UNIVERSITY OF CALIFORNIA
SANTA CRUZ

IMPACTS OF ELEVATED MANGANESE EXPOSURE ON THE DEVELOPING BRAIN AND SKELETON ACROSS THE LIFESPAN

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

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March 2021

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Impacts of Elevated Manganese Exposure on the Developing Brain and Skeleton Across the Lifespan

Travis Edward Conley

ABSTRACT

Epidemiological studies have reported associations between environmental manganese (Mn) exposure and attention-based learning and motor function deficits in children and adolescents. These studies have raised concerns about the increased vulnerability of children to the neurotoxic effects of elevated Mn exposure and underscore the need for effective exposure biomarkers to improve exposure classification, and to better detect Mn-related impairments across the lifespan. Recent animal model studies have established that elevated developmental Mn exposure can cause executive function and motor impairments, including deficits in focused and selective attention, but the specific neurobiological alterations responsible for these impairments are not well understood. Further, the impact of long-term Mn exposure on tissue accumulation of Mn in blood, brain, and bone as exposure biomarkers, and its effects on the skeleton as a potential target organ, is similarly not well understood. To address these knowledge gaps, I used an established rodent model of early life or lifelong childhood oral Mn exposure in Chapter 2 to determine 1) the relationship between oral Mn exposure and blood, brain, and bone Mn levels over the lifespan, 2) whether Mn accumulates in bone with lifelong exposure, 3) whether elevated bone Mn altered the mineral structure or physical properties of bone, and 4) bone Mn levels in aged humans (age 41-91; female, n=30; male, n=19) living in regions

impacted by historic ferromanganese alloy plant activity. In Chapter 3, I used this same rodent model of early life or lifelong oral Mn exposure to determine whether Mn causes 1) lasting disruption to the catecholaminergic system of the medial prefrontal cortex (mPFC), using quantitative protein immunohistochemistry of catecholaminergic proteins, 2) alterations to the evoked release of dopamine (DA) and norepinephrine (NE) in the PFC, and 3) whether changes in the mPFC catecholaminergic system were associated with heightened behavioral reactivity in an open field behavioral paradigm. In Chapter 2, I report that blood, brain, and bone Mn levels naturally decrease across the lifespan in the absence of elevated Mn exposure. In the presence of elevated oral exposure, bone Mn levels are strongly associated with blood and brain Mn, and that Mn did not accumulate with lifelong elevated exposure in any of the measured tissues. Additionally, elevated early life oral Mn exposures that produced high bone Mn levels up to 166 μg/g in young weanling animals caused some changes in bone mineral properties, including the local atomic structure of hydroxyapatite, and in young adult animals caused some physical changes in bone stiffness. In aged humans, bone Mn levels were universally very low (ranging from 0.014 – 0.17 μg/g), and decrease with age, but showed no relationship between gender or parity history in females. In Chapter 3, I report that postnatal Mn exposure caused heightened behavioral reactivity in the first 5-10 minutes of daily open field test sessions, consistent with deficits in arousal regulation. Mn exposure reduced the evoked release of NE, and caused lasting alteration in protein levels of tyrosine hydroxylase, DA and NE transporters, and DA D1 and D2 receptors.

These findings show that Mn does not accumulate in bone over prolonged exposure, and that the skeleton may be a relatively minor target of elevated Mn exposure. However, bone Mn levels increased to a greater extent than blood with incremental increases in blood Mn, suggesting that the skeleton may be a more sensitive biomarker of recent ongoing Mn exposure than blood. They also indicate that early postnatal Mn exposure causes broad lasting hypofunctioning of the mPFC catecholaminergic systems, consistent with the deficits in arousal regulation and attentional function. These effects are also consistent with the DA/NE dysfunction that is proposed to underlie children diagnosed with ADHD, and with the attentional deficits associated with elevated Mn exposure in children. Collectively, these findings help move the field forward by demonstrating a causal relationship between early life Mn exposure and lasting disruptions in the catecholaminergic system of the PFC that plays an important role in mediating executive function and attention, providing a mechanistic basis for how elevated Mn exposure may produce deficits in those functions in children. They also advance our understanding of Mn exposure biomarkers and identify bone Mn levels as a potentially important and sensitive biomarker of recent ongoing, but not cumulative Mn exposure.

ACKNOWLEDGEMENTS

I would like to begin by thanking my dissertation advisor, Dr. Don Smith, for his continuous scientific and professional advice, support, flexibility, and patience throughout the course of my doctoral research. His generosity in allowing me to modify the direction of my dissertation research throughout my graduate studies helped nurture my developing scientific interests and broaden my research experiences. I am also very grateful for my METX Department committee member, Dr. Manel Camps, for both his feedback on my dissertation research, and his generosity in offering me a position as Graduate Student Instructor for the Functional Human Anatomy course. Teaching that course was immensely rewarding, professionally and personally, and I learned a great deal through the process. I also want to thank my other committee members, Dr. Yi Zuo and Dr. Somshuvra Mukhopadhyay for their valuable insights and guidance that have consistently improved the design of my research.

My graduate school experience would not have been nearly as rich were it not for my wonderful lab mates and friends, past and present, in the Smith and Finkelstein labs. They have set the gold standard for how an inviting, collaborative lab environment should operate. Similarly, it has been a pleasure to work alongside my fellow graduate student colleagues throughout the METX Department who have consistently inspired me with their dedicated work ethics. I also want to thank members of greater PBSE community, including Dr. Ben Abrams of the UCSC Microscopy Center for his many dozens of hours of assistance and troubleshooting

with microscopy, image deconvolution, and Imaris image quantification. Dr. Caitlin Moyer from the Zuo lab was similarly a tremendous source of knowledge for tissue collection and image analysis. Additionally, a big thank you to Drew McDonald, a stellar undergrad I had the privilege of working with who aided in much of my brain image processing for Chapter 3.

I am infinitely grateful for the incredible friendships I have fostered and maintained throughout grad school. Both in and out of lab, my friend's professional guidance, emotional support during difficult times, collaborative filmmaking, laughter, and our shared trivia night adventures will forever be some of my happiest memories as a graduate student. Likewise, special thanks to Alok, Alec, Rick, and Erika, my older friends who have remained in my life for many years prior to starting grad school and beyond.

Finally, none are more deserving of thanks than my family: My parents, grandparents, aunts, uncles, cousins, and my furriest, four-legged, wet-nosed family member, Juneau. My mom and dad, who are the hardest working people I know, made my successes possible through their endless love and support. An extra special thanks to my Mom, who similarly earned her Ph.D. in the UC system, for continuously offering a uniquely informative perspective throughout my graduate studies. Last but not least, I am thankful for my partner, Hannah, for her enduring emotional support, patience, encouragement in my most difficult times, and for ensuring I never went a single stressful day without a hug.

