---|
| **Juvenile** | 0 mg/kg | 0.26±0.01 | 0.5±0.07 | 0.48±0.08 | 0.63±0.12 | 0.004±0.0003 |
| | 10 mg/kg | 0.49±0.06† | 1.0±0.65† | 0.47±0.32 | 1.4±0.17 | 0.009±0.0009 |
| | 30mg/kg | 0.70±0.01† | 2.5±0.45† | 1.4±0.13† | 1.1±0.51 | 0.004±0.0002 |
| **Adults** | 0 mg/kg | 0.69±0.07 | 0.48±0.18 | 0.45±0.15 | 0.50±0.21 | 0.006±0.0007 |
| | 10 mg/kg | 2.1±0.10† | 2.5±0.11 | 3.1±3.5 | 1.7±0.36 | 0.008±0.0008 |
| | 30 mg/kg | 1.1 ±0.12 | 5.6 ±2.4 | 2.05±0.43 | 1.5±0.16 | 0.015±0.0015 |
| **Juvenile +** | 0 mg/kg | 1.7±0.19 | 1.3±0.12 | 1.5±0.15 | 1.5±0.11 | 0.012±0.0007 |
| | 10 mg/kg | 1.5±0.05 | 1.8±0.13 | 1.46±0.06 | 1.4±0.10 | 0.006± 0.0004 |
| | 30 mg/kg | 1.7±0.09 | 1.3±0.15 | 1.42±0.19 | 1.3±0.20 | 0.012±0.0008 |

Table 4. **Accumulation of Fe in a variety of brain regions from mice exposed to $MnCl_2$.** 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 †.

| **Time of** | **Treatment ($MnCl_2$)** | **St** | **SNpr** | **Cortex** | **Hypothalamus** | **Serum** |
|---|---|---|---|---|---|---|
| **Juvenile** | 0 mg/kg | 6.7±1.4 | 10.1±2.02 | 14.8±9.6 | 17.2±10.4 | 2.53±0.32 |
| | 10 mg/kg | 8.05±1.9 | 18.3±12.8† | 10.7±8.5 | 23.04±8.87 | 3.32 ±1.29 |
| | 30mg/kg | 9.3±1.1 | 37.7±1.23† | 32.1±1.23† | 71.64±90.6 | 4.79±0.23 |
| **Adults** | 0 mg/kg | 26.7±2.23 | 14.9±6.3 | 13.1±4.7 | 19.8±10.1 | 3.15±0.27 |
| | 10 mg/kg | 34.6±1.32 | 41.2±10.5 | 50.4±14.1 | 28.7±5.9 | 6.23±0.62 |
| | 30 mg/kg | 33.2±1.57 | 43±24 | 44.2±3.35 | 41±3.4† | 5.13±0.35 |
| **Juvenile +** | 0 mg/kg | 26.6±1.9 | 32.5±0.35 | 23.3±2.6 | 25.4±2.28 | 2.91±0.27 |
| | 10 mg/kg | 35.4±1.2 | 34.5±1.83 | 34.3±1.18 | 31.0±1.24 | 3.4± 0.20 |
| | 30 mg/kg | 34.7±0.43 | 24.9±2.5 | 24.3±2.6 | 20.9±1.86 | 6.2±0.56 |

Table 5. **Accumulation of Cu in a variety of brain regions from mice exposed to $MnCl_2$.** 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 †.

| **Time of** | **Treatment ($MnCl_2$)** | **St** | **SNpr** | **Cortex** | **Hypothalamus** | **Serum** |
|---|---|---|---|---|---|---|
| **Juvenile** | 0 mg/kg | 1.8±0.07 | 2.0±0.13 | 4.07±0.68 | 4.1±0.75 | 0.24±0.004 |
| | 10 mg/kg | 1.9±0.16 | 3.8±0.93† | 2.8±0.50 | 5.5±0.54 | 0.22±0.002 |
| | 30mg/kg | 2.5±0.06 | 9.9±† | 7.7±1.23 | 3.04±0.81 | 0.23±0.003 |
| **Adults** | 0 mg/kg | 8.1±0.91 | 4.1±1.85 | 2.9±0.82 | 5.5±2.73 | 0.32±0.009 |
| | 10 mg/kg | 10.1±0.52 | 8.4±0.46 | 13.5±4.03 | 8.9±2.4 | 0.34±0.028 |
| | 30 mg/kg | 9.5±0.58 | 10.2±1.58 | 13.2±1.27† | 11.9±1.47 | 0.34±0.008 |
| **Juvenile +** | 0 mg/kg | 8.7±0.72 | 8.0±0.14 | 5.6±0.52 | 7.6±0.73 | 0.21±0.015 |
| | 10 mg/kg | 9.6±0.42 | 9.7±0.52 | 7.08±1.18 | 8.3±0.52 | 0.26±0.007 |
| | 30 mg/kg | 10.1±0.28 | 7.6±1.03 | 5.86±0.76 | 5.18±0.35 | 0.25±0.01 |

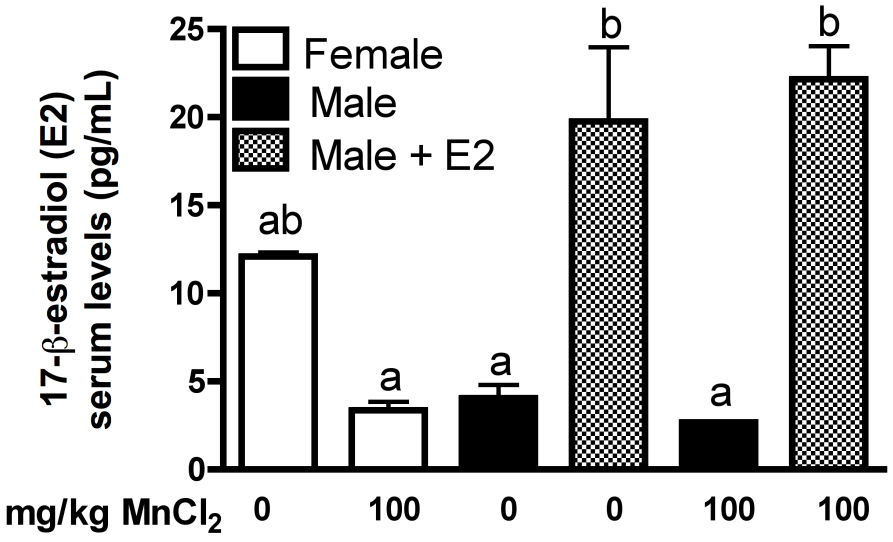


Figure 3. **Administration of exogenous E2 to juvenile male mice increases serum E2 to physiologic levels observed in age-matched female mice.** Female mice and control male mice had Silastic capsules with safflower oil (vehicle control) inserted subcutaneously at day 19 postnatal, while a subgroup of male mice received 25μg of 17-β-estradiol. No statistical change was observed between female mice and unsupplemented male mice. Supplementation of E2 in male mice caused a significant increase in serum E2 levels compared to unsupplemented males and Mn-treated females. Differences between groups were analysed by two-way ANOVA with Bonferroni's *post hoc* test. Different lettering denotes statistical differences in mean value ($p<0.001$, ± SEM).

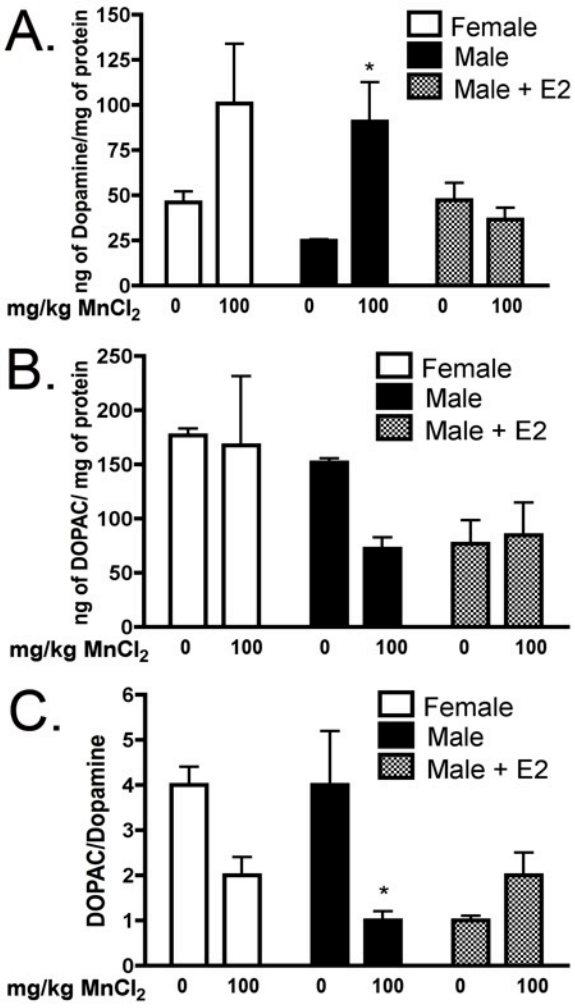


Figure 4. **E2 protects against Mn-induced changes in striatal dopamine in juvenile male mice.** (A) In the absence of E2, Mn-treated male mice had a significant increase in striatal dopamine, whereas male mice supplemented with E2 have no significant changes in dopamine levels. (B) The dopamine metabolite, DOPAC, was not significantly altered within any group, although a decreasing trend was noted in unsupplemented male mice. (C) The DOPAC/dopamine ratio was decreased in Mn-treated males in the absence of E2. Differences between groups were analysed by two-way ANOVA with Bonferroni's *post hoc* test. Statistical significance in mean value is denoted by ***** ($p<0.05$, ± SEM).

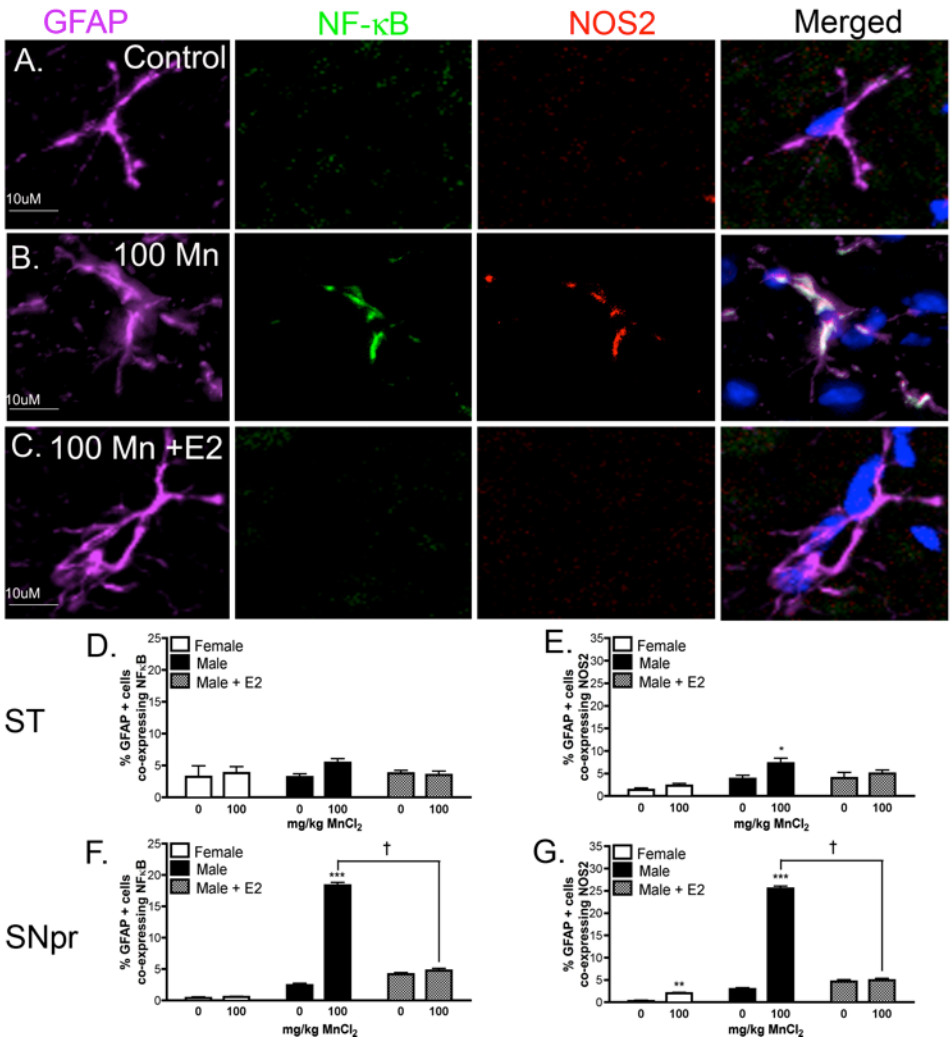


Figure 5. **E2 decreases Mn-induced activation of NF-kB and expression of NOS2 in astrocytes in transgenic NF-kB-EGFP reporter mice.** Astrocyte-specific expression of the NF-kB-EGFP reporter and NOS2 was assessed via co-immunofluorescence in the ST and SNpr of mice exposed to Mn. Images of GFAP, NF-κB-EGFP, and NOS2 expression are shown in purple, green and red channels, respectively, whereas cell nuclei are highlighted by staining with 4',6-diamidino-2-phenylindole (DAPI) in blue. Representative images from the SNpr are presented from male control mice (A), those treated with 100 mg/Kg Mn (B), or 100 mg/Kg Mn with E2 (C). Merged images indicate co-localization of NF-kB-EGFP and NOS2 expression in GFAP-positive astroglia. Quantitative analysis reveals the percent of GFAP-positive cells expressing NF-kB-EGFP in the ST (D) and SNpr (F), and the percent of GFAP-positive cells expressing NOS2 in ST (E) and SNpr (G). For statistical analysis, two-way ANOVA was used with Bonferroni's *post hoc* test to determine significance between treatment groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ± SEM) and between Mn-treated male mice († $p<0.05$, ± SEM). Images were acquired as a *z*-series using a 40X air Plan Apochromatic objective. Scale bar = 10 μm.

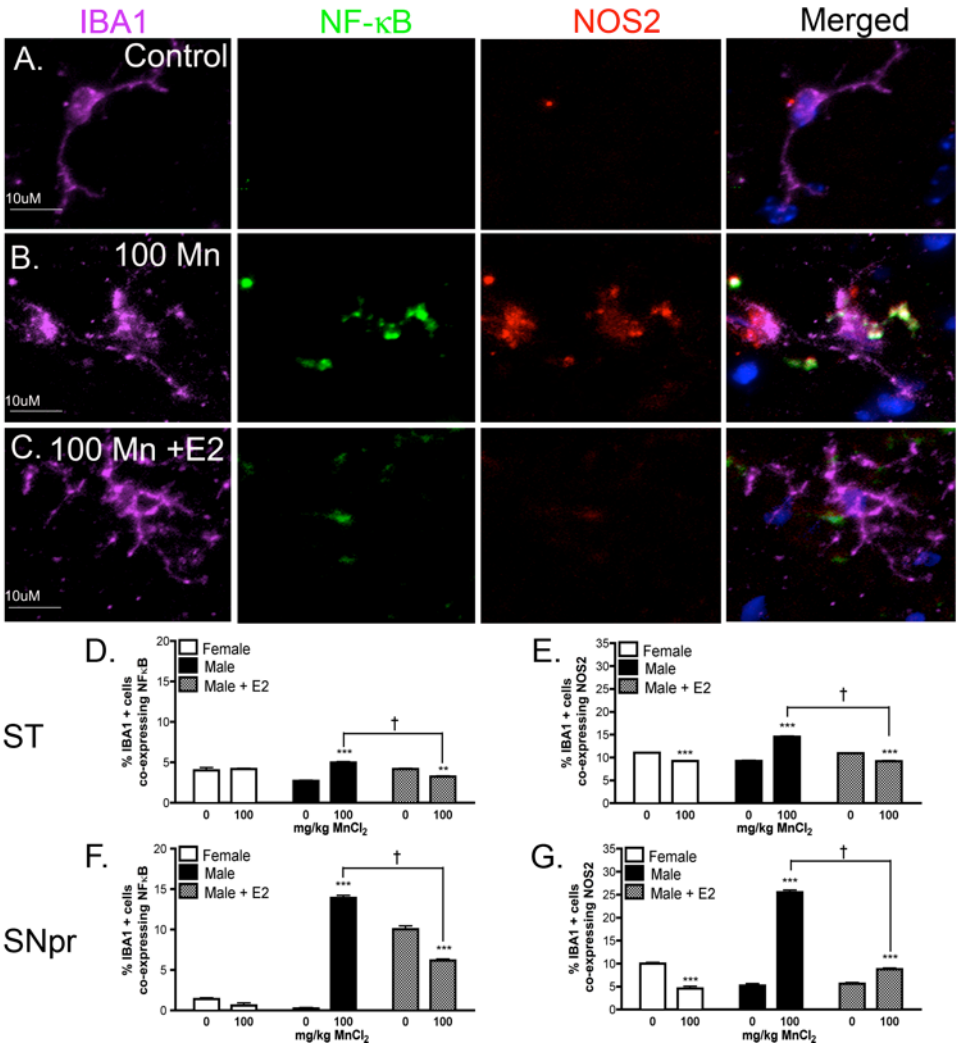


Figure 6. **E2 decreases Mn-induced activation of NF-κB and expression of NOS2 in microglia in transgenic NF-kB-EGFP reporter mice.** Microglial-specific expression of the NF-kB-EGFP reporter and NOS2 was assessed via co-immunofluorescence in the ST and SNpr of mice exposed to Mn. Images of IBA-1, NF-kB-EGFP, 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. Representative images of the SNpr are presented from male control mice (A), those treated with 100 mg/Kg Mn (B), or 100mg/Kg Mn with E2 (C). Merged images indicate co-localization of NF-kB and NOS2 expression in IBA-1 positive microglia in mice exposed to Mn. Quantitative analysis reveals the percent of IBA-1 positive cells expressing NF-kB in ST (D) and SNpr (F), and the percent of IBA-1 positive cells expressing NOS2 in ST (E) and SNpr (G). For statistical analysis two-way ANOVA was used with Bonferroni's *post hoc* test (** $p<0.01$, *** $p<0.001$, ±_SEM) and between Mn-treated male mice († $p<0.05$**,** ± SEM). Images were acquired as a *z*-series using a 40X Plan Apochromatic objective. Scale bar = 10 μm.

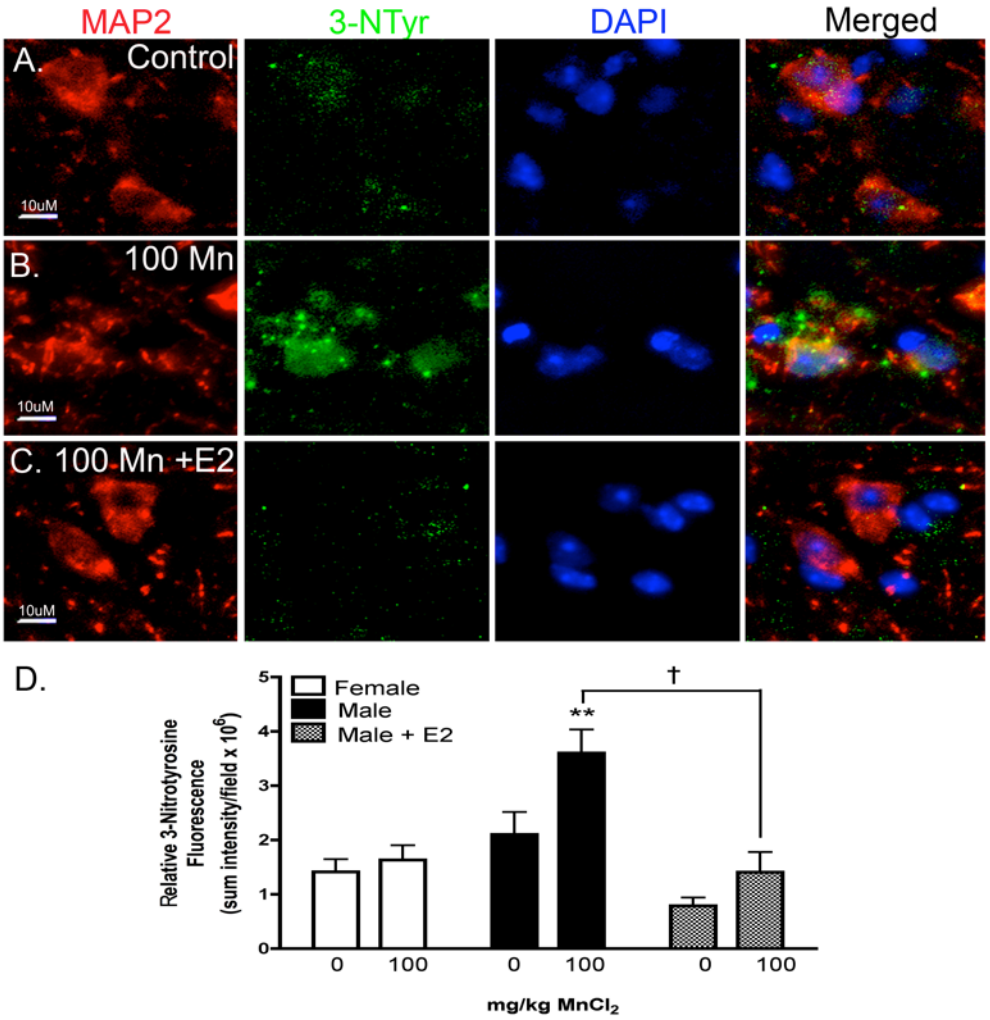


Figure 7. **Mn exposure increases levels of 3-nitrotyrosine (3-NTyr) protein adducts in SNpr neurons that are prevented by E2.** To detect modification of neuronal proteins by peroxynitrite ($ONOO^-$), an oxidative byproduct from increased production of NO by activated glia, serial sections from the SNpr of juvenile mice were stained with antibodies against the neuronal marker MAP2 (green) and 3-NTyr (red) and were counterstained with DAPI to identify cell nuclei (blue). Representative images of the SNpr are presented from control mice (A), those treated with 100 mg/Kg Mn (B), and 100 mg/Kg Mn with E2 (C). Merged images indicate co-localization of 3-NTyr adducts with both neuronal soma and dendrites in the SNpr of Mn-treated mice. Scale bar = 10 µm. Quantitative analysis reveals the sum intensity of 3-NTyr fluorescence within MAP2 positive cells (D). Data was analyzed using two-way ANOVA with Bonferroni's post *hoc* test (** $p<0.01$) and a *post hoc* test was completed between gender groups († $p<0.05$, ±_SEM). Images were acquired as a *z*-series using a 63X Plan Apochromatic objective. Scale bar = 10 µm.

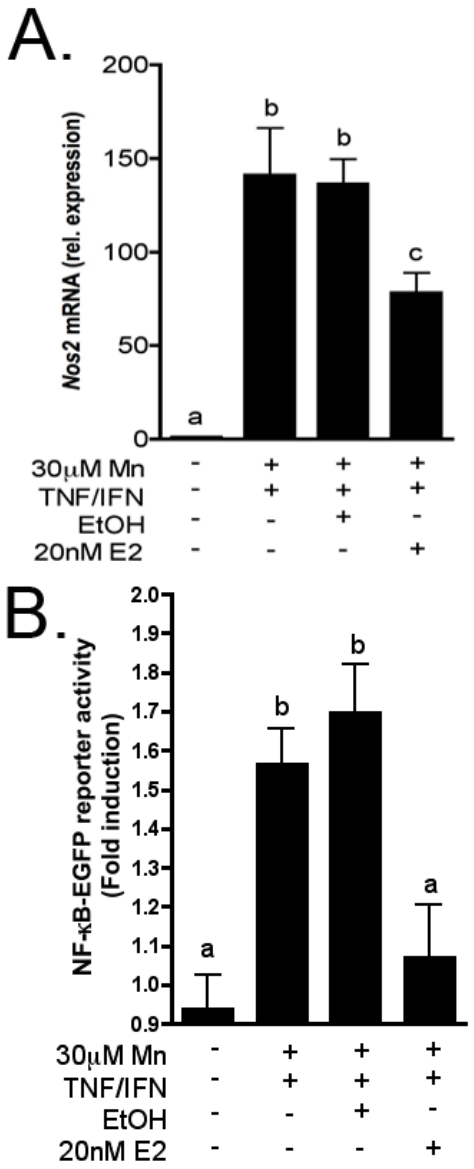


Figure 8. **E2 inhibits expression of *Nos2* and activation of NF-kB in primary astrocytes exposed to Mn and inflammatory cytokines.** (A) *Nos2* mRNA expression was increased in astrocytes exposed to 30 uM Mn + TNFα/IFNγ but was reduced by pre-treatment with 20 nM E2. (B) Activation of NF-κB was determined by live cell fluorescence imaging in astrocytes isolated from transgenic NF-κB-EGFP mice. NF-κB-EGFP reporter activity was significantly increased by treatment with Mn + TNFα/IFNγ and reduced to control levels with pre-treatment of 20 nM E2. Differences between groups were determined using one-way ANOVA followed by the Tukey-Kramer multiple comparison *post hoc* test, significance is denoted by differing letters ($p < 0.05$, ± SEM).

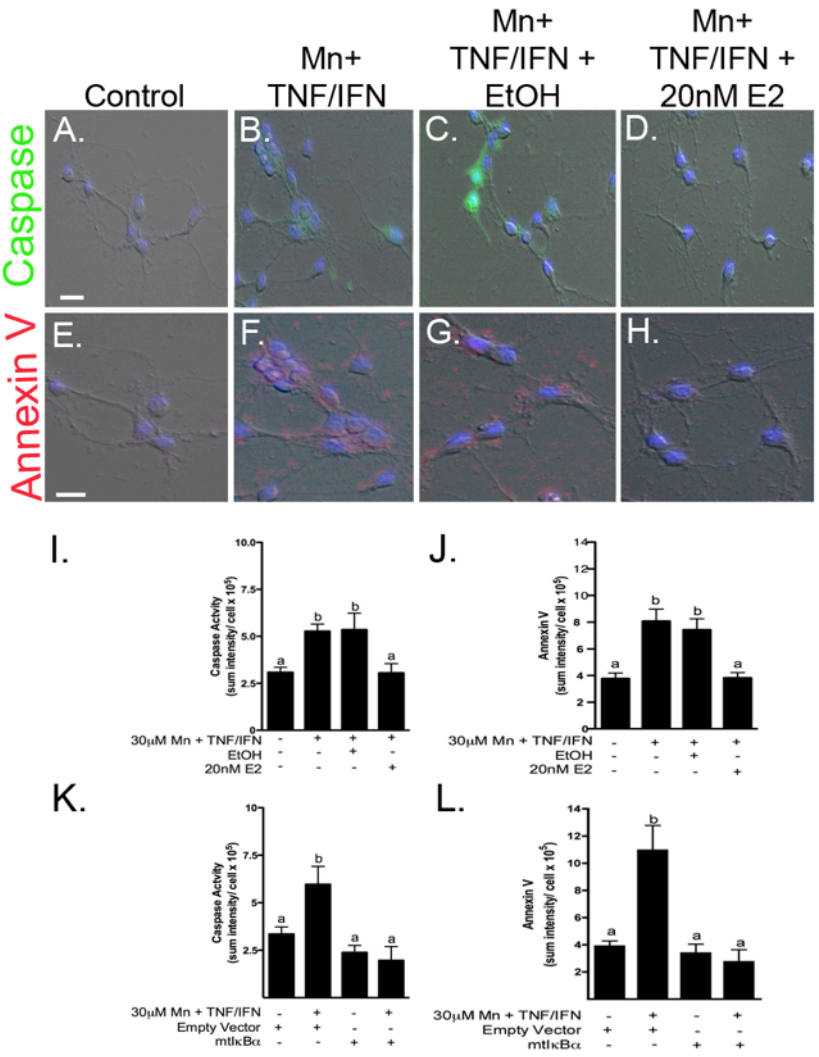


Figure 9. **E2 protects co-cultured striatal neurons from astrocyte-mediated apoptosis induced by Mn and cytokines.** Caspase activation (green) and Annexin V binding (red) in primary striatal neurons co-cultured with astrocytes is induced by exposure to Mn and TNFα/IFNγ. Representative images indicate striatal neurons incubated with astrocytes treated with saline (A & E), 30 μM Mn + TNFα/IFNγ (B & F), Mn + TNFα/IFNγ + ethanol vehicle control (C & G), and Mn + TNFα/IFNγ + 20 nM E2 (D & H). Quantitative analysis reveals a significant increase in caspase activity and Annexin V with Mn + TNFα/IFNγ and vehicle control, which was prevented by pre-treatment with E2 (I & J). Overexpression of mutant IκBα (S32/36A) in astrocytes, a dominant-negative "super-repressor" of NF-κB, significantly decreased caspase activity and Annexin V in co-cultured neurons (K & L). Differences between groups were determined using one-way ANOVA followed by the Tukey-Kramer multiple comparison *post hoc* test, significance is denoted by differing letters ($p < 0.05$, ± SEM). Images were acquired using a 40X Plan Apochromatic objective. Scale bar = 20 μm.

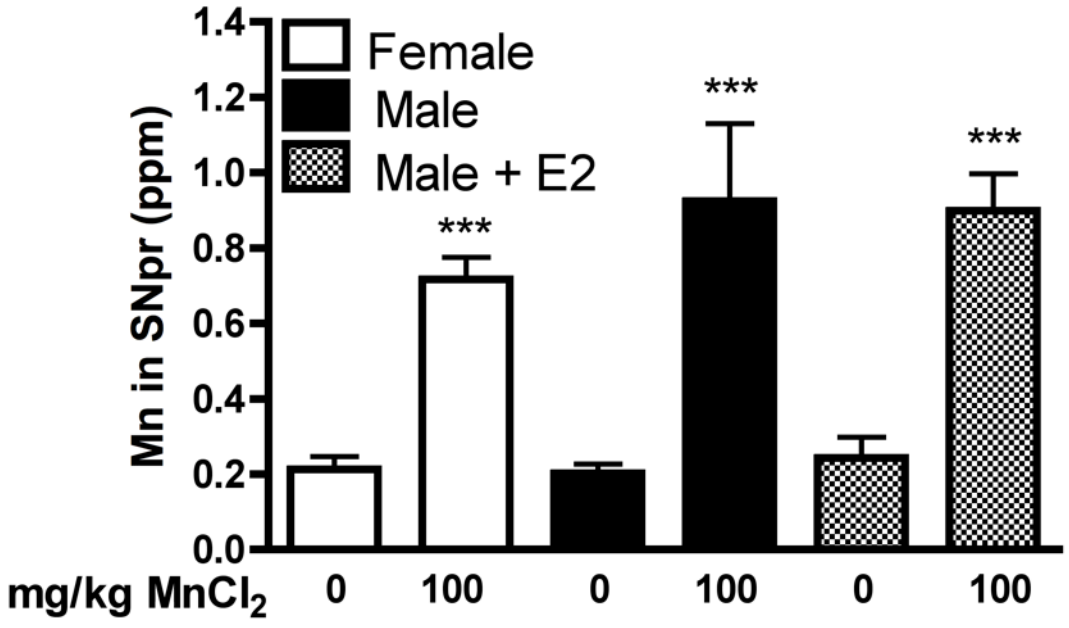


Figure 10. **Mn accumulation in the substantia nigra following oral exposure from day 21 – day 34 postnatal.** Juvenile mice were treated from day 21-34 with saline or 100 mg/Kg Mn via intragastric gavage. To control for possible differences in Mn uptake between treatment groups, the SN region of the basal ganglia was analyzed by ICP-MS as previously described (Moreno et al., 2009b). There was a significant increase in brain Mn levels in Mn-treated mice compared to saline-treated mice, demonstrating there was no difference in uptake with gender or E2 supplementation. For statistical analysis two-way ANOVA was used with Bonferroni's *post hoc* test (*** $p<0.001$, ± SEM).

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# CHAPTER 3

# GENE DELETION OF NOS2 PROTECTS AGAINST MANGANESE-INDUCED NEUROLOGICAL DYSFUNCTION IN JUVENILE MICE

The mechanisms underlying cognitive and neurobehavioral abnormalities associated with childhood exposure to manganese (Mn) are not well understood but may be influenced by neuroinflammatory activation of microglia and astrocytes that results in nitrosative stress due to expression of inducible Nitric Oxide Synthase (iNOS/NOS2). We therefore postulated that gene deletion of NOS2 would protect against the neurotoxic effects of Mn *in vivo* and *in vitro*. Juvenile NOS2 knockout ($NOS2^{-/-}$) mice were orally exposed to 50 mg/kg of $MnCl_2$ by intragastric gavage from day 21 - day 34 postnatal. Results indicate that $NOS2^{-/-}$ mice were protected against neurobehavioral alternations, despite histopathological activation of astrocytes and microglia in Mn-treated mice in both genotypes. $NOS2^{-/-}$ mice had decreased formation of 3-nitrotyrosine protein adducts within neurons in the basal ganglia that correlated with protection against Mn-induced neurobehavioral defects. Primary striatal astrocytes from wildtype mice caused apoptosis in co-cultured striatal neurons following treatment with $MnCl_2$ and tumor necrosis factor-α (TNFα), whereas $NOS2^{-/-}$ astrocytes failed to cause any increase in markers of apoptosis in striatal neurons. Additionally, scavenging nitric oxide (NO) with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) prevented the ability of Mn- and cytokine-treated wildtype astrocytes to cause apoptosis in co-cultured striatal neurons. These data demonstrate that NO plays a crucial role in Mn-induced neurological dysfunction in juvenile mice and that NOS2 expression in activated glia is an important mediator of neuroinflammatory injury during Mn exposure.

**Keywords:** astrocyte, microglia, 3-nitrotyrosine, inducible nitric oxide synthase, manganese

## Introduction

Manganese (Mn) has hormetic effects in the central nervous system (CNS), where it is required as a co-factor for numerous homeostatic and trophic functions but also causes neurotoxicity at higher doses (Aschner et al., 2009). Excessive environmental or dietary exposure leads to neuroinflammation in the CNS, particularly in the striatial-pallidum and substantia nigra *pars reticulata*, resulting in loss of striatal dopamine and motor features resembling, but distinct from, idiopathic Parkinson's disease (Newland et al., 1989, Calne et al., 1994, Perl and Olanow, 2007). Patients suffering from manganese neurotoxicity often experience neuropsychological effects such as cognitive deficiencies, irritability, and anxiety, which have been extensively documented (Woolf et al., 2002, Bowler et al., 2006, Wasserman et al., 2006, Bouchard et al., 2008). Epidemiological studies also indicate adverse neurological affects such as hyperactivity, learning, and cognitive disabilities in children consuming water with levels of Mn exceeding 300 μg/L (Zhang et al., 1995, Wasserman et al., 2006, Bouchard et al., 2007, Bouchard et al., 2011). Animal studies describing neurological and behavioral effects of early developmental Mn exposure suggest that there is a sensitive developmental period during which damage to the dopaminergic system can lead to lasting impacts into adulthood (Dorman et al., 2000, Moreno et al., 2009b, Kern et al., 2010). Studies of Mn exposure in rodents have generally focused on adult animals and there is subsequently much less information regarding the effects of Mn exposure during juvenile development.

Clinically, manganism is associated with robust astrogliosis in the basal ganglia (Olanow, 2004) and work conducted in our laboratory and others suggests that glial-derived inflammatory cytokines and nitric oxide (NO) influence the progression of

neuronal injury (Filipov et al., 2005, Liu et al., 2005, Liu et al., 2006, Moreno et al., 2009a, Moreno et al., 2009b, Verina et al., 2011). Increased expression of NOS2 by activated glial cells in response to Mn results in nitrosative stress throughout the basal ganglia (Moreno et al., 2009a) and enhanced apoptosis within selected populations of neurons in the globus pallidus and striatum (Liu et al., 2006). NOS2 is exclusively expressed in glia and produces large amounts of NO, which forms highly reactive peroxynitrite anion ($ONOO^-$) upon combining with superoxide ($O_2^{\cdot-}$), resulting in electrophilic nitration of cellular proteins that damages neurons (Ischiropoulos, 2003). Peroxynitrite-mediated nitrosative stress is implicated in a number of neurological disorders, including Alzheimer's disease (Smith et al., 1997), Parkinson's disease (Jenner, 2003), and manganese neurotoxicity (Moreno et al., 2009a).

NOS2 is predominantly regulated by the Rel-family transcription factor, nuclear factor kappa B (NF-κB) (Xie et al., 1993) and previous studies indicate that expression of NOS2 in response to Mn both *in vitro* and *in vivo* requires activation of NF-κB (Filipov et al., 2005, Moreno et al., 2008, Moreno et al., 2011). Recent studies from our laboratory exposed juvenile transgenic mice expressing a NF-κB-driven green fluorescent protein (EGFP) reporter construct to Mn and found significant increases in neuronal protein nitration that correlated with concurrent expression of NOS2 and the NF-κB-EGFP reporter in astrocytes that were inhibited *in vivo* by estrogen (Moreno et al., 2011). In studies using the dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), pharmacological or genetic inhibition of NOS2 expression in mice prevented MPTP-mediated neuronal injury (Liberatore et al., 1999, Du et al., 2001, Wu et al.,

2002). However, it is unknown whether ablation of NOS2 expression is sufficient to prevent Mn-induced neuroinflammation and neuronal injury in developing mice.