The text of this dissertation includes reprints of the following previously published material:

Conley T. E., Beaudin S. A., Lasley S. M., Fornal C. A., Hartman J., Uribe W., Khan T., Strupp B. J., Smith D. R. (2020) Early postnatal manganese exposure causes arousal dysregulation and lasting hypofunctioning of the prefrontal cortex catecholaminergic systems. *J. Neurochem.* 153, 631–649.

Peter, S.A. (2014). Exposure assessment of manganese via nutrition and inhalation in the province of Brescia. [Doctoral dissertation, University of Brescia].

CHAPTER 1: INTRODUCTION

Environmental Mn exposure is an ongoing public health concern based on studies that have established associations between environmental and occupational Mn exposure and executive and motor function deficits in children, adolescents, and adults (Bouchard *et al.* 2007; Oulhote *et al.* 2014; Lucchini *et al.* 2012a; Mora *et al.* 2018; Crinella 2012). While recent animal model studies have demonstrated a causal relationship between elevated developmental Mn exposure and attention-based and motor function deficits, the neurobiological alterations that contribute to these deficits are not well understood. Additionally, the impact of long-term Mn exposure on tissue accumulation of Mn in blood, brain, and bone as exposure biomarkers, and its effects on the skeleton as a potential target organ, is also not well understood. The goal of this dissertation research is to address the above knowledge gaps relating to environmental Mn exposure, such as the relationship between oral Mn exposure and blood, brain, and bone Mn levels over the lifespan, whether Mn accumulates in bone, and whether elevated bone Mn alters the mineral structure or physical properties of bone. Further, we also determined whether early life Mn exposure caused heightened behavioral reactivity in the open field paradigm, lasting changes in the mPFC catecholaminergic systems, and whether lifelong exposure exacerbates these effects. Before expanding on my research in Chapters 2 and 3, Chapter 1 will first focus on the present evidence and knowledge gaps regarding the need for effective exposure biomarkers of Mn to better detect Mn-related impairments across the lifespan, and the impacts of early life Mn exposure on neurobehavioral function, with an emphasis on

examining the neurochemical mechanisms of toxicity that may underlie the attentional phenotypes observed in Mn-exposed children.

Background

Fundamentals of Mn (chemistry, environmental sources, and biological roles)

Physical and chemical properties of Mn

Manganese can be found in both organic and inorganic states. As a transition metal, Mn is known to exist in 11 oxidation states, ranging from -3 to +7, but it is most commonly found as Mn^{2+} within soils and aquatic environments (Kenneth Klewicki and Morgan 1998). In biological systems, Mn is known to exist predominantly in the Mn^{2+} and Mn^{3+} oxidation states (Takeda 2003), while Mn^{4+} has been shown to exist only in plant photosystems (Kenneth Klewicki and Morgan 1998). In mammalian tissue, Mn^{2+} is the most stable ion and can be found at high concentrations in neutral solutions due to its ability to resist oxidation below pH 8 (Sigel 2000). The large ionic radius (97 pm) and small charge/radius ratio of Mn^{2+} enables it to form weak complexes with multiple ligands, such as sulfate and chloride (Saha *et al.* 2000; Sigel 2000). By comparison, Mn^{3+} has a smaller ionic radius of 78 pm, and is only able to form complexes with strong ligands, such as pyrophosphate, as a result (Sigel 2000). Mn^{2+} shares a similar valence state with other biologically important ions such as Ca^{2+} and Mg^{2+}, whereas the electron configuration of Mn^{3+} is more comparable to that of Fe^{3+}; these properties allow Mn to substitute for these other ions in various biochemical processes (Silva and Williams 1993).

Role in biological systems

Manganese plays an essential role in the metabolism of amino acids, lipids, proteins, and carbohydrates as a cofactor in various metalloenzymes are dependent on Mn as a cofactor such as arginase, required for the elimination of ammonia (Roholt and Greenberg 1956), glutamine synthetase, necessary for nitrogen metabolism and the glutamine/glutamate-γ-aminobutyric acid cycle in astrocytes (Wedler and Denman 1984; Sidoryk-Wegrzynowicz and Aschner 2013), phosphoenolpyruvate decarboxylase, needed for carbohydrate synthesis (Bentle and Lardy 1976), and manganese superoxide dismutase (MnSOD), the key mitochondrial antioxidant enzyme for metabolizing intracellular reactive oxygen species (McCord 1976; Sigel 2000).

Although Mn is needed in numerous biochemical processes, one of its most vital functions is the role it plays in inflammation defense. There are multiple forms of the SOD enzyme, including CuSOD, ZnSOD, and MnSOD, the latter of which is essential for the survival of aerobic organisms because of its ability to catalyze the disproportionation reaction of the highly reactive superoxide radical ($O_2^{\bullet -}$) to H_2O_2 and O_2 (Christianson and Cox 1999). Mn is necessary for this reaction as the transition from Mn^{2+} to Mn^{3+} at the active site of MnSOD allows for the oxidation of ambient free radicals (Sigel 2000). This process of free radical scavenging protects cells from oxidative damage and regulates cellular levels of both superoxide and other ROS that are produced during mitochondrial respiration (Christianson and Cox 1999).

Dysfunction or impaired synthesis of MnSOD leads to a cellular redox imbalance that can result in excess generation of hydroxyl, lipid peroxyl (RO_2), or alkoxyl (RO) radicals (Christianson and Cox 1999). MnSOD is particularly important in tissues with high metabolic activity as the heightened mitochondrial respiration in these regions creates a greater demand for antioxidant agents (Christianson and Cox 1999). The central nervous system is one such area with high metabolic activity, due to the ongoing synthesis of neurotransmitters and their related proteins (Thanan *et al.* 2014).

Beyond the supporting role that Mn plays in neuroinflammation defense, Mn is also necessary for the activity of glutamine synthetase, the metalloenzyme responsible for synthesizing glutamine from glutamate (Wedler and Denman 1984). Located predominantly in astrocytes, glutamine synthetase can account for roughly 80% of available Mn in the brain in order to catalyze the conversion of glutamic acid to glutamine (Prohaska 1987). This process is crucial for regulating proper synaptic function because glutamate is the primary excitatory neurotransmitter within the brain, and also serves as an important member of the glutamine/glutamate-γ-aminobutyric acid (GABA) cycle (Komuro and Rakic 1993; Bao *et al.* 2009).

Environmental sources of Mn

Manganese is found naturally in the environment where it comprises roughly 0.1% of the earth's crust, making it the fifth most abundant metal and the twelfth most abundant element (Cooper 1984). It frequently complexes with other elements in rocks and soils to form oxides, carbonates, and silicates, of which pyrolusite

(MnO_2) is the most common naturally-occurring mineral (Cooper 1984). As Mn-containing minerals erode during natural weathering processes, Mn is readily introduced into groundwater systems where it can serve as a source of human exposure for individuals who access that water using household wells. A study conducted by the U.S. Environmental Protection Agency (U.S. EPA) of Mn concentrations in groundwater found that median levels range from 5-150 μg/L, with a 99th percentile of 2,900-5,600 μg/L in certain areas (U.S. EPA, 2003). Another study by the U.S. Geological Survey (2005) found that roughly 6% of domestic household wells exceed the 300 μg Mn/L lifetime health advisory level set by the U.S. EPA, defined as the concentration below which daily consumption over a lifetime is not likely to cause adverse health effects. Food represents another natural source of Mn in the environment. Nuts, grains, rice, and teas are considered major dietary sources of Mn (Peres *et al.* 2016b; Aschner and Aschner 2005; Keen *et al.* 1999).

In addition to the natural routes through which Mn may enter the environment, there are multiple anthropogenic sources of Mn that must also be considered. Mn is widely used in industrial activities, including the manufacturing of dry-cell batteries, ferromanganese alloys, glass, textiles, cosmetics, and fertilizers (ATSDR, 2000; Srivastava et al., 1991). Occupational exposures to Mn have been known to occur for jobs involving ferromanganese alloy smelting, welding, and mining (Bast-Pettersen *et al.* 2004; Bowler *et al.* 2007; Montes *et al.* 2008; Lucchini *et al.* 1999; Lucchini *et al.* 1995; Roels *et al.* 1987). In agricultural regions, usage of

the Mn-containing fungicides, maneb and mancozeb, constitute potential sources of Mn exposure to the environment and for the residents in surrounding areas (Gunier *et al.* 2013). Mn is also incorporated into gasoline as the antiknock additive methylcyclopentadienyl manganese tricarbonyl (MMT), and combustion of this compound leads to release of Mn phosphates in the surrounding air (ATSDR, 2000). Currently, MMT is allowed only in non-reformulated gasoline (<40% of U.S. fuel supply) in the U.S. at concentrations of 31.25 mg/gal (U.S. EPA., 2015), in less than 5% of Canadian gasoline at 18 mg/L (Health Canada, 2010), and in China and Europe at 2 mg/L (CSICE, 2011; EUP, 2015).

Nutrition and development

Due to the biological requirements for Mn throughout the body, insufficient intake has the potential to cause serious health effects for mammalian systems. However, Mn deficiency seldom occurs as a result of its abundance in most diets worldwide, where dietary intake through food and water represents the major source of Mn intake in humans (Aschner and Aschner 2005). During pregnancy, Mn is needed for proper formation of bone, cartilage, and tendons for the developing fetus. This growth window creates a greater than normal demand for Mn by the Mn-dependent proteoglycans that are necessary for synthesis of healthy skeletal connective tissue (Keen and Zidenberg-Cherr 1996). In the rare cases in which an individual does not receive enough Mn in their diet during fetal development, this deficiency can lead to impaired bone growth, birth defects, lowered fertility, and

altered metabolism of lipids and carbohydrates (Freeland-Graves and LLanes 1994; Keen *et al.* 1999).

Adequate intake for Mn in adults, established by the National Academy of Sciences in 2001, is currently listed at 2.3 mg/day for men and 1.8 mg/day for women, but this amount differs with age depending on physiological need (Aschner and Aschner 2005). While these adequate intake values for food have not changed since their establishment in 2001, recent epidemiological studies have raised concerns over the amount of Mn in drinking water that may be safe for consumption by children, based on age-related differences in the rate of Mn metabolism (Ljung and Vahter 2007; Bouchard *et al.* 2011; Wasserman *et al.* 2006a). These concerns are addressed later in this chapter.

Uptake and elimination at the organismal level

The extent that Mn is absorbed, distributed, metabolized, and excreted varies somewhat over the lifespan of the individual. Healthy adults typically absorb about 1-5% of ingested Mn across the gastrointestinal tract (Davis *et al.* 1993). When separated based on sex, women absorb more Mn on average than men, and it has been proposed that reduced gastrointestinal absorption in men coincides with the iron status and greater levels of serum ferritin in males (Finley *et al.* 1994; Finley 1999; Aschner and Aschner 2005). Inhalation represents an additional route of Mn exposure, where 60-70% of inhaled Mn is cleared from the respiratory tract through mucocilliary action or swallowing (Mena 1974). Direct absorption of respired Mn can

occur through the alveolar ducts in the lung, and Mn can be transported directly into the brain when inhaled through nasal passages and absorbed into the olfactory tract (Vitarella *et al.* 2000; Dorman *et al.* 2002). Once taken up through the gastrointestinal tract, Mn is transported to the liver via the portal vein, where depending on body Mn status, much of the absorbed Mn may be transported via hepatocytes into the biliary canal for biliary excretion into the large intestine (this excretory pathway is incompletely developed in infants compared to older children and adults). Roughly 80% of Mn is excreted through the bile for subsequent fecal elimination (Malecki *et al.* 1996), but a small portion (<0.1%) is excreted through the pancreas and kidneys (Davis *et al.* 1993). Mn that is not taken up into hepatocytes is deposited in the central blood, where average blood Mn concentrations in adults range from 4-15 μg Mn/L under normal conditions (Dobson *et al.* 2004). Once in blood, Mn can cross the blood-brain barrier through the capillary endothelium of the choroid plexus (Rabin *et al.* 1993).

While adults have homeostatic mechanisms in place for regulating Mn uptake, these homeostatic controls are not fully developed in infants or young children, potentially making them more vulnerable to the harmful effects associated with elevated manganese exposure. Both absorption and retained levels of ingested Mn are higher in infants than in older children and adults. Specifically, the few studies on this subject indicate that infants absorb approximately 20% of consumed Mn, much more than adults or older children (Lönnerdal 1997). Rodent studies support these findings in which neonate rats less than 15 days of age are able to absorb and retain over 40%

of ingested Mn (Keen *et al.* 1986; Miller *et al.* 1975b; Pappas *et al.* 1997), compared to less than 5% retained by adult rats (Mena 1974; Ballatori *et al.* 1987). This early postnatal window represents a period of increased vulnerability to Mn exposure because the biliary pathway necessary for effective excretion and the blood-brain barrier are not fully established until later stages of development. In rats, this period does not occur until weaning at approximately postnatal day (PND) 17 - 21 (Miller *et al.* 1975b). Human studies have shown that the timeframe for this physiological milestone also occurs during infant development, where the blood-brain barrier remains semi-permeable to Mn and other potential toxins until its complete formation around 3-4 months of age (Obermeier *et al.* 2013; Daubing 1968). An additional source of concern over the immature blood-brain barrier is based on the fact that crucial neurochemical systems also develop during this period, as demonstrated by work from Broaddus and Bennett, showing that dopaminergic synapses experience the highest period of growth during the first 2-3 weeks of postnatal life (Broaddus and Bennett 1990). Due to the limited data regarding the effects of developmental Mn exposure on the central nervous system, further studies are needed to more closely examine the recognized windows of susceptibility to Mn neurotoxicity through use of early postnatal exposure models.