In this study, we used transgenic mice expressing a truncated NOS2 protein lacking the calmodulin binding domain (Laubach et al., 1995) to address the question of whether NOS2 is directly involved in the glial inflammatory response leading to neuronal nitration and behavioral dysfunction in juvenile mice orally exposed to Mn. We postulated that mice lacking NOS2 ($NOS2^{-/-}$) would be protected against the neurotoxic effects of Mn. Our results indicate that loss of NOS2 function attenuates peroxynitrite adduct formation in the striatal-pallidum and substantia nigra *pars reticulata* associated with alterations in neurobehavioral function and neurochemistry *in vivo*, as well as prevents astrocyte-mediated neuronal apoptosis *in vitro*. Collectively, these data implicate Mn-induced production of NO in the mechanism of neurotoxicity in developing mice.

## Methods

*Animal model*.

Three-week-old male wildtype (C57BL/6) and NOS2-deficient mice ($NOS2^{-/-}$; B6.129P2-$Nos2^{tm1Lau}$/J, bred to congenicity onto a C57Bl/6 background) were obtained from the Jackson Laboratory (Bar Harbor, Maine) and littermates were exposed to 0.9% saline (control) or 50 mg/kg $MnCl_2$ by daily intragastric gavage. Only male mice were used, given their increased sensitivity relative to females, as we previously reported (Moreno et al., 2009b). The dose of Mn was adjusted for the molar stoichiometry in the tetrahydrate form and administered at 50 mg/kg based upon daily weighing of each animal. The dosing regimen was based upon previous data from our group and others demonstrating that juvenile mice and rats exposed to similar doses (30 - 50 mg/kg, *per oral*) displayed behavioral alterations and increased expression of neuroinflammatory biomarkers (Moreno et al., 2009a, Kern and Smith, 2010). Mice (n= 7-10 animals per group) were treated for two weeks (day 20-34 postnatal) prior to termination for neurochemical and immunohistochemical analysis. Animals were kept on a 12-hour light/dark cycle and provided *ad libitum* with water and standard laboratory chow (2018 Harlan-Teklad Global 18% Protein Rodent Diet). This diet contains low levels of essential trace elements, including 118 μg/g Mn and 225 μg/g Fe, yielding a baseline consumption of approximately 0.2 - 0.3 mg Mn/day for juvenile mice (juvenile mice consume 2 - 3 g chow/day), in addition to the delivered dose of Mn, which was 0.3 - 0.75 mg Mn/day for mice ranging from 10 - 25 g during this period of juvenile development. All procedures were performed under an IACUC-approved protocol at Colorado State University under the care of veterinary staff at the Laboratory Animal Resources Facility.

*Neurobehavioral analysis*.

Mice were preconditioned one day prior to open field activity parameters using Versamax behavior chambers with an infrared beam grid detection array to assess animals movements in X, Y, and Z planes. Multiple behavioral parameters pertaining to basal ganglia function were collected and analyzed using VersaDat software (Accuscan Instruments, Inc.), including total distance traveled, number of movements, rearing activity, and margin time as previously studied in our laboratory (Liu et al., 2006). Activity measured juvenile exposure every other day for two weeks during the period of oral gavage. Anxiety behavior was determined using the elevated plus maze for mice according to (Walf and Frye, 2007) that was conducted prior to termination on day 34. Briefly, mice were individually assessed in a 30cm x 5cm x 15cm open/closed arm chamber for 5 minutes and recorded by video for data collection, analysis recorded observed time spent in open arms versus closed arms. Catecholamines and monoamines were determined by high performance liquid chromatography with electrochemical detection (Vanderbilt University, Center for Molecular Neuroscience core facility).

*Immunohistochemistry and immunofluorescence*.

Mice were anesthetized by inhalation of isofluorane and perfused intracardially as published previously by our laboratory (Moreno et al., 2009a). Briefly, brains were collected and fixed in 4% paraformaldehyde for 4-6 hours and stored in graded sucrose and 0.1M $NaKPO_4$ buffer at 4°C. Brains were embedded in OCT and cut in 10 μM coronal serial sections for examination for immunohistochemistry. For gliosis, primary

antibodies to GFAP (1:500; Sigma, St. Louis MO) and IBA1 (1:500; Wako, Osaka Japan) were used. The substantia nigra *pars reticulata* (SN) and striatum-pallidum (ST) regions were evaluated due to their known susceptibility to Mn neurotoxicity (Olanow, 2004, Moreno et al., 2009a, Verina et al., 2011). Sections were developed using horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine reagents from the Vectastain ABC Kit. Bright field images were collected from each region and scored blinded by a veterinary pathologist for glial morphology as previously described (Moreno et al., 2009a). For co-immunofluorescence studies, sections from the ST and SN were incubated with Anti-NeuN (1:500; Millipore, Bedford, MA) and 3-NT (1:100; Millipore) antibodies in combination to assess levels of protein nitration in neurons. Specific protein epitopes were visualized with secondary antibodies labeled with AlexaFluor- 488 or -647 (Molecular probes, Eugene OR) and slides were mounted in media containing 4',6-diamidino-2-phenylindole (DAPI) to identify cell nuclei. Serial sections were systematically imaged using common anatomical landmarks and fields were selected based upon unbiased acquisition of fields using DAPI. Fluorescence intensity was quantified by masking the sum intensity per field of 3-NT fluorescence in areas expressing NeuN. Images were acquired using a Zeiss 20x or 40x Air PlanApochromat objective with 6 microscopic fields examined per region from three serial sections, for a total of 18 microscopic fields that were averaged for each 4-6 animals per group.

*Astrocyte-neuron co-culture*.

Neurons were isolated from the striatum of 1 day-old C57BL/6 mice as previously described (Carbone et al., 2009) and were grown for 4-6 days on poly-D-lysine coated

glass coverslips to permit axonal development and phenotypic maturation. Neurons were maintained in neurobasal media supplemented with B27, L-Glutamine and 1% 50 units/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin (PSN). Primary striatal astrocytes were isolated from 1-3 day-old C57BL/6 and $NOS2^{-/-}$ mice as described by (Aschner and Kimelberg, 1991), modified according to our previous studies (Carbone et al., 2008, Moreno et al., 2008) 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), 1% PSN at 37˚C with 5% $CO_2$. After reaching confluence, astrocytes were subcultured onto permeable cell culture inserts (pore size= 0.4 μM) at $1\times10^4$/well approximately 7 days before treatments. Astrocytes were exposed for 24-hours prior to co-culture with saline or 30 μM $MnCl_2$ and 10 pg/ml TNFα, washed with phosphate buffered saline (PBS, pH 7.4), and then inserted over neurons in the presence or absence of 100 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) for 6 hours. Thereafter, inserts containing astrocytes were removed and neurons were assessed for multiple indices of apoptosis by live-cell fluorescence imaging. Caspase activity was determined using 100 μM rhodamine-110 bis-(L-aspartic acid amide) (488 nm excitation), phosphatidylserine appearance on the cell surface was determined with 50 nM annexin V-Alexafluor 647 conjugate, and nuclei were identified using the cell-permeable DNA stain, Hoescht 33342 (2 μM final concentration in imaging medium). Using a 20x Plan Apochromat air objective, 10-12 fields per treatment were blindly captured by differential interference contrast and analyzed by sum intensity for fluorescent signal intensity, which was normalized to the number of cells in each field. Data were acquired and analyzed using

SlideBook v 5.0 (Intelligent Imaging Innovations, Denver CO).

*Statistical Analysis*.

Comparison of two means was performed by Student's t-test. Ranking of glial pathology were analyzed by one-way ANOVA followed by Kruskal-Wallis *post hoc* test. *In vitro* studies comparing three or more means were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison *post hoc* test with Prism software (v4.0c, Graph pad Software, Inc., San Diego, CA). Results are expressed as the mean $\pm$ SEM from a minimum of 3 independent studies and for all experiments, $p < 0.05$ was considered significant.

## Results

### NOS2$^{-/-}$ mice are protected against Mn-induced behavioral disinhibition.

Neurobehavioral parameters were evaluated in order to determine if sub-chronic Mn exposure led to differential alterations in neuromotor function between wildtype and NOS2$^{-/-}$ mice. Our previous studies in juvenile C57BL/6 mice reported elevated novelty seeking and hyperactive behaviors following oral exposure to 30 mg/kg Mn from day 21 - day 34 postnatal (Moreno et al., 2009b). In the present studies, behavioral assessments in open-field activity chambers indicated that 50 mg/kg Mn in wildtype mice increased rearing movements after 14 days of exposure (Figure 1a; $p<0.006$). There was also a significant increase in the total number of movements ($p<0.007$) with Mn treatment compared to wildtype controls, additionally a trend to increased total distance traveled was observed ($p<0.06$) and no significant change in the time spent in the margin. However, there was no change in behavioral parameters in NOS2$^{-/-}$ mice between control and Mn-treated groups (Figure 1b). We assessed the anxiety-related behavior by the elevated plus maze, which demonstrated a decrease in time spent in the open arms of the chamber in Mn-treated wildtype mice (Figure 2a; $p<0.05$), whereas no change was observed in NOS2$^{-/-}$ mice (Figure 2b). Levels of multiple neurotransmitters were determined in the striatum in parallel with behavioral assessments, including dopamine, DOPAC, 3MT, HVA, serotonin, 5HIAA, noradrenaline, and adrenaline. However, there was no difference between any neurotransmitter or metabolite in wildtype or knockout mice (Table 1). The HVA/dopamine ratio was decreased in wildtype mice exposed to Mn ($p<0.05$) (Figure 3a) but no change was detected in the DOPAC/DA ratio (Figure 3c). There were no significant changes in the HVA/DA or DOPAC/DA ratios in knockout

mice (Figure 3b and d). Additionally, no change was observed the ratio of 3MT/DA in either genotype (data not shown).

**Mn-induced glial activation is not directly regulated by NOS2.**

Activation of astrocytes and microglia was determined using blinded pathological scoring by a veterinary pathologist based upon immunohistochemical staining for the astrocyte marker, glial fibrillary acidic protein (GFAP) (Figure 4), or the microglial marker, ionized calcium binding adaptor molecule (IBA1) (Figure 5), in the striatal-pallidum (ST) and substantia nigra *pars reticulata* (SN). In the ST of wildtype mice, Mn treatment caused an increase in the level of astrocyte activation, whereas in the SN of mice exposed to 50 mg/kg Mn astrocyte activation was decreased (Figure 4e). Interestingly, hypertrophy of cytoplasmic processes in astrocytes in SN was not observed in wildtype mice in response to Mn treatment and there was a diminished presence of reactive astrocytes in this brain region in Mn-treated wildtype mice compared to controls (Figure 4 a and b). Mn treatment in $NOS2^{-/-}$ mice did not cause a significant change in astrogliosis in the ST but did cause an increase in astroglial activation in the SN compared to $NOS2^{-/-}$ controls (Figure 4e). Astrocytic hyperplasia and hypertrophy was observed in the SN of $NOS2^{-/-}$ Mn-treated mice (Figure 4c and d).

Activation of microglia were determined by pathological scoring based on immunohistochemical staining for IBA1, due to the up-regulation of this protein during inflammatory activation of microglia and macrophages. Sections were ranked by pathological scores similarly to astrocyte analysis. In the ST of wildtype mice, there was no indication of microglial activation with Mn treatment at this time point (Figure 5e).

However, in mice exposed to 50 mg/kg Mn, there was a significant increase in the number of activated microglia in the SN (Figure 5 and b). The same trend was observed in the SN in $NOS2^{-/-}$ mice (Figure 5 c and d), whereas no change was detected in the ST following Mn treatment (Figure 3e).

**Mn-induced neuronal protein nitration is attenuated in $NOS2^{-/-}$ mice.**

Levels of neuronal 3-nitrotyrosine (3NT) protein adducts were assessed by immunofluorescence in wildtype and $NOS2^{-/-}$ mice to determine the extent of nitrosative stress resulting from Mn-induced production of NO/peroxynitrite (Figure 6). Representative low power images of 3NT immunofluorescence reveal extensive areas of peroxynitrite adduct formation throughout the striatum and cortex. Co-localization of 3NT adducts within neuronal soma was determined in the ST and SN by immunofluorescence (Figure 6 a-d). Sections were masked on NeuN (green, FITC) and the intensity of neuron-specific 3NT fluorescence (red, CY5) in co-localized images was determined in saline- and Mn-treated mice for each genotype. In wildtype mice, a significant increase in neuronal 3NT adducts was observed in both the ST and SN following Mn treatment (Figure 6e) that was prevented in $NOS2^{-/-}$ mice (Figure 6f).

**Astrocyte-derived NO mediates neuronal apoptosis.**

In previous studies, we demonstrated that astrocytes exposed to low levels of Mn and inflammatory cytokines induce apoptosis in co-cultured neurons (Liu et al., 2005, Moreno et al., 2011). We therefore exposed primary striatal astrocytes isolated from $NOS2^{-/-}$ mice to 30 μM $MnCl_2$ and 10 pg/mL TNFα (Mn + TNFα) to determine whether

loss of NOS2 expression in astrocytes would protect primary striatal neurons from apoptosis (Figure 7). Wildtype astrocytes were treated with saline or Mn + TNFα and exposed to neurons for 6 hours. Caspase-3 activation and annexin V were used as indicators of apoptosis and determined by live-cell fluorescence imaging. Following treatment with Mn + TNFα, both caspase activity and annexin V staining (Figure 7e and f) were significantly increased in striatal neurons following co-culture with wildtype astrocytes. However, $NOS2^{-/-}$ astrocytes exposed to Mn + TNFα did not cause an increase in either caspase activity or annexin V labeling in co-cultured striatal neurons (Figure 7e and f).

To further determine the etiological role of astrocyte-derived NO in Mn-dependent apoptosis in striatal neurons, wildtype astrocytes were exposed to Mn + TNFα and then incubated with striatal neurons in the presence or absence of the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Figure 8). The presence of 100 μM PTIO completely attenuated caspase-3 and annexin V staining in striatal neurons following co-culture with Mn-treated wildtype astrocytes, whereas wildtype astrocytes exposed to Mn + TNFα in the presence of vehicle control (DMSO) significantly increased the number of apoptotic neurons (Figure 8c and d).

**Discussion**

Nitrosative stress is implicated in neuronal injury during Mn intoxication (Aschner et al., 2009) and aberrant expression of NOS2 by activated glia may be critical to this response by enhancing oxidative damage to neurons and promoting a cycle of neuroinflammation that ultimately leads to synaptic dysfunction, loss of neurons, and deprecations in neurological function (Liu et al., 2006, Moreno et al., 2009a, Verina et al., 2011). The present studies support this model, because neurobehavioral changes observed in juvenile C57Bl/6 wildtype mice exposed to Mn were absent in $NOS2^{-/-}$ mice (Figure 1). These results are consistent with previous studies that identify juvenile development as a particularly vulnerable time to Mn-induced behavioral dysfunction, associated with increased spontaneous movement, hyperactivity and behavioral disinhibition (Brenneman et al., 1999, Moreno et al., 2009b, Kern et al., 2010). Likewise, clinical studies associate childhood exposure to Mn with hyperactivity and behavioral disinhibition (Bouchard et al., 2007, Ericson et al., 2007). The elevated plus maze assay revealed that Mn-treated wildtype mice spent less time in the open arms of the chamber compared to $NOS2^{-/-}$ mice (Figure 2), indicating an increase in anxiety-related behavior. This observation was not reported in other studies that exposed pre-weanling rats to a similar dose of Mn (Kern et al., 2010), although this may be a species-specific observation reflective of a greater degree of dysfunction in Mn-treated mice. Protection against these behavioral alternations in $NOS2^{-/-}$ mice suggests that overproduction of NO by activated glial cells plays a direct role in the observed alterations in neurobehavioral function in Mn-treated mice.

Although there were changes in locomotor function and neurobehavioral activity

in Mn-treated mice, neurotransmitter levels were unaltered by exposure in either genotype (Table 1). However, there was a significant decrease in the HVA/dopamine ratio (Figure 3), suggesting either a decrease in DA catabolism or possibly an increase in DA release . We previously reported that mice orally exposed to 30 mg/kg Mn during this period of juvenile development had increased striatal dopamine levels (Moreno et al., 2009b), whereas adult mice orally exposed to 100 mg/kg Mn for 8 weeks displayed loss of striatal dopamine (Liu et al., 2006), suggesting a biphasic response where low-dose or short duration exposure to Mn causes an increase in synaptic release that causes increased dopamine turnover and neuronal injury over longer duration exposures. These earlier studies also reported that juvenile mice exposed to 30 mg/kg Mn had a decrease in the DOPAC/DA ratio in the striatum that was not observed in adult animals exposed to the same dose of Mn for 8 weeks (Moreno et al., 2009b), suggesting that dopamine catabolism is more vulnerable in juveniles than adults. Similarly, in the present studies we observed a decrease in the HVA/dopamine ratio in Mn-treated wildtype mice that was prevented in $NOS2^{-/-}$ mice, which could explain the locomotor differences between the two genotypes. Excess NO that occurs during neuroinflammation alters dopamine neurotransmission and regulation of locomotor activity in the striatum (West et al., 2002, Del Bel et al., 2005), possibly through inhibition of dopamine re-uptake (Kiss and Vizi, 2001, West et al., 2002). During conditions of oxidative stress, NO can also reduce apparent dopamine release, whereas under physiological conditions NO facilitates dopamine neurotransmission (Trabace and Kendrick, 2000). These data suggest that age and duration of exposure to Mn are important factors in modulating behavioral outcomes and neurotransmitter metabolism in the basal ganglia.