Cellular uptake and elimination

There are multiple mechanisms responsible for maintaining Mn homeostasis at the cellular level. Following absorption into blood plasma, Mn can be present as a

free ion (Takeda 2003), and transported through two different serum complexes, based on oxidation state. The majority of Mn, present in the Mn^{2+} form, is transported into the cell via the divalent metal transporter 1 (DMT1), a solute carrier protein that has also been shown to transport divalent iron (Fe^{2+}), zinc (Zn), cobalt (Co), copper (Cu), cadmium (Cd), nickel (Ni), and lead (Pb) (Garrick *et al.* 2006; Salazar *et al.* 2008). DMT1 is expressed in the brain basal ganglia, striatum, substantia nigra, globus pallidus, and subthalamic nucleus, all regions that correspond with Mn deposition (Williams *et al.* 2000; Burdo *et al.* 2001; Huang *et al.* 2004). Despite the ability of DMT1 to transport multiple ions, its transport affinity is not equivalent for all metals. Garrick et al. (2006) established that DMT1 transports Mn more readily than Fe, and that low Fe levels can increase DMT1 expression (Crossgrove and Yokel 2004). In contrast to Mn^{2+}, Mn^{3+} is transported by plasma transferrin, which may comprise up to 20% of plasma Mn (Aisen *et al.* 1978), enabling cell entry via interaction with the transferrin receptor. Plasma transferrin protein is synthesized in the liver, and expressed in cells throughout the body, especially in neurons, astrocytes, and microglia (Moos and Morgan 2000). Additional influx of Mn, albeit likely minor, has been observed through interactions with the dopamine transporter (DAT) (Ingersoll *et al.* 1999; Kim *et al.* 2002) and Zn transporters, most notably SLC39A8 (ZIP8) and SLC39A14 (ZIP14) (Dalton *et al.* 2005; He *et al.* 2006). In particular, SLC39A14 is a transmembrane transporter that can uptake Zn, Fe^{2+}, and Mn, and deficiency in this protein has been recently reported to cause brain Mn

accumulation and motor deficits in mice (Tuschl *et al.* 2016; Mukhopadhyay 2018; Jenkitkasemwong *et al.* 2018).

Similar to Mn influx, the efflux of Mn is known to occur via multiple cellular exporters, but recent research on this topic has focused on SLC30A10, a cell-surface Mn efflux transporter initially detected in the brain and liver, that has been demonstrated to reduce cellular Mn levels and protect against toxicity (Leyva-Illades *et al.* 2014; Zogzas *et al.* 2016). SLC30A10 is noteworthy because loss-of-function mutations in this transporter represent the only known cause of hereditary Mn-induced parkinsonism (Mukhopadhyay 2018), prior to which, the association between occupational exposure to Mn and parkinsonism had been well established (Chen *et al.* 2015). Individuals that possess a genetic loss-of-function mutation in the SLC30A10 gene have displayed blood Mn levels 10-20 times higher than those with a functional version of the gene, even under normal Mn exposure conditions (Tuschl *et al.* 2012; Quadri *et al.* 2012; Mukhopadhyay 2018). A 2019 study from Carmona and colleagues found that this form of Mn-induced parkinsonism in individuals with the SLC30A10 mutation resulted from Mn accumulation within nanovesicles of the Golgi apparatus, suggesting that dysfunction of the vesicular trafficking machinery led to the disease onset (Carmona *et al.* 2019). Mukhopadhyay and colleagues further investigated the role of SLC30A10 in regulating brain Mn homeostasis using tissue-specific SLC30A10 knockout mice, and found that while the brain-specific knockouts showed no elevation in brain Mn levels, they determined that SLC30A10 was also expressed in the gastrointestinal tract (Taylor *et al.* 2019). Interestingly, using

endoderm-specific knockouts which lacked SLC30A10 in the liver and gastrointestinal tract, elevated Mn levels were detected in the blood, brain, and liver, suggesting that under basal physiological conditions, SLC30A10 regulates brain Mn via expression in the liver and gastrointestinal tract. Further, Taylor et al. showed that under conditions of elevated Mn exposure, SLC30A10 brain-specific knockouts had greater brain Mn levels than control, indicating that SLC30A10 protects against Mn neurotoxicity in the presence of elevated exposure (Taylor *et al.* 2019). Other proteins implicated in Mn transport include ATPase13A2 (Tan *et al.* 2011), ferroportin (Choi *et al.* 2019), and the secretory pathway Ca^{2+}-APTase1 (SPCA1) (Ton *et al.* 2002; Madejczyk and Ballatori 2012), although the importance of these transporters has yet to be determined.

Genetic polymorphisms in Mn influx and efflux transporters have also recently been reported to play a role in the neurodevelopmental susceptibility to the neurotoxic effects of Mn. Specifically, Wahlberg et al. (2018) examined a cohort of Italian children and reported that common single-nucleotide polymorphisms in SLC39A8 (influx) were associated with 7-15% reductions in blood Mn, whereas a polymorphism in SLC30A10 (efflux) corresponded with an increased average blood Mn of 41%. Importantly, children with this SLC30A10 polymorphism associated with higher blood Mn also scored lower on certain IQ subtests, and increased ADHD-related behaviors (Wahlberg *et al.* 2018). Broberg et al. (2019) additionally studied this same Italian cohort to determine the influence of sex and genetics on sensitivity to environmental Mn exposure using associations with soil Mn, and reported that girls

with genotypes linked to high blood Mn showed strong positive associations with soil Mn and Conners' scores of ADHD-type behaviors; in contrast to girls, boys only showed a positive linear relationship with soil Mn for Conners' scores of hyperactivity (Broberg *et al.* 2019). Collectively, these studies provide support for the ability of Mn transporter genetics to effect sensitivity to elevated environmental Mn exposure.

Mn exposure biomarkers

The increased understanding of the health risks associated with elevated Mn exposure have led to a greater need for effective exposure biomarkers to help detect and diagnose Mn-related impairments, especially given that children are more vulnerable to elevated exposures and the neurotoxic effects of Mn than adults, and exposure risk changes over the lifespan (Ljung and Vahter 2007; Erikson *et al.* 2007; Kern *et al.* 2010; Mora *et al.* 2015a). Reported biomarkers for Mn exposure have included blood, hair, saliva, urine, nails, and teeth (Arora *et al.* 2012b; Haynes *et al.* 2015; Laohaudomchok *et al.* 2011; Ward *et al.* 2018; Claus Henn *et al.* 2010; Gil *et al.* 2011; Butler *et al.* 2019; Mora *et al.* 2018; Lucchini *et al.* 2012a; Mora *et al.* 2015a; Oulhote *et al.* 2014). Blood and urine Mn levels appear to reflect, if anything, only recent exposures over the span of several days to weeks (Järvisalo *et al.* 1992; Cowan *et al.* 2009; Smith *et al.* 2007), while hair and nail Mn levels have been reported to reflect exposures on the scale of ~3-12 months (Eastman *et al.* 2013; Reiss *et al.* 2015; Jursa *et al.* 2018; Laohaudomchok *et al.* 2011; Ward *et al.* 2018).

Teeth or skeletal Mn levels may reflect exposures over longer periods of months to years, based on recent studies (Claus Henn *et al.* 2018; Rolle-McFarland *et al.* 2018; Austin *et al.* 2017; Horton *et al.* 2018; Arora *et al.* 2012a).

The extent that candidate Mn exposure biomarkers are associated with adverse health outcomes, such as cognitive and behavioral deficits in humans, is mixed (Haynes *et al.* 2015; Mora *et al.* 2015a; Lucchini *et al.* 2012a; Lucchini *et al.* 1999; Smith *et al.* 2007). For example, some studies have reported associations between Mn levels in hair, blood, and teeth with cognitive or behavioral impairments (Menezes-Filho *et al.* 2011; Haynes *et al.* 2015; Mora *et al.* 2015a), while others have reported no association between blood and urinary Mn with health outcomes (Lucchini *et al.* 2012b; Lucchini *et al.* 1999; Smith *et al.* 2007). In the case of tooth Mn levels, recent studies suggest that higher prenatal dentine Mn levels are associated with improved visual spatial abilities, impulse control and attentional function, whereas higher postnatal dentine Mn levels are associated with no, or adverse neurobehavioral effects, in children depending on age, sex, and outcome (Bauer *et al.* 2017; Horton *et al.* 2018; Claus Henn *et al.* 2018; Mora *et al.* 2015a). These differences across studies in the extent that the Mn exposure biomarker(s) are associated with adverse health effects may result in part from exposure misclassification, further underscoring the need for an improved understanding of Mn exposure and exposure biomarkers over the lifespan.

Bone represents a potential candidate as a long-term biomarker of Mn exposure, since Mn levels have been shown to increase in bone during developmental

periods, and Mn in bone has been estimated to account for roughly 40% of body Mn (Aschner and Aschner 2005; Andersen *et al.* 1999; O'Neal *et al.* 2014). The basis for Mn incorporation into bone mineral may be due in part to Mn^{2+} serving somewhat as a biologic analog to Ca^{2+} (Frausto da Silva and Williams 2001). This Mn^{2+} - Ca^{2+} relationship in mineralized tissues may be similar to the well-established relationship between $Pb^{2+}$and Ca^{2+}, which leads to the accumulation of lead in mineralized tissues and the utility of bone and tooth lead levels as biomarkers of cumulative lead exposure (Hu 1998; Smith *et al.* 1996; Téllez-Rojo *et al.* 2004; Specht *et al.* 2016; Arora *et al.* 2012b; Mora *et al.* 2015a). Moreover, recent technological advances have led to the development of portable neutron activation systems for *in vivo* assessment of bone Mn levels in humans (Pejović-Milić *et al.* 2009; Liu *et al.* 2013; Rolle-McFarland *et al.* 2018), suggesting the emerging feasibility of assessing bone Mn levels as an exposure biomarker to complement other tissue measures currently in use. Additionally, the fact that Mn is an essential nutrient that plays a role in skeletal development and maintenance, while lead serves no essential biological function may inform differences in bone-lead vs. bone-Mn interactions (Andersen *et al.* 1999; O'Neal *et al.* 2014; Aschner and Aschner 2005). If Mn accumulates in bone with elevated exposure, similar to lead, bone Mn may prove to be an informative biomarker to assess Mn body burden over the lifespan.

Mn and cognition/behavior

Neurobehavioral and cognitive deficits observed in humans

Over the past decade and a half, a growing number of epidemiological studies have reported associations between Mn in children's hair, blood, teeth, and drinking water with deficits in learning, Full Scale IQ and verbal comprehension, and behavioral issues such as hyperactivity, inattention, oppositional behavior, and impulsivity, among others (Bauer *et al.* 2017; Arora *et al.* 2011; Arora *et al.* 2012b; Mora *et al.* 2018; Oulhote *et al.* 2014; Ericson *et al.* 2007; Bouchard *et al.* 2011; Coetzee *et al.* 2016). Regarding impairments of memory and learning, a study by Wasserman et al. (2006) investigated an association between Mn levels in Bangladesh's well water and intellectual function in a cohort of 10-year-old children, which revealed a significant association between elevated well-water Mn concentrations and reduced Full-Scale, Performance, and Verbal raw IQ scores in a dose-response manner. Wright and colleagues (2006) explored the relationship between hair-Mn concentrations and general intelligence in children that resided near a hazardous waste site, showing that greater hair Mn levels were associated with reduced verbal IQ and working memory scores, once those scores were adjusted for arsenic exposure (Wright *et al.* 2006). These results have been further supported by more recent studies with a combined focus on assessing water- and hair-Mn levels with respect to memory (Oulhote *et al.* 2014; Sanders *et al.* 2015), which demonstrate a linear association between log10 hair-Mn and a nonlinear association between log10 water-Mn and impaired memory (Oulhote *et al.* 2014). Additional research by Claus Henn and colleagues reported that increased maternal blood Mn during pregnancy was associated with reduced childhood neurodevelopment scores in 2-

year-old children residing near Superfund site (Henn *et al.* 2017), and similar cognitive deficits have been reported by others (Bauer *et al.* 2017; Haynes *et al.* 2015; Claus Henn *et al.* 2010; Horton *et al.* 2018; Bauer *et al.* 2020).

Other studies focused on aberrant behavior, in addition to lower academic skills, in response to Mn exposure. In addition to memory assessments, Oulhote et al. (2014) also examined the manner by which hair/water-Mn was related to attention and motor function. By using the California Verbal Learning Test-Children's Version (CVLT-C), Conners' Continuous Performance Test (CPTII), Digit Span, Santa Anna Test, and manual Fingertapping evaluations completed by teachers and parents, investigators determined that greater log10 Mn levels were linearly associated with poorer attention and motor function (Oulhote *et al.* 2014). Other researchers have used similar methods of behavioral assessment to show comparable results; a Canadian study (Bouchard *et al.* 2007) of ADHD-like behaviors in Mn-exposed children revealed a significant association between Mn levels and attention-related impairments. Further associations have been demonstrated between elevated Mn exposure and lower visual-spatial ability (Bauer *et al.* 2017; Claus Henn *et al.* 2018), along with impaired motor function (Chiu *et al.* 2017; Dion *et al.* 2016; Lao *et al.* 2017).