Gliosis was determined by immunohistopathological examination of GFAP and IBA1 protein expression in the basal ganglia. In wildtype mice, 50 mg/kg Mn caused a significant increase in activation of GFAP-positive astrocytes in the striatum and decreased astrocyte activation in the SN (Figure 4). We previously observed a similar trend toward a decrease in GFAP staining in the SN at 30 mg/kg Mn in juvenile mice (Moreno et al., 2009a), suggesting the basal level of proliferative activity of astrocytes is inhibited by Mn exposure during post-weaning juvenile development, a period during which a high level of astrocyte proliferation occurs (Rice and Barone, 2000). These findings indicate that the effects of juvenile exposure to Mn could result in lasting dysfunction in the development of the SN that could promote greater susceptibility to secondary neurotoxicant exposures during adulthood, as we reported in adult mice orally exposed to 30 mg/kg Mn for 8 weeks that had also been exposed to Mn as juveniles (Moreno et al., 2009a, Moreno et al., 2009b). Because astrocytic regulation of neuronal development and maturation is critical through postnatal day 55 (Riol et al., 1992), inhibition of astrocyte proliferation during juvenile development in Mn-treated mice could have lasting consequences on basal ganglia circuits regulated by dopamine. Excessive NO has cytostatic effects on neuronal growth and it is possible that proliferating astrocytes respond similarly with a reduction in growth due to Mn-induced NO (Peunova and Enikolopov, 1995). In $NOS2^{-/-}$ mice exposed to Mn there was a significant increase in GFAP activation in both the ST and SN, indicating astrocyte activation still occurred despite loss of function of NOS2. This suggest that glial-derived NO is critical to Mn-induced neurological dysfunction, because alterations in both neurobehavioral function and neurochemistry were prevented by gene deletion of NOS2

despite the increase in astrocyte activation.

The progression of Mn neurotoxicity has also been associated with activation of microglia and subsequent release of inflammatory cytokines and NO (Verina et al., 2011). Notably, we observed an increase in abundance of IBA1-positive microglia in the SN with Mn treatment in both wildtype and $NOS2^{-/-}$ mice (Figure 5). Microglia are particularly dense in this region (Lawson et al., 1990) and the observed activation of microglia following Mn exposure is consistent with earlier studies from our laboratory, which reported a similar increase in microglial activation in juvenile mice exposed to Mn at 30 mg/kg Mn (Moreno et al., 2009a). Collectively, these data indicate that gene deletion of NOS2 does not alter microglial activation in Mn-treated mice but rather differentially affects activation and proliferation of astrocytes in the basal ganglia. This finding supports a direct role for astrocytes in neuroinflammatory injury and nitrosative stress following Mn exposure.

Mn-induced activation of astrocytes and microglia results in increased levels of NOS2 and NO (Bae et al., 2006, Moreno et al., 2009a, Verina et al., 2011). However, glial activation in $NOS2^{-/-}$ mice exposed to Mn did not increase neuronal protein nitration (Figure 6), indicating that NOS2 is responsible for Mn-induced nitrosative stress and peroxynitrite formation, rather than NOS1 (nNOS) or NOS3 (eNOS). These results support that nitrosative stress in the basal ganglia is an important pathological feature of Mn neurotoxicity that influences neuronal injury and locomotor activity in exposed mice. The apparent increase in 3NT adducts observed in the cortex of Mn-treated mice was also prevented in $NOS2^{-/-}$ mice (Figure 6), suggesting that this model may be relevant to neuropathological changes that have been reported in the cortex of *Cynomolgus*

macaques chronically exposed to Mn (Guilarte et al., 2008b). Nitration of tyrosine residues inhibits the enzymatic activity of tyrosine hydroxylase (TH), which can alter dopaminergic neurotransmission in response to MPTP treatment (Ara et al., 1998). A similar mechanism may underlie the changes in neurobehavioral function observed in Mn-treated mice, because ablating NOS2 prevented formation of nitrotyrosine-labeled proteins in knockout mice and reversed the behavioral changes that occurred in Mn-treated wildtype animals.

Co-culture studies using primary striatal astrocytes and neurons indicated that wildtype astrocytes exposed to Mn and TNFα caused apoptosis in striatal neurons (Figure 7) that was prevented in the presence of $NOS2^{-/-}$ astrocytes, indicating that NO is required for astrocyte-mediated neuronal injury. These findings confirm our previous observations that pharmacologic inhibition of NOS2 in Mn- and cytokine-treated astrocytes prevented apoptosis in PC12 neurons (Liu et al., 2005). Glial-derived NO causes neuronal injury in part by inhibiting mitochondrial Complex IV, resulting in loss of ATP and increased production of superoxide (Bal-Price and Brown, 2001, Brown and Neher, 2010). The present data suggest a similar mechanism, because the NO scavenger, PTIO, completely prevented neuronal apoptosis following incubation with wildtype astrocytes stimulated with Mn and TNFα (Figure 8). These data are consistent with our previous findings that Mn promotes NOS2 expression in astrocytes in orally exposed mice associated with neuronal apoptosis in striatal and pallidal interneurons expressing enkephalin, ChAT, and NOS1 (Liu et al., 2006).

In summary, these studies demonstrate that juvenile mice lacking functional NOS2 are protected from Mn-induced locomotor dysfunction and glial-mediated

nitrosative stress, indicating that production of NO by activated glia contributes to neurotoxicity. Further studies are necessary to determine the temporal pattern of NOS2 expression in astrocytes and microglia, as well as the cellular signaling mechanisms leading to inducible expression of NOS2. It will also be important to understand how the activation of NOS2 during juvenile Mn exposure relates to susceptibility to secondary exposures and neurological disease during aging.

# CHAPTER 3

# FIGURES

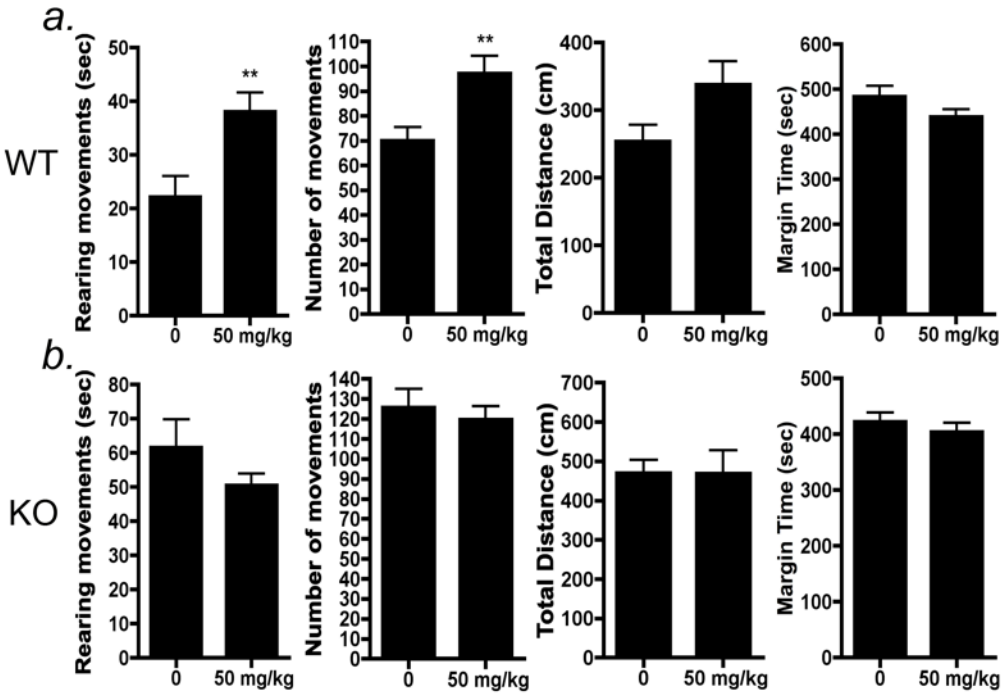


Figure 11. **NOS2$^{-/-}$ mice are protected against Mn-induced behavioral disinhibition.** (a) Treatment by oral gavage for 14 days with 50 mg/kg Mn significantly increased rearing movements in wildtype mice, an indicator of vertical movement. Number of movements increased significantly in Mn-treated wildtype mice in addition to a trend to increase total distance travels. There was no change seen in margin time. (b) There was no indication of affect by treatment on NOS2$^{-/-}$ mice in vertical movement or other tested perimeters. Significance *$p$< 0.05.

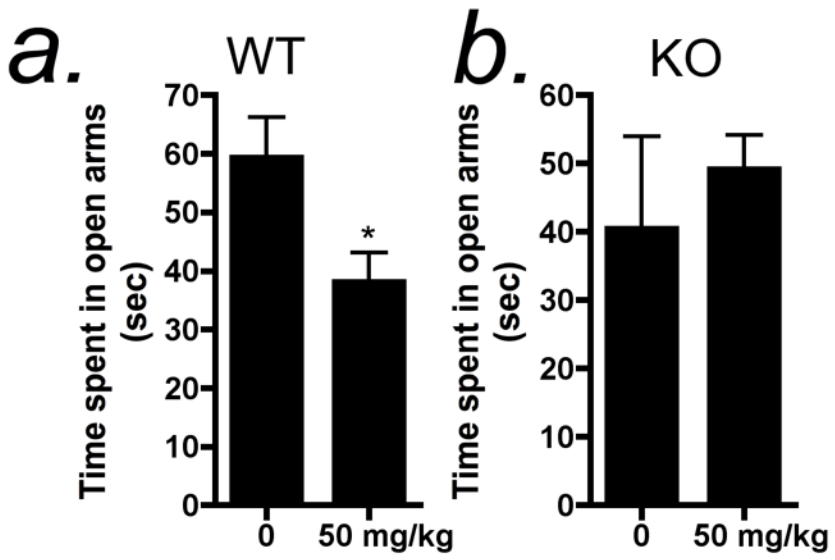


Figure 12. **NOS2$^{-/-}$ mice were protected from increased anxiety-related behavior with Mn exposure.** (a) In the elevated plus maze Mn treated wild animals spent significantly less time in the open arms compared to controls. (b) NOS2$^{-/-}$ had no measurable difference between control and treated. Significance *$p$< 0.05.

Table 6. **Striatal catecholamine and monoamine levels (ng/mg) in wildtype and NOS2$^{-/-}$ mice.**

| | Wild type | | NOS2$^{-/-}$ | |
|---|---|---|---|---|
| Treatment | 0 | 50 mg/kg | 0 | 50 mg/kg |
| Dopamine | 98.09±11.8 | 97.24±5.9 | 113.96±7.9 | 92.87±12.5 |
| DOPAC | 8.36±0.75 | 7.97±0.64 | 9.15±1.06 | 8.48±1.49 |
| 3MT | 3.74±0.86 | 3.31±0.22 | 3.43±0.18 | 2.94±0.45 |
| HVA | 10.63±1.21 | 7.88±0.70 | 9.27±0.74 | 8.08±0.28 |
| 5HT | 8.89±1.4 | 7.46±0.78 | 7.80±0.57 | 8.21±0.28 |
| 5HIAA | 3.30±0.61 | 2.51±0.24 | 3.33±0.14 | 2.68±0.14 |
| NE | 3.31±0.68 | 2.95±0.72 | 2.9±0.94 | 3.55±0.23 |
| E | 2.57±0.08 | 2.52±0.20 | 2.85±0.27 | 3.12±0.29 |

Concentrations expressed as ng of neurotransmitter/mg of protein
Data represented are mean ± SEM ($n$ = 3-4 animals per group).

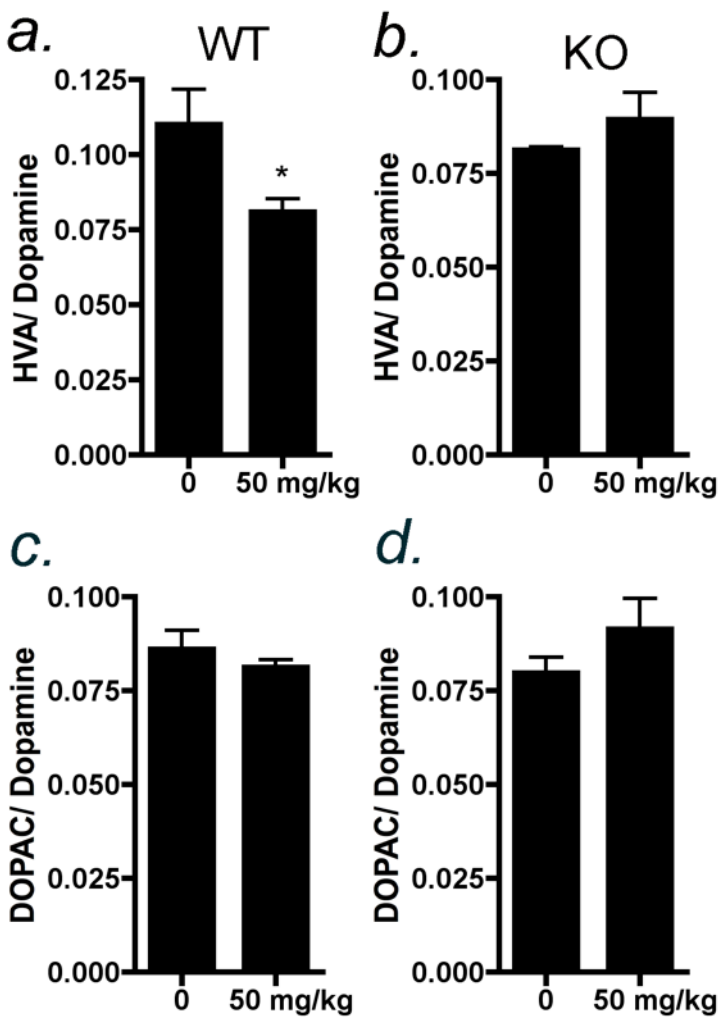


Figure 13. **Changes in striatal HVA/dopamine ratios are prevented in NOS2$^{-/-}$ mice.** Wildtype mice (a) treated with Mn had a significantly decreased ratio of HVA/dopamine compared to saline treated controls. NOS2$^{-/-}$ (b) had no change in the HVA/dopamine ratio. DOPAC/dopamine ratios also indicated no measurable change in wildtype or NOS2$^{-/-}$ mice (c-d). *$p$< 0.05.

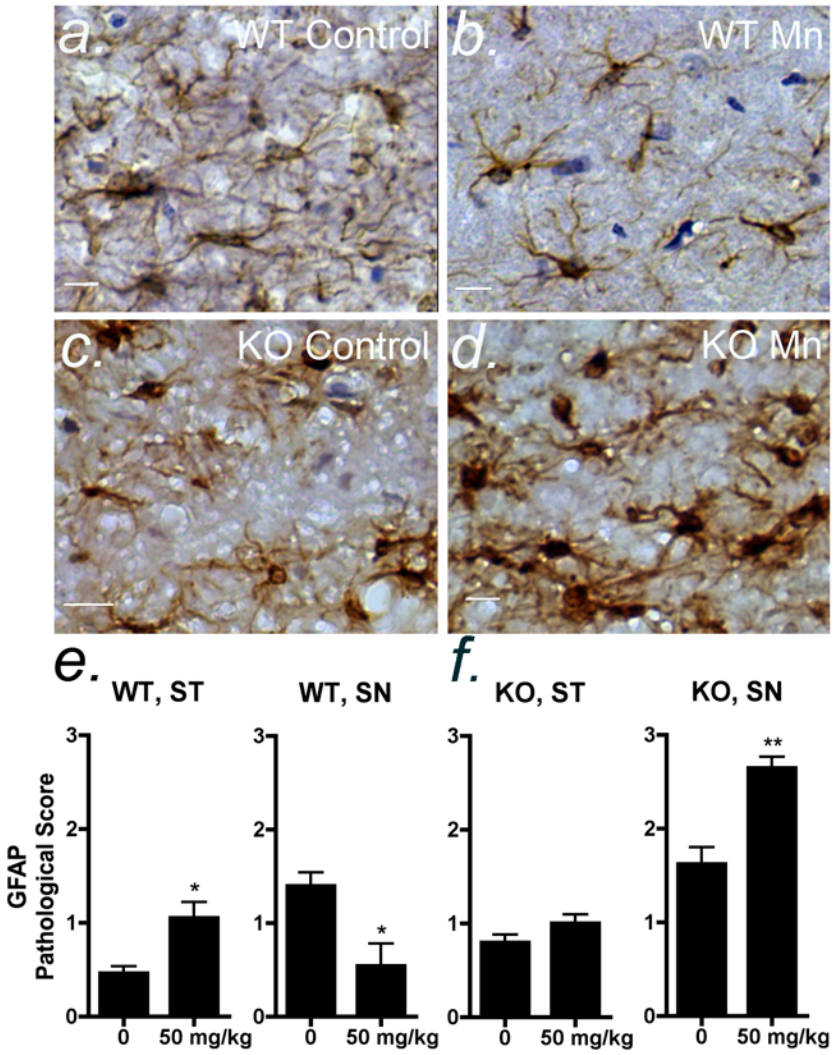


Figure 14. **Mn-induced astrocyte activation in the SN is differentially regulated between wildtype and NOS2$^{-/-}$ mice.** Mice were exposed to 50 mg/kg Mn by oral gavage for 14 days, and tissue assessed by immunohistochemistry for GFAP intensity. Representative images of the SN are depicted for control and Mn treated mice for wildtype (a-b) and NOS2$^{-/-}$ (c-d) groups. Analysis of pathological scoring data in the SN and ST of wildtype (e) and NOS2$^{-/-}$ (f) mice was performed by one-way ANOVA with Krusal-wallis post test. Scale bar = 10 μm. *$p$< 0.05, **$p$< 0.01.

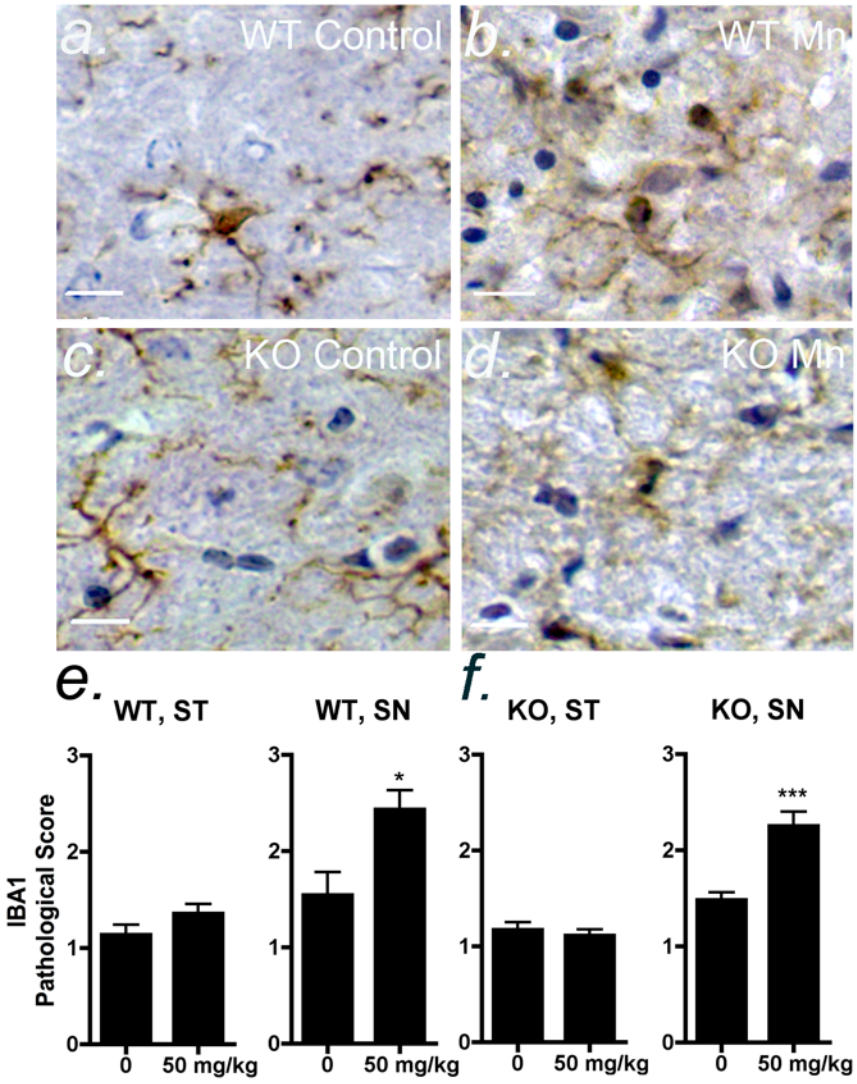


Figure 15. **Mn-induced microglial activation is not directly regulated by NOS2.** Mice were exposed to 50 mg/kg Mn by oral gavage for 14 days, and tissue assessed by immunohistochemistry for IBA1 staining. Representative images of the SN are depicted for control and Mn treated mice as (a-b) wildtype and (c-d) $NOS2^{-/-}$ mice. Qualitative analysis in the SN and ST of (e) wildtype and (f) $NOS2^{-/-}$ mice are represented by a one-way ANOVA with Krusal-wallis post test. Scale bar = 10 μm. Significance *$p< 0.05$, ***$p< 0.001$.