In addition to concerns over Mn-contaminated well water, some researchers have expressed worries about the effects of soy-based infant formula on the development of ADHD in children (Crinella 2012; Golub *et al.* 2005; Ericson *et al.* 2007). Available evidence suggests that approximately 20-30% of infants in the U.S.

consume some form of formula within the first year of life, and that higher rates are consumed in communities with lower socioeconomic status (CDC 2006; Merewood *et al.* 2005). About 20% of these infants on formula consume soy-based formula, which is known to contain elevated levels of Mn L (Golub *et al.* 2005; Lönnerdal *et al.* 1981; Cockell *et al.* 2004). Frisbie et al. (2019) recently examined Mn concentrations in various infant formulas available in the U.S. and France, including products with cow-milk, goat-milk, soy, and rice, and reported that Mn levels ranged from 160-2,800 μg Mn/L compared to concentrations found in human breast milk of only 3-6 μg Mn/L (Frisbie *et al.* 2019). Given the observed attention-based deficits that have been previously described following developmental Mn exposure, Crinella (2012) concluded that non-breastfed infants are at greater risk for developing ADHD-like symptoms later in life. While these findings and the ones presented above detail persuasive evidence regarding the risks of early life Mn exposure, they are restricted by their correlational nature and limited ability to control for confounding variables. This issue highlights the importance of targeted animal-model studies that allow researchers to administer Mn doses to neonates under controlled conditions, and ultimately establish a causal link between developmental Mn exposure and neurological dysfunction.

Neurobehavioral and cognitive deficits observed in animals

Animal model studies are an essential aspect of toxicological research for Mn based on their ability to test the effects of specific doses in individuals of known age

over defined durations of time. Studies in rodents and non-human primates have demonstrated that early developmental Mn exposure produces behavioral and attentional deficits. However, multiple studies have relied on behavioral tests that are not specifically designed to evaluate the learning or emotional deficits similar to those observed in Mn-exposed children; these tests include the negative geotaxis, acoustic startle, and burrowing detour (Tran *et al.* 2002; Reichel *et al.* 2006; Pappas *et al.* 1997; Brenneman *et al.* 1999). A few studies have implemented more effective tests for assessing learning and emotional regulation in rats and non-human primates that better reflect the deficits in executive function reported in Mn-exposed children. Notably, our group was the first to demonstrate that oral elevated early postnatal Mn exposure of 25 or 50 mg Mn/kg/day over PND 1-21 can directly cause attentional dysfunction in the 5-choice serial reaction time task (5-CSRTT) in a manner consistent with arousal dysregulation (Beaudin *et al.* 2017b).

Other studies have also assessed the impact of Mn exposure on cognitive function. For example, McDougall et al. (2008) reported that adult rats given 750 μg Mn/day from PND 1-21 required more time to reach criterion on a fixed ratio 1 operant learning task relative to controls. Similarly, Golub et al. (2005) observed that non-human primates fed Mn-supplemented soy formula for the first 4 months of life displayed reduced play behavior, increased impulsivity, and more affiliative clinging in social dyadic interactions relative to controls. A study from our group found that preweanling rats exposed to 25 or 50 mg Mn/kg/day from PND 1-21 experienced deficits in spatial learning and memory associated with increased frequency of

stereotypic behavior in an 8-arm radial maze (Kern *et al.* 2010). Shukakidze et al. (2003) observed similar learning deficits in rats assessed in a radial maze test following Mn exposure during adulthood. A more recent study from Peres et al. (2015) reported that adult rats exposed to Mn early in life (20 mg Mn/kg/day from PND 8-12) had short-term memory impairments based on their inability to distinguish between a familiar and novel object in a novel object recognition task.

Studies have also provided evidence that Mn exposure can contribute to gross and fine motor dysfunction in animals. Impairments in motor coordination and balance due to Mn exposure have been demonstrated by a number of investigators (Bouabid *et al.* 2014; Peres *et al.* 2015; Beaudin *et al.* 2013). For example, our group showed that neonate rats exposed to 25 or 50 mg Mn/kg/day in early life (PND 1-21) or throughout life exhibited deficits in fine motor control in the Montoya staircase test, and that rats exposed throughout life (25 mg Mn/kg/day) were more severely impaired than those exposed only in early life (Beaudin *et al.* 2013; Beaudin *et al.* 2015). Others reported Mn-induced balance deficits in adult rats (exposed to 10 or 20 mg Mn/kg/day from PND 8-12) during the rotarod test (Peres *et al.* 2015). Gross locomotor activity in the open field paradigm has also been assessed in response to Mn exposure, but the results have been less consistent. One study found that neonatal Mn exposure in rats (25 or 50 mg Mn/kg/day from PND 1-21) resulted in hyperactivity and behavioral disinhibition in the open field (Kern *et al.* 2010). Other researchers have observed similar cases of behavioral disinhibition (Calabresi *et al.* 2001) or hyperactivity in rodent models (Chandra *et al.* 1979; Brenneman *et al.* 1999;

Pappas *et al.* 1997), while others have reported hypoactivity (Cordova *et al.* 2013a; Betharia and Maher 2012; Reichel *et al.* 2006), or no difference in locomotor activity (Dorman *et al.* 2000). While these inconsistencies may result from variations in Mn exposure duration, dose, route, and age of animals at the time of exposure, they nonetheless suggest that more research is needed to clarify the nature of cognitive and neurobehavioral deficits that result from early developmental Mn exposure.

Pharmaceutical therapies in animals exposed to Mn

In light of the growing concern for children who have been developmentally exposed to elevated levels of Mn, pharmaceutical compounds represent a potential therapy for treating behavioral and attentional learning deficits. However, very little research has been conducted in animal models on the efficacy of such therapies. At present, only our group has reported on this topic, specifically by testing the ability of methylphenidate (MPH) to alleviate the motor dysfunction caused by developmental Mn exposure. In that study, rats were orally exposed to 0 or 50 mg Mn/kg/day throughout life (PND 1-145), and fine motor function was assessed using the Montoya staircase test. Results showed that oral MPH administration (2.5 mg/kg/day given 1 hour before testing for 16 days) completely alleviated the fine motor deficits caused by Mn exposure (Beaudin *et al.* 2015). Pharmacological studies in animals have shown that the therapeutic value of MPH for treating ADHD-like disorders results from the mechanism through which MPH increases synaptic catecholaminergic signaling in the prefrontal cortex and striatum by blocking DAT

and NET and inhibiting the reuptake of synaptic dopamine and norepinephrine (Gamo *et al.* 2010; Szobot *et al.* 2008; Volkow *et al.* 2005). Other pharmaceutical compounds such as atomoxetine and guanfacine are known to increase norepinephrine/dopamine signaling to improve PFC function (Gamo *et al.* 2010; Bymaster *et al.* 2002). Atomoxetine does this by selectively inhibiting the NET to increase synaptic DA and NE levels, while guanfacine acts as an α_{2A} receptor agonist to inhibit cAMP signaling in dendritic spines, thereby strengthening synaptic inputs onto pyramidal neurons (Arnsten 2009a; Gamo *et al.* 2010; Bymaster *et al.* 2002). Despite the demonstrated ability of these compounds to improve PFC function, they have not yet been evaluated as a means for alleviating the neurological deficits associated with Mn exposure. Future research should address this issue to identify additional therapies and mechanisms by which developmental Mn exposure may contribute to cognitive and behavioral dysfunction.

Mn and the brain

Mechanisms of toxicity

Multiple hypotheses have been proposed regarding the mechanism(s) through which Mn may function as a neurotoxicant. One of the most prominent hypotheses relates to the ability of Mn to increase cellular oxidative stress, which some have suggested may partially involve mitochondrial dysfunction (Gunter *et al.* 2009; Gavin *et al.* 1999; Sarkar *et al.* 2017). Mn^{2+} is easily taken up by mitochondria into the mitochondrial matrix via the mitochondrial calcium uniporter (Gavin *et al.* 1999;

Kamer *et al.* 2018), where it may affect several mitochondrial processes, including impairing enzymatic activity of MnSOD, inhibiting other mitochondrial metabolic enzymes, or limiting mitochondrial respiration, such as complexes 1 and 2 of the electron transport chain (Fernandes *et al.* 2017; Galvani *et al.* 1995; Gunter *et al.* 2009; Neely *et al.* 2017; Yin *et al.* 2008). However, some have noted that many of the above in vitro studies on Mn-mitochondria interactions may have limited relevance to environmental Mn exposures due to the Mn concentrations used, which are substantially higher than those demonstrated to be neurotoxic in other studies (Bowman and Aschner 2014). To address this, Warren et al. (2020) recently used two Huntington's disease striatal cell lines with varying sensitivities to Mn exposure to determine Mn toxicity thresholds. Using doses ranging from 0-300 μM, above and below the cytotoxic threshold, Warren et al. observed no effect on markers of mitochondrial function for concentrations below the threshold, but did report declines in mitochondrial function only above the cytotoxic threshold (Warren *et al.* 2020). Notably, the authors concluded that mitochondrial dysfunction did not precede Mn cytotoxicity because impairment of mitochondrial function only occurred at Mn concentrations high enough to initiate cell death (Warren *et al.* 2020).

Alternative hypotheses to mitochondrial dysfunction have focused more directly on the ROS-generating potential of Mn in the brain. In brain regions with high concentrations of DA, for example, Mn^{3+} has been demonstrated in vitro to be effective at oxidizing DA, a process that can lead to the generation of radical quinones (Archibald and Tyree 1987). Brain regions where catecholamines are

synthesized can be especially prone to the production of ROS, in particular superoxide radicals, because of the adjacent hydroxyl groups on the catechol structure that are susceptible to autoxidation (Miller *et al.* 1996; Cohen and Heikkila 1974). Other studies, including those from our group, have reported that Mn^{3+} can inhibit aconitase (Reaney *et al.* 2002), and also disrupt iron regulation and may contribute to oxidative stress via higher levels of labile iron (Kwik-Uribe and Smith 2006; Kwik-Uribe *et al.* 2003). In vivo studies have also demonstrated the ability of Mn exposure to cause oxidative stress. For example, Desole et al. (1994) showed that a 7-day consumption of oral Mn by adult rats led to heightened concentrations of ascorbic acid and glutathione (GSH), suggesting a greater need for antioxidants in response to larger amounts of ROS. Non-human primates exposed to inhaled Mn experienced decreased GSH levels, accompanied by increases in metallothionine, another marker of oxidative stress (Erikson *et al.* 2008; Erikson *et al.* 2002). Mn exposure can also activate apoptosis in in vitro models, which may contribute to Mn-induced cell death or neurodegeneration in brain regions prone to Mn accumulation (Chen *et al.* 2019; Schrantz *et al.* 1999; Hirata *et al.* 1998).

Brain regions impacted by Mn exposure

Following decades of research into the mechanisms of Mn neurotoxicity, there is substantial evidence that certain brain regions are targeted more selectively than others in the presence of Mn. Once absorbed, Mn tends to disproportionately affect the basal ganglia, a collection of nuclei composed of the caudate nucleus, putamen,

globus pallidus, subthalamic nucleus, and substantia nigra, as well as the hypothalamus and cerebral cortex (Yamada *et al.* 1986; Karki *et al.* 2013); within these subregions, the globus pallidus has been reported as the principal area for accumulation (Aschner *et al.* 2007; Yamada *et al.* 1986). These brain regions are noteworthy because of their associated domains of function with respect to neurobehavioral and cognitive processes. The basal ganglia plays an important role in the regulation of motor coordination and synthesis of the neurotransmitters that underlie movement-related neuronal circuitry (Struve *et al.* 2007).

Recent research into the neurobiological causes of attention-based disorders, like those seen in individuals developmentally exposed to Mn, has highlighted the importance of prefrontal cortical function in cognitive processes (Arnsten and Li 2005; Arnsten 2009a). Neurocircuitry within the prefrontal cortex (PFC) is hypothesized to be largely dependent on the catecholaminergic systems that innervate this brain region (Arnsten and Dudley 2005; Lee and Solivan 2008).

Impact of Mn exposure on the catecholaminergic system

Before describing the evidence that links Mn exposure with catecholaminergic dysfunction, it is important to first understand the role of catecholamines, such as dopamine (DA) and norepinephrine (NE), in relation to the PFC. DA is synthesized from the precursor molecule 3,4-dihydroxyphenylalanine (Levodopa) following decarboxylation by the enzyme aromatic amino acid decarboxylase (Lachowicz and Sibley 1997). Within the brain, the ventral tegmental area (VTA) and substantia nigra

are especially high in DA, as they are the primary regions where DA synthesis occurs (Seamans and Yang 2004). Once synthesized, DA primarily binds to the two classes of DAergic receptors: D1-like (D1, D5) and D2-like (D2, D3, D4) (Arnsten and Li 2005). All DA receptors are G-protein-coupled receptors (GPCRs) that are metabotropic in nature and contain seven transmembrane domains (Lachowicz and Sibley 1997). Binding of the DA molecule to a DA receptor triggers different metabolic pathways depending on which receptor is activated. For example, stimulation of D1-like receptors creates an excitatory input that activates adenylyl cyclase and subsequently increases intracellular levels of cyclic adenosine monophosphate (cAMP); Stimulation of D2-like receptors has an opposing effect in which adenylyl cyclase activity in inhibited, thereby inhibiting production of cAMP (Lachowicz and Sibley 1997). Synaptic DA concentrations are primarily regulated by levels of release and the action of the DA transporter (DAT) to reuptake DA into the presynaptic neuron (Reith, 1997). DA receptor distribution studies of the rodent and non-human primate PFC have established that levels of mRNA and receptor binding sites are significantly higher for the D1 receptor than any other DA receptor subtype (Lidow *et al*. 1991; Goldman-Rakic *et al.* 1992; Farde *et al.* 1987; Gaspar *et al.* 1995).