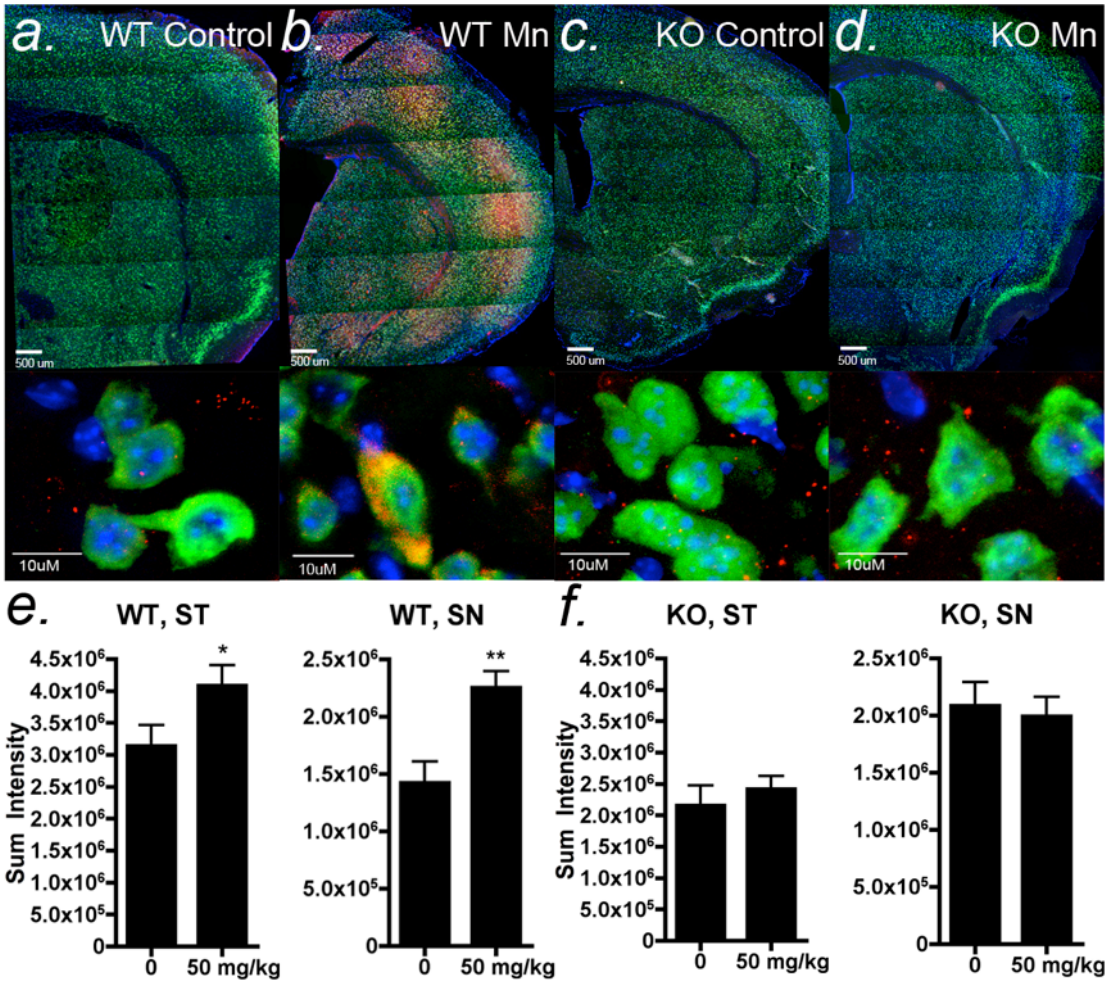


Figure 16. **Mn-induced neuronal protein nitration is attenuated in NOS2$^{-/-}$ mice.**

Increases of NO in the presence of superoxide, leads to peroxynitrite oxidation of proteins, which is detected by immunofluorescence. Serial sections were masked on a general neuronal antibody, NeuN (green, FITC) while a accounting for peroxynitrite adducts, 3NT (red, CY5) only present within FITC and counter stained with 4',6-diamidino-2-phenylindole, DAPI (blue). Representative images of 3NT modified proteins indicate co-localization of neuronal soma that are depicted for control and Mn treated mice as (a-b) wildtype and (c-d) NOS2$^{-/-}$ mice. Quantitation is representated in the (e) wildtype and (f) NOS2$^{-/-}$ mice by student-t test. Scale bar = 10

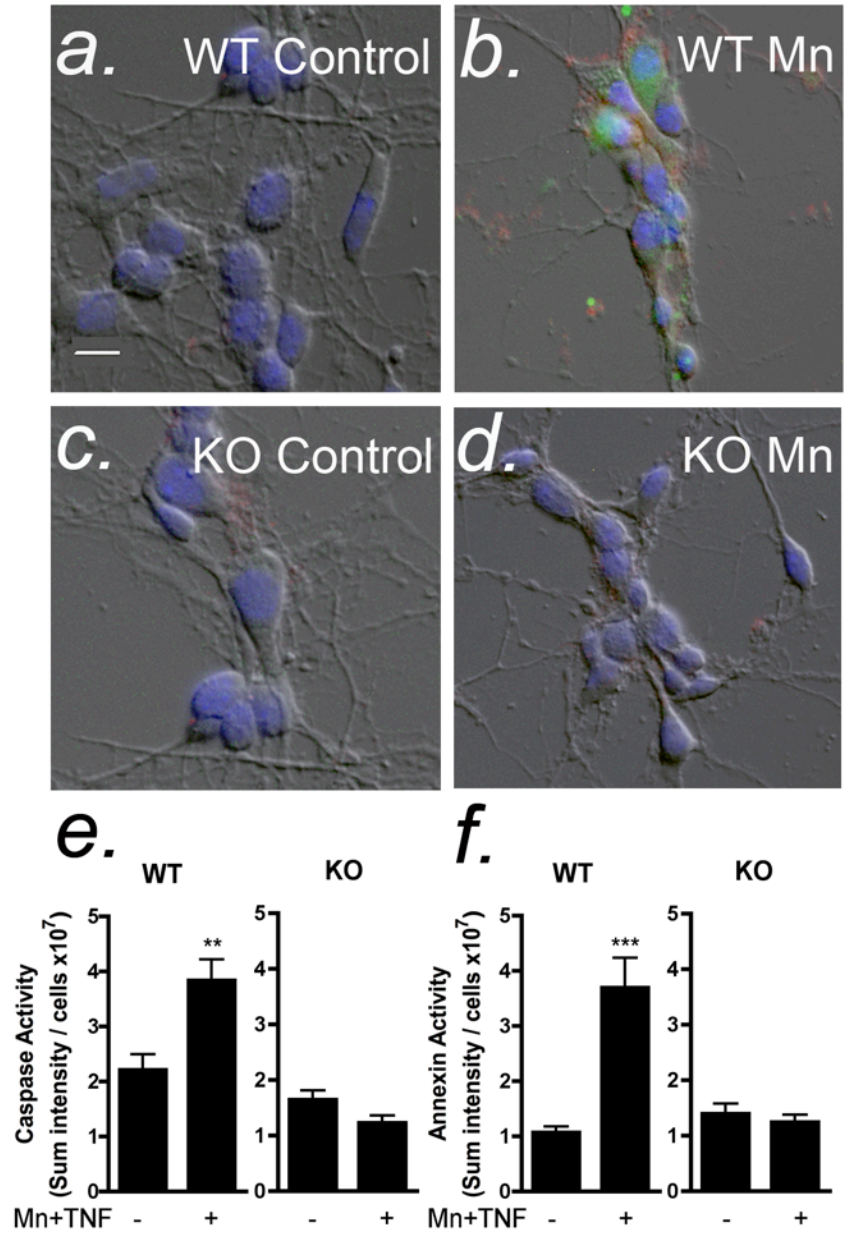


Figure 17. **Astrocyte-derived NO mediates neuronal apoptosis.** Apoptosis was determined in primary striatal neurons by live-cell fluorescence imaging of phosphatidylserine (annexin V, red) and caspase-3 (green) after 6 hours (h) of co-culture with astrocytes that had treated for 24 h with 30 μM with Mn and 10 pg/ml TNFα. Representative images are depicted of neurons co-cultured with control and Mn-treated astrocytes cultured from wildtype (a-b) and $NOS2^{-/-}$ (c-d) mice. Mn-treated wildtype astrocytes (e) co-cultured with neurons caused an increase in caspase activity and annexin V staining. Mn-treated $NOS2^{-/-}$ astrocytes (f) had no change in levels of caspase activity or annexin V staining. Scale bar = 10 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

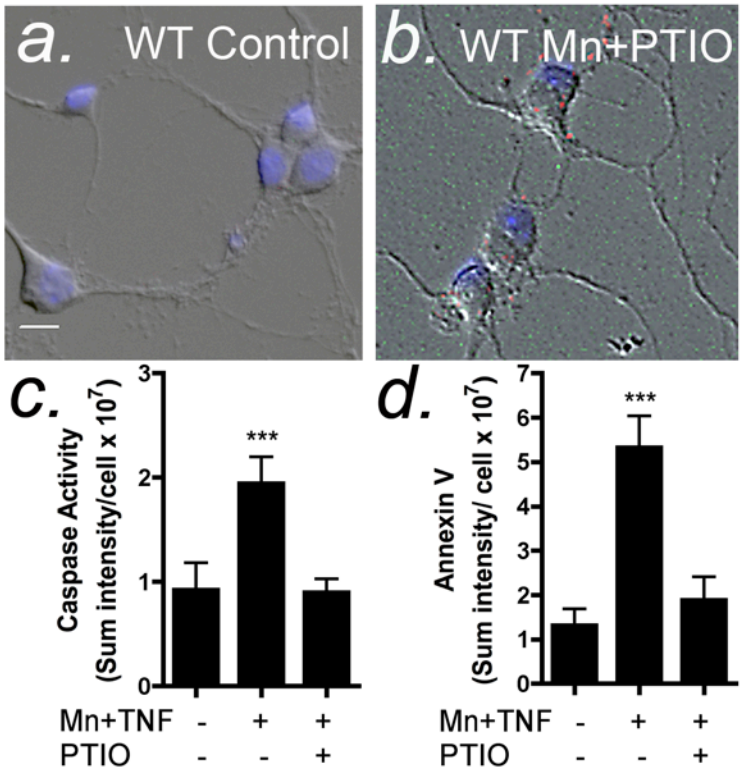


Figure 18. **Chemically scavenging NO prevents apoptosis in striatal neurons co-cultured with wildtype astrocytes exposed to Mn and TNFα.** Striatal astrocytes cultured from wildtype mice were exposed to 30 μM with Mn and 10 pg/ml TNFα for 24 h and incubated with striatal neurons in the presence or absence of the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). Representative images are shown from neurons co-cultured with control (a) and Mn-treated (b) wildtype astrocytes in the presence of PTIO, which prevented both caspase activation (c) and annexin V staining (d). Scale bar = 10 μm. ***$p < 0.001$.

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# CHAPTER 4

# ATP-DEPENDENT CALCIUM SIGNALING IN STRIATAL ASTROCYTES IS ACUTELY SENSITIVE TO INHIBITION BY STRUCTURALLY DIVERSE CATIONIC NEUROTOXICANTS

The basal ganglia are group of midbrain nuclei important for control of motor function that are highly sensitive to damage from oxidative stress, inflammation, and environmental neurotoxicants. Here we propose that inhibition of transmitter-evoked calcium signaling in astrocytes may contribute to this sensitivity because this pathway modulates diverse trophic functions in the CNS, including metabolism, synaptic activity, and regional cerebral blood flow (rCBF). To examine mechanisms underlying alterations in $Ca^{2+}$ signaling in astrocytes, we postulated that several structurally diverse neurotoxicants of the basal midbrain, all of which are cationic, would inhibit transmitter-induced calcium ($Ca^{2+}$) signaling in cultured astrocytes: $MPP^{+}$, the active metabolite of the model parkinsonian neurotoxicant, 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP); Paraquat (PQ); 6-Hydroxydopamine (6-OHDA); and Manganese ($Mn^{2+}$). Using $Ca^{2+}$ imaging in primary cultured striatal astrocytes, we investigated the effect of acute treatment with each neurotoxicant on agonist-induced intracellular Ca2+ transients. We observed a dose-dependent decrease in ATP-induced intracellular Ca2+ transients and mechanically stimulated $Ca^{2+}$ waves in cultured astrocytes following acute application of MPP+, 6-OHDA, PQ and $Mn^{2+}$. These compounds also acutely inhibited OAG-induced intracellular Ca2+ transients, suggesting that a receptor-operated cation channel such as the transient receptor potential (TRP) channel might be targeted. The TRPC3 channel antagonist, Pyr3, blocked OAG-induced intracellular Ca2+ transients similarly to MPP+, PQ, and Mn2+, but not 6-OHDA. Moreover, acute application of MPP+ also inhibited TRPC3-like currents, as determined by whole cell patch clamp experiments. These findings indicate that endogenous and exogenous chemicals that are structurally diverse but that have cationic properties inhibit ATP-induced $Ca^{2+}$ signaling in astrocytes and

may therefore share a common mechanism of neurotoxicity in their capacity to disrupt trophic functions reliant on this signaling phenomenon.

Keywords: Astrocytes, calcium signaling, purinergic receptors, transient receptor potential channels

## Introduction

The basal midbrain is vulnerable to neuronal injury from oxidative stress, neuroinflammation, and environmental compounds. A common mechanism of many such stressors in neurons is dysregulation of calcium signaling but this phenomenon is less well understood in glial cells. Cellular calcium signals in the CNS control a wide range of physiological processes from neuronal exocytosis of neurotransmitters and $Ca^{2+}$ - dependent long-term potentiation (Malenka et al., 1988, Heidelberger et al., 1994), to astrocyte intercellular communication and regulation of regional cerebral blood flow (Cornell-Bell et al., 1990, Pascual et al., 2005). Astrocytes are non-excitatory cells that communicate through intercellular $Ca^{2+}$ propagations, which regulate the neuronal microenvironment in part by maintaining the metabolic and trophic support for neurons. Dysfunction in astrocytic $Ca^{2+}$ signaling could therefore adversely impact neuronal function and survival.

Neurodegenerative diseases are associated with inflammatory activation of microglia and astrocytes that contributes to neuronal injury. 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine /1-methyl-4-phenylpyridinium (MPTP/MPP+) and the endogenously formed 6-hydroxydopamine (6-OHDA) are toxin-induced neurodegenerative models of the basal ganglia both indicating pathological states of the astrocyte (O'Callaghan et al., 1990, Rodrigues et al., 2001). Furthermore, the herbicide paraquat and excessive manganese exposure, are basal ganglia neurotoxicants that result in astrocyte and neuronal dysfunction. The chronic mechanisms of these neurotoxicants are associated with astrogliosis and neuronal oxidative stress (Chen and Liao, 2002, Drechsel and Patel, 2008), however acute mechanisms by which essential $Ca^{2+}$ transients in astrocytes are

susceptible to perturbation by these exogenous and endogenous neurotoxic agents are unclear.

Adenosine Triphosphate (ATP) is the primary extracellular gliotransmitter that activates purinergic receptors (P2) on astrocytes (Guthrie et al., 1999). P2 receptors are divided into two subfamilies; ionotropic (P2X) receptors and metabotropic (P2Y) receptors P2Y receptors are activated by low physiological ATP concentrations (<100 μM), whereas P2X receptors are activated by high concentrations of ATP that are released during ischemia and oxidative stress (James and Butt, 2002). Neuronal transmission can activate glutamate and P2 receptors expressed by astrocytes, increasing intracellular $Ca^{2+}$ levels that stimulate ATP exocytosis, producing a $Ca^{2+}$ wave that propagates outward through networks of astrocytes through subsequent paracrine release of ATP (Pascual et al., 2005, Perea and Araque, 2005, Koehler et al., 2006). P2Y receptors are G-protein-coupled receptors that initiate phospholipase-C (PLC)-dependent cleavage of phosphatidylinositol 4,5-bisphosphate, resulting in inositol 1,4,5-triphosphate ($IP_3$)-induced release of $Ca^{2+}$ stores from the endoplasmic reticulum. Formation of $IP_3$ results in an initial transient from stored $Ca^{2+}$ but also generates the second messenger, diacylglycerol (DAG) which stimulates a more prolonged and sustained extracellular $Ca^{2+}$ influx through an ion channel, resulting in a biphasic calcium response. Receptor operated channels have been linked with $Ca^{2+}$ influx in astrocytes (Grimaldi et al., 2003), but what is unclear is how they are affected under neurotoxic conditions.

Transient receptor potential (TRP) channels are a family of non-selective cation channels that are widely expressed in diverse cell types, including astrocytes (Grimaldi et al., 2003). DAG is a known agonist for TRP channels, specifically TRPC3, C6 and C7

(Hofmann et al., 1999, Okada et al., 1999). Previous research in smooth muscle cells has indicated that TRPC3 channels (Grimaldi et al., 2003) are associated with ATP-dependent $Ca^{2+}$ signaling through activation of P2Y receptors instigating the PLC-$IP_3$-DAG pathway (Montell et al., 2002, Reading et al., 2005). However, the activation and role in intracellular calcium influx of this non-selective cation channel in astrocytes in unknown. Furthermore, TRPC3 is found in high abundance in the basal ganglia of the mouse (Kunert-Keil et al., 2006) and in the human (Riccio et al., 2002), possibly associated with the selective vulnerability to neurotoxicants in these regions. Finally, previous research has indicated that cationic molecules can modulate $Ca^{2+}$ signaling and that extracellular $Ca^{2+}$ channels play a critical role in the observed $Ca^{2+}$ alterations in glia (Wildman et al., 2003, Ko et al., 2004, Tjalkens et al., 2006, Lee et al., 2011).

Therefore we propose a novel mechanism in astrocytes in which $Ca^{2+}$ transients are acutely affected by exogenous and endogenous neurotoxic molecules. To examine mechanisms by which structurally diverse neurotoxicants of the basal midbrain could modulate ATP-induced $Ca^{2+}$ signaling in astrocytes, we acutely exposed cultured striatal astrocytes to each compound and determined the effect on ATP- or OAG-induced $Ca^{2+}$ transients using real-time fluorescence imaging, as well as the capacity to inhibit TRPC3 currents in HEK cells over-expressing the channel. The compounds were: MPP+, the active metabolite of the model parkinsonian neurotoxicant, 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP); the herbicide paraquat (PQ); 6-hydroxydopamine (6-OHDA); and manganese ($Mn^{2+}$). The results of these studies indicated that acute application of each compound prior to stimulation with ATP or OAG decreased the amplitude of evoked transients, in addition to diminishing TRPC3-dependent

electrophysiological currents induced by OAG. These data suggest that TRPC3 is a receptor-operated channel in astrocytes linked to activation of metabotropic purinergic receptors and that several structurally diverse cationic neurotoxicants decrease $Ca^{2+}$ transients in astrocytes by inhibiting this channel, implicating TRPC3 channels are a common target that may be relevant to the mechanism of neurotoxicity.

**Materials and Methods**

All reagents were ordered form Sigma Aldrich (St. Louis MO) unless otherwise stated.

*Cell culture.*

Primary striatal astrocytes were isolated from 1 - 3 day old C57BL/6J mice, as previously described (Aschner and Kimelberg, 1991, Moreno et al., 2008). Striatal hemispheres were rapidly dissected, extracted and maintained in Minimal Essential Media (MEM) supplemented with Earle's Salts and L-glutamine (Hyclone), with 10% Fetal Bovine Serum and 1% penicillin-streptomycin-neomycin (Invitrogen). Cells were grown to confluence at 37˚C, 5% $CO_2$ in a humid atmosphere for approximately three weeks. Cells were subcultured onto 4-well poly-d-lysine-coated cover glass chambered slides and allowed to grow to semi-confluence. In our laboratory, cultures consistently yield >98% astrocytes as determined by immunofluorescence staining for glial fibrillary acidic protein (Tjalkens et al., 2006). All procedures involving animals were conducted under a protocol approved by the Animal Care and Use Committee at Colorado State University.

*Calcium Imaging.*

Astrocytes were grown to approximately 75% confluence on four-well chamber slides and incubated with 2 μM of Fluo-4, AM (Invitrogen) for 15 minutes at 37˚C prior to imaging. Cells were imaged in media (MEM, without phenol red or sodium bicarbonate) supplemented with 10mM HEPES (pH 7.4) at 25 ˚C. Groups of approximately 15-30 contiguous cells per field of view were identified for imaging. Cells were stimulated with 1 μM ATP to selectively activate metabotropic purinergic receptors, rather than

ionotropic receptors (see supplemental Figure 1; (James and Butt, 2002)) or with 1-oleoyl-2-acetyl-sn-glycerol (OAG; 100 μM), a TRPC channel agonist. Toxicants ($MPP^+$, 6-OHDA, PQ, or $Mn^{2+}$) were added 30 seconds prior to each agonist. Images of Fluo-4 fluorescence were collected every 500 milliseconds for 120 seconds with camera binning set at 4x4 pixels and an exposure time of approximately 20 milliseconds. Images were collected on a Zeiss Axiovert 200M microscope equipped with a Hammatsu ORCA-ER cooled charge-coupled device camera. Fluorescent intensity was expressed relative to the baseline image ($F/F_0$), where $F_0$ is the fluorescence level prior to stimulation, and analyzed using Slidebook software (v4.1 and 5.0; Intelligent Imaging Innovations, Inc., Denver, CO) .

*Mechanical stimulation.*

Astrocytes for wave propagation studies were seeded on poly-d-lysine coated 30 mm round coverslips and placed in an incubation chamber (Zeiss). After collection of baseline Fluo-4 AM (Invitrogen) intracellular $Ca^{2+}$ intensity for 10 seconds wave studies were mechanically-induced with a 5 μm diameter glass micropipet using a micromanipulator. Each toxicant, either $MPP^+$, 6-OHDA, PQ, or $Mn^{2+}$ was added approximately 30 seconds prior to stimulation. Images for wave studies were acquired every 500 milliseconds for 60 seconds. Fluorescent intensity was expressed relative to the baseline image ($F/F_0$) 75 nm from the site of initiation.

*Inhibitors U73122, Pyr3, $Ca^{2+}$ free media.*

Astrocytic $Ca^{2+}$ transients were determined by real-time fluorescence imaging in the presence of the phospholipase C (PLC) inhibitor, U73122 (10 μM), or its inactive analog, U73433 (10 μM). After a 10 minute incubation period with the inhibitor or analog, the cells were stimulated with 1 μM ATP and imaged as described above. Pyrazole 3 (Pyr3), a selective extracellular inhibitor of TRPC3 (1-10 μM), was incubated with astrocytes for 30 minutes prior to imaging. Following incubation with Pyr3, cells were stimulated with ATP or OAG agonists as described. For experiments without extracellular $Ca^{2+}$, $Ca^{2+}$-free imaging medium supplemented with 2 mM EGTA (pH 7.4) was used in replacement of imaging medium.

*Electrophysiology*

HEK 293 cells were cultured in Dulbecco's 1× high glucose modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.5% penicillin-streptomycin (Gibco). Cells were incubated at 37°C with 6% $CO_2$, media was changed every 2–3 days, and cells were subcultured when confluent using 0.25% trypsin-EDTA (Gibco). Currents were recorded using an AxoPatch 200B amplifier equipped with an Axon CV 203BU headstage (Molecular Devices). Recording electrodes (1–3 MΩ) were pulled, polished, and coated with wax to reduce capacitance. GΩ seals were obtained in Mg-PSS. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. Clampex and Clampfit versions 10.2 (Molecular Devices) were used for data acquisition and analysis, respectively. All patch-clamp experiments were performed at room temperature (22–25 °C). The extracellular bath solution for TRPC3 contained (mM) 140 NaCl, 5 CsCl, 0.1 $MgCl_2$, 0.05 $CaCl_2$, 10 Glucose, 10 HEPES (pH 7.4).

Pipette solution contained (mM) 130 CsOH, 110 aspartic acid, 15 CsCl, 1 $MgCl_2$, 3.6 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.4). The calculated reversal potentials for monovalent and divalent cations are 0 mV and -43.2 mV, respectively. Reversal potentials for monovalent anions are 1.6 mV for the TRPC3 solution. HEK cells were transfected by electroporation with a plasmid that encodes TRPC3 channels tagged with yellow fluorescent protein (TRPC3-YFP). Transfected cells were plated on 12-mm glass coverslips and allowed to adhere for approximately 2–3 h at 37 °C, 6% $CO_2$. Transfected cells expressing TRPC3-YFP were identified using fluorescent microscopy, initially voltage clamped at 0 mV, and voltage ramps from −100 mV to +100 mV were applied every 4 seconds. After baseline currents were recorded, the membrane permeable diacylglycerol (DAG) analogue, OAG (10 μM), was administered to mediated TRPC3 currents. When the evoked current reached a steady state, MPP+ (100 μM) was administered to examine the effect of the compound on TRPC3 currents.

*Expression of P2X/Y receptors and TRP channels*

Total RNA from cultured astrocytes were prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase digestion. RNA was quantified using a nanodrop technology, with the yield being no less then 75 ng/μL. Whole brain total RNA were prepared with Qiagen RNeasy Midi Kit and used for positive control. cDNA was synthesized using reverse transcriptase (BioRad) and reverse transcriptase-PCR was conducted using an Eppendorf Mastercycler. PCR products were visualized with a 1.5 % agarose gel with ethidium bromide staining on a BioRad Gel Doc imager. Primers indicated in supplemental Table 1.

*Statistical analysis*

Comparisons three or more means with one independent variable was performed using one-way analysis of variance (ANOVA) followed by the tukey's multiple *post hoc* test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). Comparison of two-group comparisons were analyzed using the Student's t-test. Results are expressed as the mean $\pm$ SEM from a minimum of 3 independent studies and for all experiments, $p<0.05$ was considered significant.

**Results**

*Acute application of cationic toxicants significantly decrease ATP-induced $Ca^{2+}$ transients.*

Astrocytes communicate through increases in intracellular $Ca^{2+}$ that leads to exocytosis of the gliotransmitter, ATP, further propagating intracellular $Ca^{2+}$ through paracrine signaling (Guthrie et al., 1999, Pascual et al., 2005). P2 receptor mRNA expression is present in cortical and striatal astrocytes (Supplemental Figure 2; (Franke et al., 2001, Franke et al., 2004)). In order to assess if endogenous and exogenous molecules interfere with astrocytic signaling we acutely applied the neurotoxicants and subsequently activated purinergic receptors with 1 μM ATP, visualizing the $Ca^{2+}$ transients with real-time fluorescence microscopy. Application of increasing concentrations of the endogenous neurotoxin 6-OHDA exhibited a dose-dependent decrease in intracellular response and representative traces indicated the loss of sustained $Ca^{2+}$ after purinergic receptor activation. DOPAC, the non-cationic metabolite of dopamine was assessed indicating no change in $Ca^{2+}$ transients with 10 μM application (Figure 1a). Similarly, addition of MPP+ indicated a significant concentration dependent decrease in $Ca^{2+}$ transients in striatal astrocytes (Figure 1b). Additionally, $Mn^{2+}$ and paraquat had a decrease in intracellular $Ca^{2+}$ transients in astrocytes with of increasing concentrations of the toxicants (Figure 1c and d).

*Acute application of toxicants alters $Ca^{2+}$ waves in striatal astrocytes.*

Astrocytic $Ca^{2+}$ waves propagate through activation of purinergic receptors and paracrine signaling of extracellular ATP (Guthrie et al., 1999). Mechanical stimulation

of astrocytes initiates the rise in intracellular $Ca^{2+}$ that propagates to adjacent glial cells through release of gliotransmitters, creating a $Ca^{2+}$ wave (Newman, 2001). We acutely applied the neurotoxicants and evoked a single astrocytic soma to illicit $Ca^{2+}$ transient to propagate the wave with real-time fluorescence microscopy. In order to induce a control wave, 100 μL of imaging media was applied 30 seconds prior to mechanical activation (Figure 2a). Prior to mechanical stimulation, 10-100 μM 6-OHDA was added to the imaging media. Increasing doses of 6-OHDA diminished the intracellular $Ca^{2+}$ levels 75 μm from site of activation compared to control waves (Figure 2b). Application of MPP+ decreases intracellular $Ca^{2+}$ in cells adjacent to the site of mechanical stimulation (Figure 2c). However, PQ and $Mn^{2+}$ application did not significantly alter $Ca^{2+}$ wave propagation in a dose-dependent manner (Figure 2d and e). The rate and area of the wave propagation were assessed and there was no change in either parameters (data not shown). Kymographs depicting a similar wave line intensities with each of the neurotoxicants indicate no change in the rate of propagation depicted by distance versus time.

*ATP-induced intracellular $Ca^{2+}$ transients in astrocytes require PLC and extracellular $Ca^{2+}$.*

To examine the mechanism of ATP-induced intracellular $Ca^{2+}$ transient inhibition by cationic neurotoxins in primary striatal astrocytes, we pharmacologically inhibited the ATP-induced PLC pathway with the purpose to determine the necessity for mobilization of intracellular $Ca^{2+}$ and the role that extracellular $Ca^{2+}$ plays in the signal transduction pathway. In order to prevent the production of $IP_3$ and DAG, an inhibitor of PLC, U73122 (10 μM), was administered resulting in a significant inhibition of $Ca^{2+}$ transients

in astrocytes. The inactive analog, U73343, did not alter $Ca^{2+}$ transients in comparison to controls (Figure 3a and b). Metabotropic pathways are known to produce a biphasic rise in intracellular $Ca^{2+}$ through $IP_3$ generation, concomitant to extracellular $Ca^{2+}$ influx through DAG production. To decipher the role of extracellular $Ca^{2+}$ in striatal astrocytes, $Ca^{2+}$ was removed from the media resulting in a significant decrease in peak amplitude (Figure 3c). Representative traces from these results further suggested that sustained levels of $Ca^{2+}$ require extracellular $Ca^{2+}$ sources due to the decrease in phase plateau (Figure 3d).

*OAG activates extracellular $Ca^{2+}$ influx in astrocytes independently of the phospholipase C - $IP_3$ pathway and from intracellular stores.*

OAG is a membrane permeable analog of secondary messenger, DAG, which has been specifically shown to activate TRPC subunits 3, 6 and 7 (Hofmann et al., 1999, Okada et al., 1999). However, since mRNA expression of only TRPC3 was present in astrocytes it can assumed that TRPC6 and 7 are not present (Supplemental Figure 3). Addition of OAG to primary astrocytes resulted in an increase in intracellular $Ca^{2+}$ (Figure 4a) indicating activation of TRPC channels. Moreover, in the presence of U73122, there was no change in OAG-induced $Ca^{2+}$ signals, which supports DAG-induced extracellular $Ca^{2+}$ influx after ATP-PLC activation (Figure 4a and b). OAG application in $Ca^{2+}$-free media had a significant decrease in the maximum amplitude in the influx of extracellular $Ca^{2+}$ (Figure 4c). Additionally the absence of extracellular $Ca^{2+}$ further inhibited $Ca^{2+}$ transients but independently from the depletion of $Ca^{2+}$ from intracellular stores by Thapsigargin (10 μM) and caffeine (5 mM; Figure 4d).

*Pyr3 significantly diminishes ATP and OAG-induced $Ca^{2+}$ transients in striatal astrocytes.*

Pyr3, a specific inhibitor of TRPC3 (Kiyonaka et al., 2009), was utilized in order to determine its role in extracellular $Ca^{2+}$ influx in response to stimulation with ATP and OAG in astrocytes. ATP-induced $Ca^{2+}$ influxes were diminished with the pre-incubation of 1 and 10 μM Pyr3 (Figure 5a and b) further suggesting the ATP-DAG-induced pathway leading to activation of TRPC3. Additionally, OAG application in the presence of cells pretreated with 10 μM Pyr3 similarly resulted in diminished agonist-induced $Ca^{2+}$ response and no change in $Ca^{2+}$ amplitude (Figure 5c and d).

*Acute application of cationic neurotoxins inhibits intracellular $Ca^{2+}$ transients induced by TRPC3 agonist.*

To address the question as whether cationic neurotoxicants altered TRPC3 signaling in astrocytes, OAG was applied in the presence of the cationic neurotoxicants. OAG-induced $Ca^{2+}$ transients were significantly attenuated by MPP+, $Mn^{2+}$ and PQ (Figure 6a). However, 6-OHDA did not elicit a complete diminishment of $Ca^{2+}$ transients from OAG-activated controls (Figure 6a).

*Electrophysiology*

To study the effects of MPP+ on TRPC3 currents, recombinant TRPC3 channels were expressed in HEK cells. Transfected cells were patch clamped in the conventional whole cell configuration. Currents in unstimulated transfected cells were small, (Figure 7a) however, large TRPC3-dependent currents were evoked by administration of the

membrane permeable 10 μM OAG (Figure 7a and b). Furthermore, these current were attenuated with the application of Pyr3 (data not shown). TRPC3-like currents were inhibited by administration of 100 μM MPP+ to the bathing solution (Figure 7a and b), indicating that TRPC3 $Ca^{2+}$ influx are blocked by the compound.

**Discussion**

$Ca^{2+}$ signaling in astrocytes is essential for regulation of metabolic and trophic support of in the CNS. Research in neurotoxin-induced models of the basal ganglia have focused on chronic exposures leading to astrogliosis and concomitant neuronal death. Acute affects of cationic neurotoxins upon astrocytes and physiological $Ca^{2+}$ signaling have not been observed. To examine alterations by which structurally diverse neurotoxicants of the basal midbrain modulate ATP-induced $Ca^{2+}$ signaling in astrocytes, we acutely exposed cultured striatal astrocytes to each compound and determined the effect on ATP- or OAG-induced $Ca^{2+}$ transients, in addition to the capacity to inhibit TRPC3 currents in HEK cells over-expressing the channel. Together these results indicate that acute treatment with these cationic neurotoxins, alter intracellular $Ca^{2+}$ signals in astrocytes via extracellular cation channels, specifically TRPC3.

ATP is the primary gliotransmitter in astrocytes (Guthrie et al., 1999) and low concentrations of ATP (<100 μM) selectively activate P2Y receptors (Supplemental data Figure 1 and 2; (James and Butt, 2002)). To assess changes in intracellular $Ca^{2+}$, Fluo-4 AM dye was used to visualize real-time fluorescence of $Ca^{2+}$ transients in primary striatal astrocytes. Results indicate that the endogenous and exogenous neurotoxins, 6-OHDA and MPP+, attenuate astrocytic intracellular $Ca^{2+}$ amplitudes in a dose-dependent manner (Figure 1a and b). Mn and PQ-induced $Ca^{2+}$ amplitudes are also suppressed in astrocytes upon the acute exposure (Figure 1c and d). Because P2Y receptors have been shown to be coupled to receptor-operated $Ca^{2+}$ channels (Patterson et al., 2005; Reading et al., 2005), we postulated that the observed inhibitory effect from these toxicants could involve direct inhibition of extracellular $Ca^{2+}$ channels.

Physiologically relevant astrocyte-to-astrocyte $Ca^{2+}$ propagation can be recapitulated in primary astrocytic cultures using a micropipet to mechanically stimulate a single cell eliciting a rise in intracellular $Ca^{2+}$ spreading a wave of $Ca^{2+}$(Guthrie et al., 1999, Newman, 2001, Peters et al., 2005). Acute exposure of confluent astrocytes to 6-OHDA at increasing concentrations of 10 – 100 μM, resulted in an attenuation of intracellular $Ca^{2+}$ wave amplitudes 75 μm from the site of initiation (Figure 2b). Similarly, MPP+ decreased the intensity of intracellular $Ca^{2+}$ (Figure 2c) although the area and rate of propagation were not decreased with either toxicant. Mn and PQ inhibit $Ca^{2+}$ wave intensity but not in a dose-dependent manner as observed with 6-OHDA and MPP+ (Figure 2d and e). Notably, acute treatment with MPP+ and 6-OHDA inhibited ATP-induced intracellular $Ca^{2+}$ transients (Figure 1) at lower doses than intercellular $Ca^{2+}$ waves (Figure 2), we attributed this dosage difference to the relative simplicity of the single-cell imaging approach using direct application of ATP. During mechanical stimulation and $Ca^{2+}$ wave propagation, astrocytes are known to release transmitters such as glutamate, in addition to ATP, that can augment wave activity (Pascual et al., 2005). This is likely why increasing the concentration of MPP+ was required for inhibition of $Ca^{2+}$ waves, nevertheless the presence of these cationic neurotoxicants modulated the $Ca^{2+}$ wave propagation in astrocytes.

Metabotropic receptor activation and the requirement for extracellular $Ca^{2+}$ were confirmed in striatal astrocytes (Figure 3), in accordance to previously published studies(Grimaldi et al., 2003), implicating the presence of a plasma membrane channel facilitating entry of extracellular $Ca^{2+}$. Similarly, propagation of mechanically-induced $Ca^{2+}$ waves were dramatically reduced in $Ca^{2+}$-free medium (data not shown),

demonstrating the functional importance of extracellular $Ca^{2+}$ for cell-to-cell signaling in cerebrum-derived astrocytes. OAG stimulation increased intracellular $Ca^{2+}$ under multiple experimental manipulations such as in the presence of the PLC inhibitor, U73122 (Figure 4a and b) and upon depletion of $Ca^{2+}$ from intracellular stores, however OAG-induced increases were not observed with the absence of extracellular $Ca^{2+}$ (Figure 4c and d). Collectively, these studies indicate the role of $Ca^{2+}$ influx through a plasma membrane cation channel directly activated by OAG and that activation of metabotropic P2 receptors is likely coupled to this signaling pathway. Due to mRNA expression data we selectively obtained the specific TRPC3 inhibitor, Pyr3, in order to directly suppress this channel among TRPC subfamily members (Kiyonaka et al., 2009), which were not present in primary astrocytes. Treatment of astrocytes with Pyr3, robustly attenuated ATP-induced intracellular $Ca^{2+}$ transients, indicating that ATP is coupled with TRPC3 in astrocytes (Figure 5a-c). Additionally, OAG-induced intracellular $Ca^{2+}$ transients were suppressed verifying the OAG-dependent TRPC activation (Figure 5d-f).

Finally, OAG-induced rises in intracellular $Ca^{2+}$ in the presence of MPP+, $Mn^{2+}$ and PQ were significant attenuated in astrocytes (Figure 6). However, 6-OHDA was only partially inhibited the $Ca^{2+}$ transients, possibly by interactions with another $Ca^{2+}$ channel or decrease in the extracellular toxicant concentration due to a transporter uptake in the astrocytes. Therefore, we tested the selective suppression of TRPC3 by over expressing recombinant TRPC3 in HEK cells. These data indicated that TRPC3-dependent $Ca^{2+}$ influx is inhibited by MPP+ (Figure 7). We used these higher doses to illicit inhibition due to the possibility that over expression of channels leads to alterations in constitutive

$Ca^{2+}$ signaling (Kiyonaka et al., 2009) and the possibility that the native TRPC channel is composed of heteromeric assemblies, altering signaling properties (Earley et al., 2007).

These data indicate that ATP-induced intracellular $Ca^{2+}$ transients in primary astrocytes are acutely inhibited in the presence of cationic neurotoxins, notably through interactions with TRPC3. This is a novel mechanism in which physiological signaling in astrocytes are altered by extracellular exogenous and endogenous. Further observations in $Ca^{2+}$ oscillations in astrocytes and sub-chronic impacts upon the astrocytic functions in terms of secondary signaling events would be relevant in the presence of these exogenous and endogenous neurotoxins. These observed alterations in $Ca^{2+}$ homeostasis have could have implications astrocytic function, therefore, modulation these cellular dysfunctions could progress neurological demise associated with lack of physiological signals and coupling communication.

# CHAPTER 4

# FIGURES

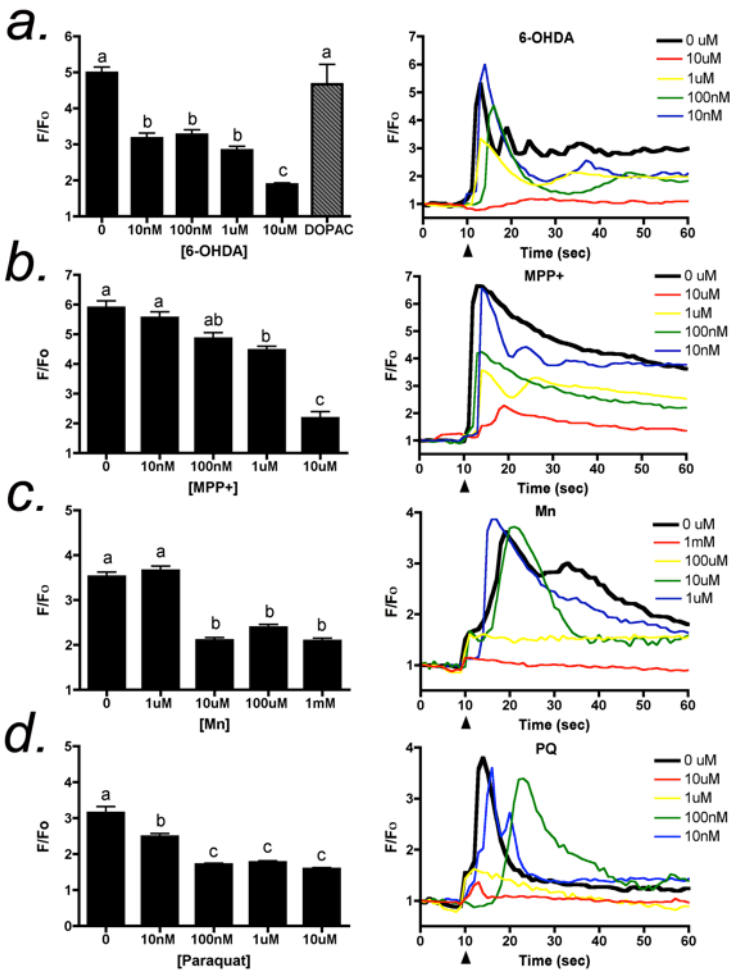


Figure 19. **Acute exposure to toxicants inhibits calcium transients in striatal astrocytes**. Quantitative analysis and representative calcium response traces. (a-d) Calcium transients were initiated by the addition of 1uM ATP (denoted by ) either without toxin, with a 30-second incubation of 10nM, 100nM, 1uM, or 10uM, or non-cationic control, DOPAC. Different lettering indicates statistical significance $p<0.05$ ANOVA.

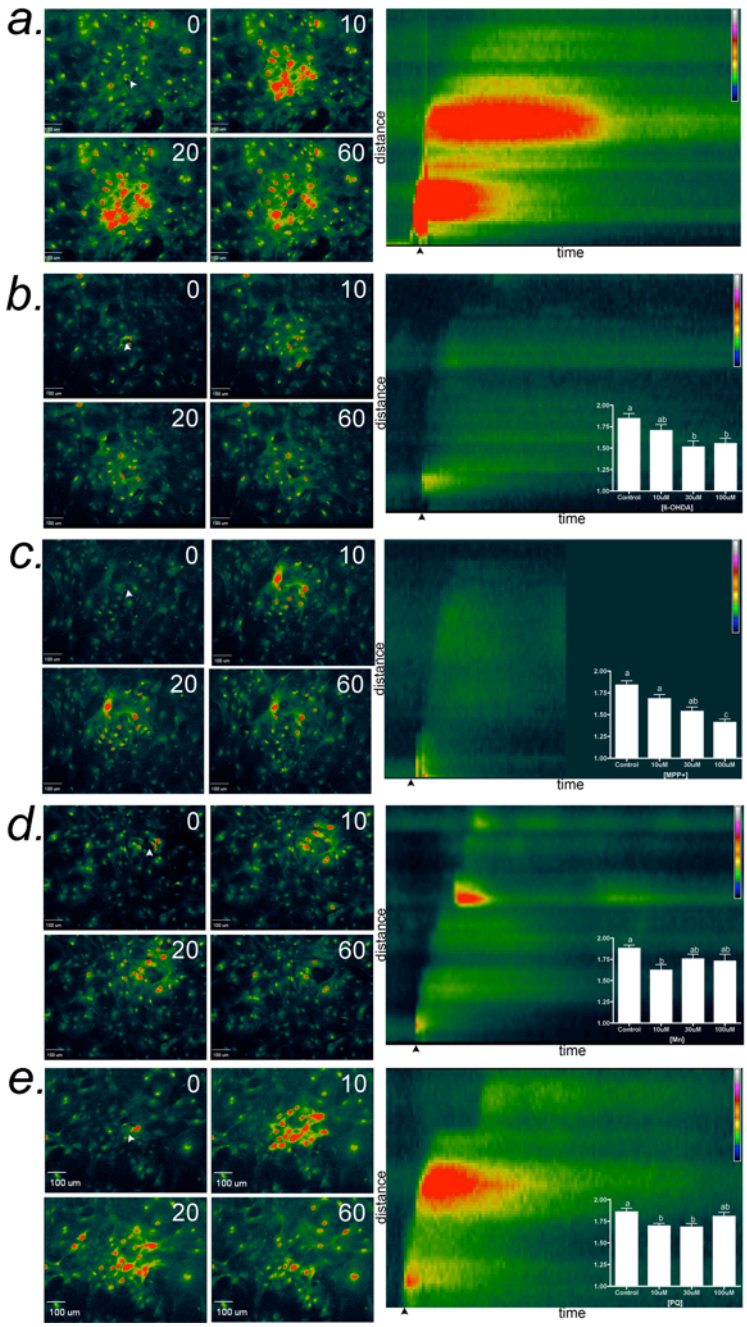


Figure 20. **Acute exposure to toxicants attenuates the propagation of $[Ca^{2+}]_i$ waves in primary astrocytes.** Calcium waves were initiated in primary striatal astrocytes by mechanical stimulation of single cell using a glass micropipet (arrows) and visualized by real time fluorescence microscopy. Images show a representative wave propagation and a kymograph of the wave front. (a) Control wave (b-e) toxicants Mechanical initiation denoted by

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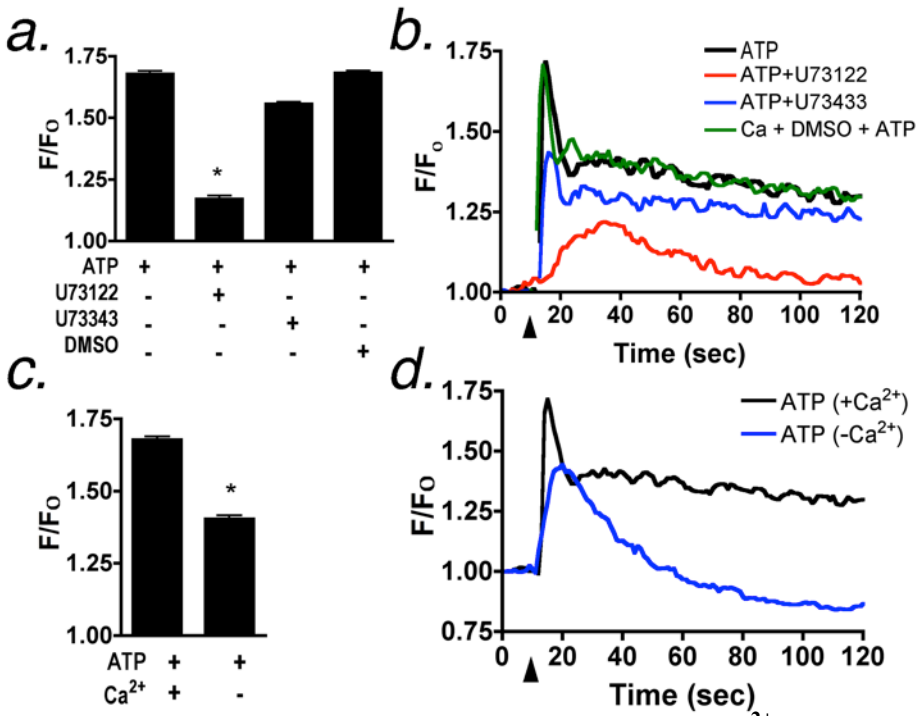


Figure 21. **Metabotropic receptors mediate ATP-induced $Ca^{2+}$ transients in cultured astrocytes. Quantification** of peak amplitudes from (a) indicates that U73122 markedly inhibited ATP-induced $Ca^{2+}$ transients. Removal of extracellular $Ca^{2+}$ inhibited both the peak amplitude and sustained phase of ATP-induced $Ca^{2+}$ transients (c). ATP application denoted by .

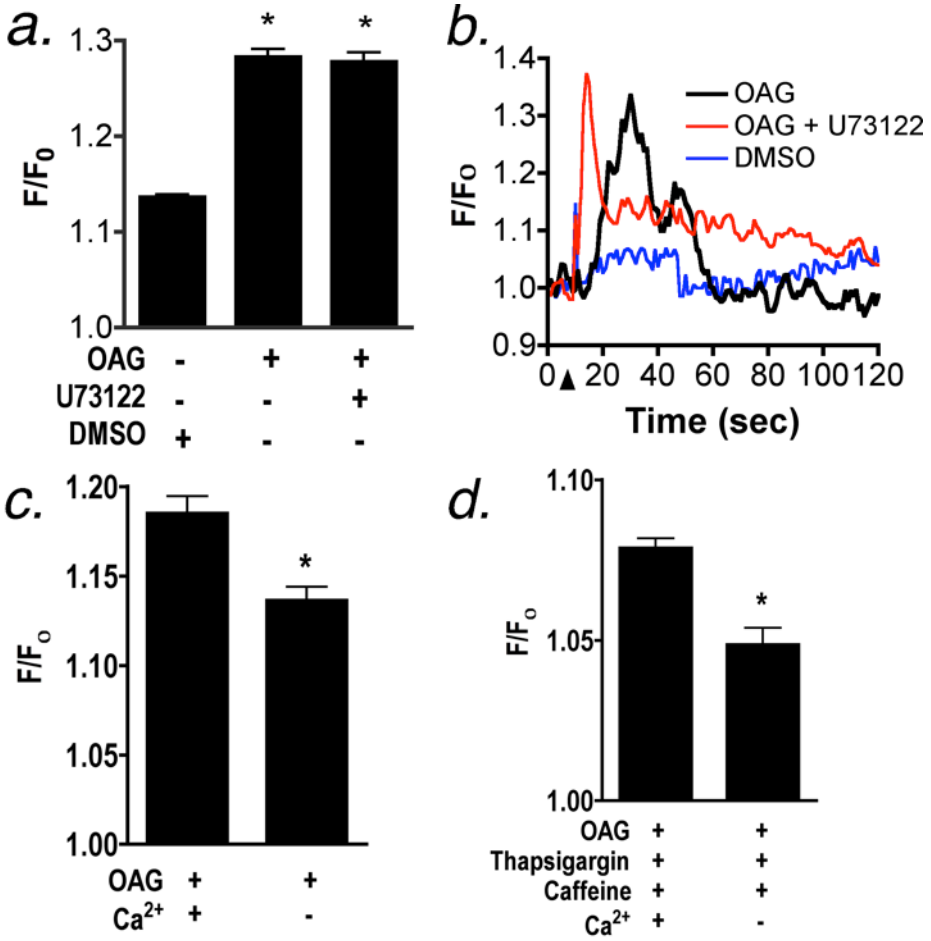


Figure 22. **OAG activates $Ca^{2+}$ transients in astrocytes independently of the phospholipase C - $IP_3$ pathway that require extracellular $Ca^{2+}$ but do not require $Ca^{2+}$ release from intracellular stores.** $Ca^{2+}$ transients were stimulated with 100uM OAG in the presence or absence of U73122 or DMSO vehicle control (a-b), in the presence or absence of extracellular $Ca^{2+}$ (c), intracellular $Ca^{2+}$ stores were depleted by additions of 10uM thapsigargin and 5mM caffeine and then OAG-induced $Ca^{2+}$ transients were determined in the presence or absence of extracellular $Ca^{2+}$ (d). ATP application denoted by .

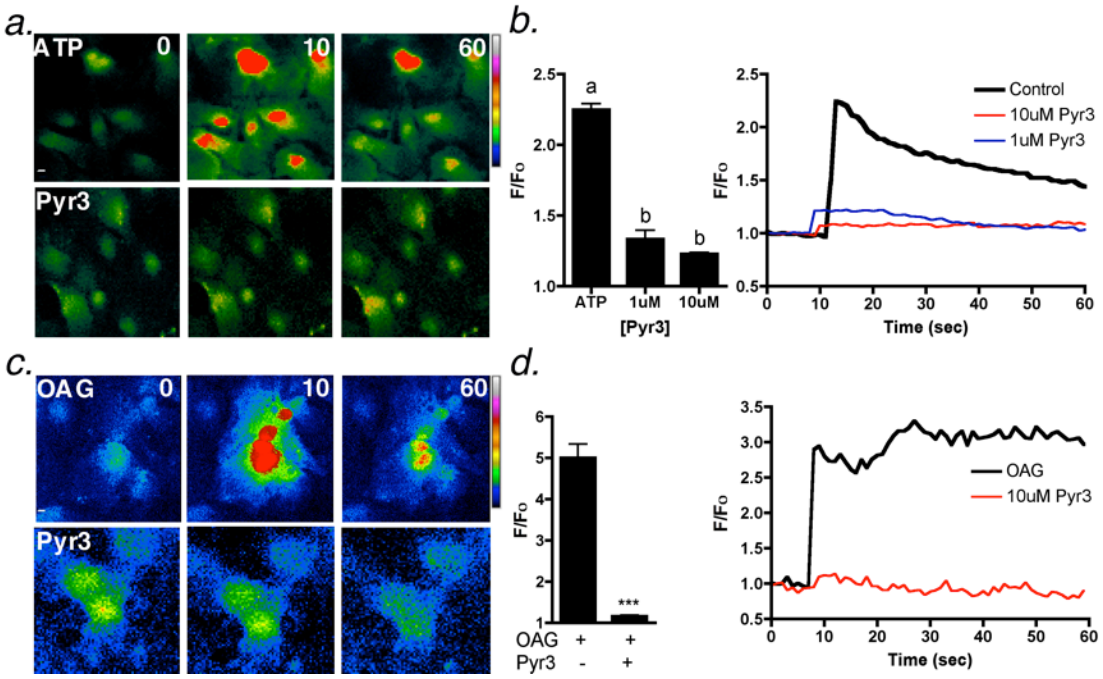


Figure 23. **$Ca^{2+}$ transients are inhibited with agonist activation of metabotropic and TRPC channels in the presence of TRPC3 antagonist, Pyr3.** Representative images (a-b, e-f), quantitative analysis (c, g), and calcium response traces (d, h) display the suppression of $Ca^{2+}$ signals with Pyr3. $Ca^{2+}$ transients were initiated by the addition of 1uM ATP or 100uM OAG either with or without cells pre-incubated with 10 uM Pyr3.

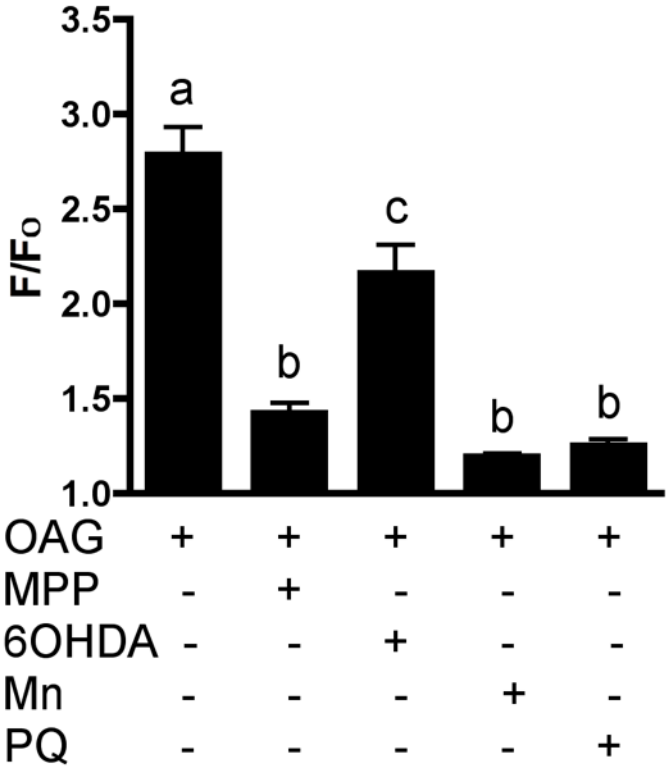


Figure 24. **OAG activation with acute exposure to toxicants inhibits calcium transients in striatal astrocytes.** Quantitative analysis shows that OAG activation of TRPC channels in astrocytes are attenuated by the presence of MPP+, $Mn^{2+}$ and PQ. Calcium transients were initiated by the addition of 100uM OAG either without toxin, whereas acute activation of intracellular calcium through the TRPC channels were modulated by these toxicants. 6-OHDA did not significantly suppress $Ca^{2+}$ influx through TRPC mediated entry, however could modulate an upstream 2$^{nd}$ messenger to $Ca^{2+}$ influx in astrocytes.

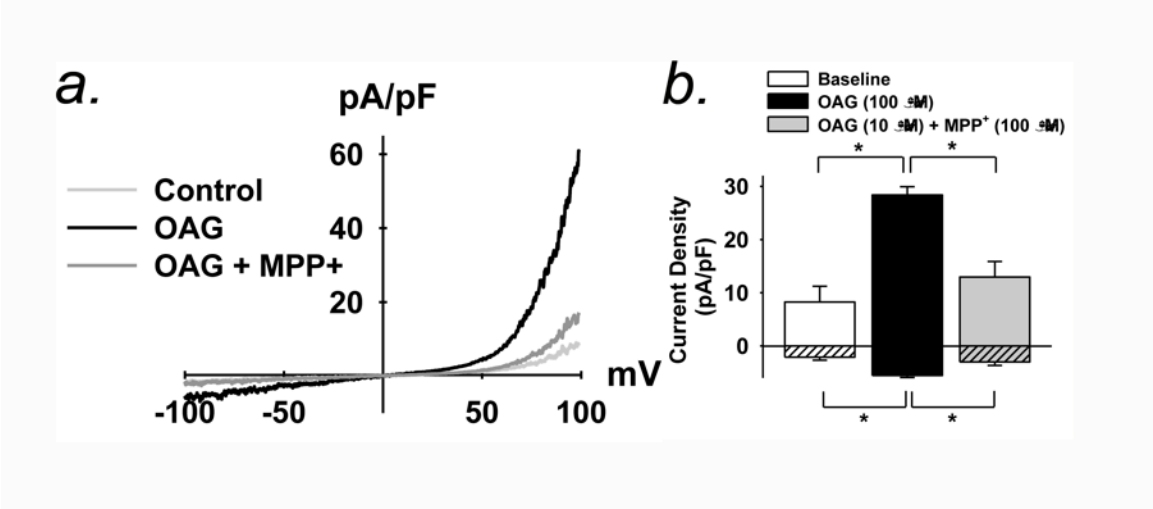


Figure 25. **OAG- induced TRPC3 currents are blocked by MPP+.** Normalized current (I) vs voltage (V) relationship of baseline and OAG-induced currents in human embryronic kidney (HEK) cells overexpressing TRPC3-YFP recorded in absence and presence of 1-methyl-4-phenylpyridinium (MPP+; 100 μM)(a). Summary data of average current density at +80 mV before and following 10 μM OAG stimulation and after administration of 100 μM MPP+ in the presence of ANG II (b). n = 4

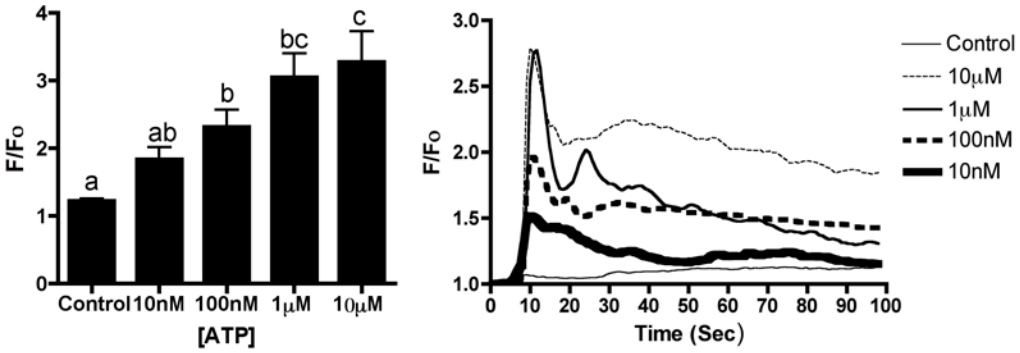


Figure 26. **ATP dose response.** Addition of 10uM-10nM concentrations of ATP was applied to activate purinergic receptors on striatal astrocytes. 1uM ATP was deciphered to activate metabotropic receptors according to initial peak from internal storage while having sustained calcium influx.

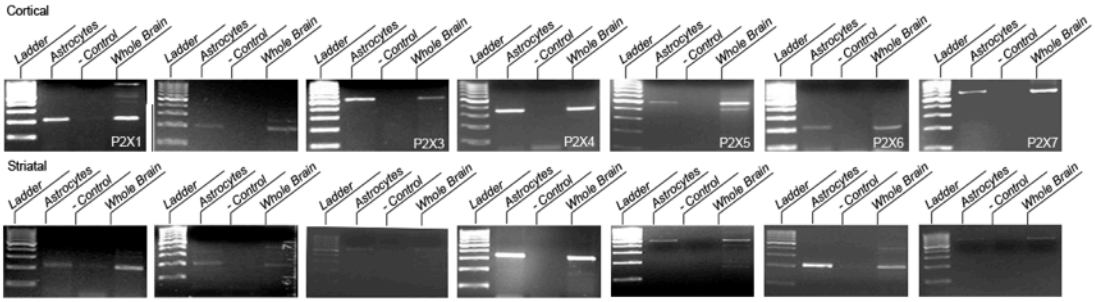


Figure 27. **Ionotropic P2X receptor expression.** Reverse transcriptase-PCR indicates expression of all P2X receptors in cortical and striatal astrocytes.

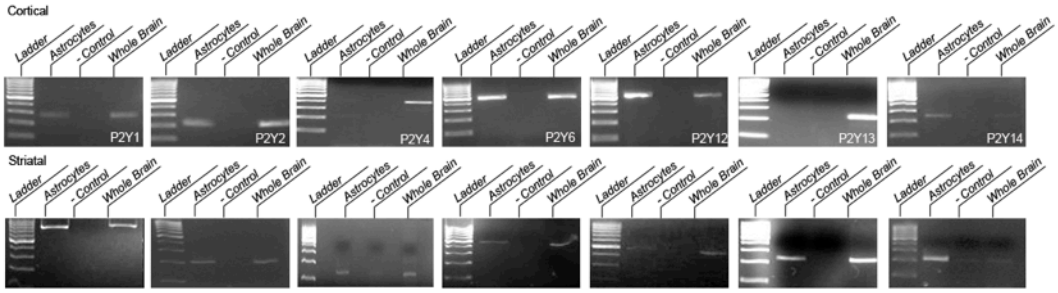


Figure 28. **Metabotropic P2Y receptor expression.** Reverse transcriptase-PCR indicates expression of all P2Y receptors in cortical except P2Y4 and P2Y13. Whereas striatal astrocytes express all P2Y receptors.

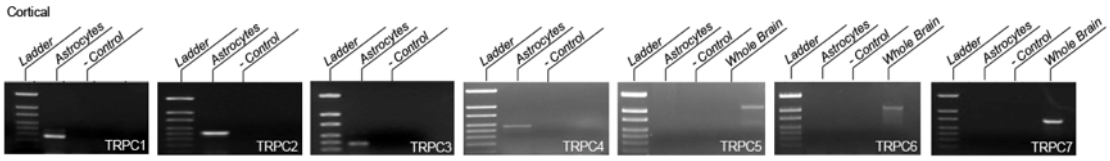


Figure 29. **TRPC receptor expression in cortical astrocytes.** Expression of subfamily TRPC receptors were assessed by reverse transcriptase PCR. All TRPC receptors 1-4 were present in astrocytes.

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# CHAPTER 5

# DISCUSSION

The most common neurological diseases in the elderly are Alzheimer's disease (AD) and Parkinson's disease (PD); both are chronic, progressive and multifactorial neurodegenerative diseases. As life expectancy increases there is an association with the number of people with neurodegenerative diseases because aging is one of the greatest risk factors. By 2050 it is expected there will be five times as many people over the age of 85 suggesting that over 13 million Americans will have AD, with the annual cost to Medicare rising above $1 trillion for AD alone (CNDR, 2011). The etiology of these disorders is unclear, but the majority of the cases are idiopathic. However, for PD the onset is associated both with age and with environmental risk factors such as rural living, drinking well water and chemical exposures (Drechsel and Patel, 2008). The sporadic onset of PD and other parkinsonian-like disorders has been attributed to gene-environment interactions suggesting susceptibility from environmental exposures throughout life. PD and other neurodegenerative diseases may share certain mechanisms of etiology and disease progression, such as neuroinflammation, and therefore understanding these processes may help identify better targets for therapeautic intervention.

Neuroinflammation is one of the common observations in a number of neurodegenerative diseases such as AD, PD, and amyotrophic lateral sclerosis. Microglial activation, astrogliosis, increased expression of iNOS/NOS2, excitotoxicity, oxidative and nitrosative stress, hypoperfusion, and protein aggregates, all ultimately resulting in apoptosis, are some of the mechanisms of neuroinflammation and are not distinct to any one neurodegenerative disease further suggesting a common pathway to

neuronal demise (Ischiropoulos and Beckman, 2003, Zhong et al., 2008, Hirsch and Hunot, 2009, Borghammer et al., 2010, D'Haeseleer et al., 2011). Among these mechanisms our research has focused on the glia, particularly astrogliosis, in order to observe the cellular and molecular inflammatory signaling that leads to nitrosative stress in the basal ganglia. Previous *in vitro* research in our laboratory has demonstrated that Mn potentiates the cytokine activation of Nf-kB and expression of NOS2, inducing the production of NO in astrocytes (Moreno et al., 2008). My contributions to this work indicate that NOS2 expression through the Nf-kB pathway in glia results in the free radical accumulation, production of NO, and nitrosative stress following exposure to Manganese (Mn) (see Chapter 2).

Chronic exposure to high levels of Mn causes a progressive neurodegenerative disorder of basal ganglia resembling certain neurological characteristics of Parkinson's disease, yet distinct in symptoms and pathological features (Calne et al., 1994). Although Mn toxicity is generally well documented with respect to effects in occupationally exposed adults, there are few studies of environmentally relevant exposures in juveniles. Therefore, we chose to study environmentally relevant concentrations of Mn in juvenile mice, adult mice, and mice exposed both as juveniles and adults. We postulated that juvenile exposure would distinctly activate microglia and astrocytes, and then subsequent re-exposure to Mn would result in greater glial NOS2 expression and induction of nitrosative stress in the basal ganglia. These studies revealed that developing mice exposed to Mn had a distinct neuroinflammatory reaction of the glia compared to Mn treated adults (see Chapter 2A). Additionally, juvenile Mn exposure increased NOS2 expression in the glia, therefore enhancing production of NO and subsequenty nitrosative

stress, further demonstrating a greater neuroinflammatory effect upon secondary exposure during aging (Moreno et al., 2009a). These studies identified low doses of environmental Mn as a potential risk factor for juvenile neuroinflammation. Developmental neuroinflammatory events can predispose the CNS to enhanced susceptibility upon exposures during aging decreasing neuronal integrity; this is possibly due to the reactive gliosis present. Whereas clinical and epidemiological reports correlate early inflammatory events, implying gliosis, are critical in the development of neurodegenerative diseases (Liu and Hong, 2003).

Additionally, this study design observed neurobehavioral and neurochemical modification with Mn upon differing stages of age (see Chapter 2B). These studies indicated that Mn exposure as juveniles had a differential dopaminergic impact, showing that juveniles had a significant increase in hyperactivity and dopamine concentrations, while pre-exposed adults have hypoactivity and loss of striatal dopamine (Moreno et al., 2009b). These data correlate with the epidemiological literature regarding Mn exposed adults with symptoms including hypokinesia (Calne et al., 1994), while childhood symptoms include hyperactivity (Bouchard et al., 2007, Bouchard et al., 2011). Additionally, childhood attention deficit disorder, a rising public health concern, has been linked to manganese exposure (Woolf et al., 2002) with both disorders sharing symptoms. These *in vivo* findings in dopaminergic activity could correlate with recent studies demonstrating that Mn treatment differentially alters tyrosine hydroxylase (TH) activity, the enzyme that synthesizes dopamine. This *in vitro* data has demonstrated that acute treatment with Mn increases the activity of TH, whereas chronic treatment results in a decrease in TH activity (Zhang et al., 2011). These outcomes suggest that timing and

duration of Mn exposure is an important indicator in the change in gliosis and modulation of dopaminergic activity. However, additional time point studies are necessary in order to determine the developmentally relevant changes in Mn-induced neurotransmission.

Epidemiological and clinical studies have indicated a sex dependence in the incidence of neurological diseases which correlate the female hormone estrogen with neuroprotective effects. In Parkinsonian models, such as MPTP and 6-OHDA, estrogen (E2) has shown diminished activation of the glia and is positively associated dopaminergic integrity (Morale et al., 2006, Tripanichkul et al., 2006). The previous studies with Mn exposure also indicated a sex-dependent response to neurobehavioral affects (Moreno et al., 2009b). Therefore, we supplemented juvenile males to E2 to observe the neuroprotective effect in Nf-kB reporter mice (see Chapter 2C). The results of this study strongly suggest that E2 decreases the Mn-induced activation of Nf-kB and expression of NOS2 (Moreno et al., 2011). These mice had significant decreases in neuronal protein nitration and *in vitro* studies indicated an attenuation of apoptosis with E2 treatment in striatal neurons. These data add to the other neuroinflammatory models of neurodegeneration indicating the E2 does suppress glial inflammation and decreases neuronal injury. Collectively, these studies demonstrated that the severity of Mn neurotoxicity is age and sex-dependent, and that early exposure predisposes the basal ganglia to greater neuroinflammatory susceptibility upon subsequent re-exposure during aging (see Chapter 2).

A common factor in these studies was neuroninflammatory expression of NOS2. Therefore, my next study used NOS2 knockout mice (see Chapter 3) to determine the functional relevance of this inflammatory gene in the mechanism of Mn neurotoxicity *in*

*vivo*. These results indicated that gene deletion of NOS2 attenuates peroxynitrite adduct formation in the basal ganglia associated with modification of behavioral markers. Furthermore, *in vitro* evidence indicated that NO causes astrocyte-dependent neuronal apoptosis indicating that Mn-induced production of NO contributes to neurotoxicity. This study is consistent with the MPTP model that demonstrated that the NOS2 gene is essential for the neuronal demise in the substania nigra (Liberatore et al., 1999) yet gliosis is still present in both neurotoxic models.

If early neuroinflammatory reactions sensitize the basal ganglia to later exposure to environmental neurotoxicants, then potentially exposure to such agents as bacterial endotoxins, viral episodes and/or environmental neurotoxins could increase susceptibility to the development of neurodegereative diseases. Therefore, I also examined astrocytic communication in the presence of neurotoxins (see Chapter 4) and found that cationic endogenous and exogenous neurotoxins alter physiological calcium signaling in astrocytes. These observed alterations in $calcium^{+}$ homeostasis could have implications with lack of physiological signals and coupling communication regulated by astrocytes, therefore, modulation these cellular dysfunctions could progress neurological demise.

My research contributed to the understanding that 1) Mn neurotoxicity is age- and sex-dependent, and that early exposure to Mn predisposes the basal ganglia to greater neuroinflammatory susceptibility upon subsequent re-exposure during aging, 2) juvenile mice lacking functional NOS2 gene are protected from Mn-induced locomotor dysfunction and glial-mediated nitrosative stress indicating that production of NO contributes to Mn neurotoxicity, and 3) ATP-induced calcium currents in primary astrocytes exhibit inhibition of TRPC3 in the presence of cationic exogenous and

endogenous neurotoxins. Overall, these studies indicate that astrocytes have a direct role in neuroinflammatory injury and nitrosative stress following Mn exposure and astrocytic signaling can be modulated by extracellular chemicals, potentially indirectly influencing neuronal integrity and viability.

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