There is clear evidence that developmental Mn exposure has the ability to modulate and impair catecholaminergic systems, in which altered DAergic function has been demonstrated in numerous studies (Kern *et al.* 2010; Kern and Smith 2011). Miller et al. (1996) first noted that in vitro Mn could lead to the direct autoxidation of

DA. Studies in non-human primates indicate that very elevated exposures to Mn via inhalation of concentrations greater than 300 mg/kg can lead to reductions in striatal DA (Bird *et al.* 1984). Research from our group has shown that postnatal Mn exposure from PND 1-21 in rats caused altered expression of D1, D2, and DAT in various brain regions including the striatum, nucleus accumbens, and PFC (Kern *et al.* 2010). Under these conditions, D1 and DAT expression was significantly reduced in the striatum and nucleus accumbens, whereas D2 expression was significantly increased in these same areas, as well as the PFC (Kern *et al.* 2010). McDougall et al. (2008) used an identical window of exposure at a higher dose of 750 μg Mn/day, and similarly found that reduced DAT expression in the striatum and nucleus accumbens. DAT may be especially important for mediating Mn accumulation in the brain based on studies involving inhibition of the DAT protein. One study using adult DAT-knockout (DAT-KO) mice demonstrated that intraperitoneal injection of 50 mg Mn/kg body weight resulted in a 40% reduction of accumulated Mn in the striatum of DAT-KO mice relative to wild-type individuals (Erikson *et al.* 2005). A similar study showed that Mn-exposed mice treated with the DAT-inhibitor, GBR12909, accumulated 60% less Mn in the globus pallidus, relative to Mn-exposed mice that did not receive the same inhibitor, providing further evidence that the DAT can contribute substantially to Mn uptake in certain brain regions (Anderson *et al.* 2007).

It is also noteworthy that a number of Mn-DA studies have reported some inconsistent results, possibly due to differences in exposure regimens (i.e., duration, dose, and route, along with species and age of experimental animals). For example,

Eriksson et al. (1992) observed a decrease in postsynaptic D2-like receptors in the brains of non-human primates following inhalation of Mn, while Nam and Kim (2008) found that striatal D2 receptor levels in mice increased in a dose-dependent manner following intraperitoneal Mn exposure. Inconsistencies have also been reported in studies of evoked DA release in Mn-exposed animals, and these may similarly result from varying exposure regimens. Despite the substantial amount of evidence on the topic of Mn-induced toxicity of DAergic neurons, more research focused on the use of neonatal exposure regimens in the PFC is necessary to address the knowledge gap regarding the specific mechanisms by which DAergic insult, and altered D1 receptor levels in particular, may result from developmental Mn exposure.

In contrast to DA, the majority of NE is synthesized in the locus coeruleus by DA β-hydroxylase and interacts principally with noradrenergic α_1, α_2, β_1, β_2, and β_3 receptor families, all of which are GPCRs (Samuels and Szabadi 2008). Synaptic concentrations of NE are regulated by levels of release and subsequent reuptake by the norepinephrine transporter (NET), a transmembrane protein that has also been shown to uptake DA in regions with low levels of DAT (Mandela and Ordway 2006; Moron *et al.* 2002). The α_2 receptor subtypes have been the focus of many studies involving PFC function, with a particular emphasis on the role of α_{2A} receptors, which are primarily postsynaptic to NE-producing cells on the dendritic spines of PFC pyramidal neurons (Wang *et al.* 2007; Arnsten 2009a). Part of the beneficial function of α_{2A} receptors in the PFC has been attributed to their ability to strengthen synaptic connections by closing "leaky" ion channels adjacent to the synapses on

dendritic spines (Wang *et al.* 2007; Arnsten and Dudley 2005). Multiple studies have shown that inhibition of α_{2A} receptors has been linked to PFC dysfunction relating to deficits in working memory, attention, and hyperactivity in a profile comparable to ADHD (Ma *et al.* 2005; Ma *et al.* 2003; Li *et al.* 1994). Based on the similarities in attentional and cognitive deficits observed in both Mn-exposed children and individuals with inhibited α_{2A} receptor function, there is reason to believe that α_{2A} receptor dysfunction may partially underlie the attentional deficits seen in Mn exposed children and animal models (Arnsten and Li 2005; Arnsten and Dudley 2005; Arnsten and Pliszka 2011).

Studies examining the effect of Mn exposure on NEergic function are scarce compared to those on DAergic systems, but some informative observations have been made. Several investigators have reported elevated tissue levels of NE in the rat whole brain (Chandra *et al.* 1979) and brain stem/hypothalamus (Autissier *et al.* 1982) in response to intraperitoneal injection of Mn. Alternatively, some *in vitro* studies have shown that Mn exposure decreased DA but not NE-uptake in a dose-dependent manner in synaptosomes collected from rat forebrains (Lai *et al.* 1982) (Lai et al. 1982) and wholebrains (Chandra and Shukla 1981). The only study to examine the specific association between Mn exposure and noradrenergic α_2 receptors showed that rats exposed to Mn after weaning for five weeks exhibited a two-fold decrease in both protein and mRNA levels of the α_2 receptor in the locus coeruleus and substantia nigra, along with a decrease in extracellular NE in the caudate-putamen (Anderson *et al.* 2009). Given the limited and inconsistent evidence

between Mn exposure and NEergic dysfunction, more research is needed in order to address the knowledge gap on the impact of developmental Mn exposure on NEergic function in the PFC, with a particular focus on the α_{2A} receptor.

Research Questions

My research aims to determine the potential for using bone as a biomarker of long-term Mn exposure, and whether bone mineral is altered by such exposures, in addition to furthering the understanding of how developmental postnatal Mn exposure causes attentional and behavioral deficits involving the catecholaminergic systems. As a result of my research, I have identified specific neuronal and glial cell subtypes that are altered by elevated Mn exposure, as well as changes to bone tissue properties, both structural and functional in nature, with strong relevance to Mn exposure in humans. These findings provide novel insights related to improving public knowledge of the risks of elevated Mn exposure, both in early postnatal life and throughout the lifespan, that should be considered when shaping policies to protect human health in the future.

In my first research chapter, we used the our established rodent model of early childhood oral Mn exposure to investigate the relationship between oral Mn exposure and tissue Mn levels in blood, brain, and bone in the context of early postnatal vs. lifelong Mn exposure. Specifically, this study examined 1) whether Mn accumulates in bone tissue over the lifespan in early postnatal vs. lifelong Mn exposure, 2) the extent to which bone Mn levels were associated with elevated Mn levels in the brain,

and 3) whether elevated bone Mn altered the mineral structure or physical properties of bone. We also measured the tissues levels of skeletal Mn in an aged human population from regions impacted by historic ferromanganese alloy industrial activity to determine the average bone Mn levels in environmentally-exposed adults. In the latter example, we contrasted bone Mn with bone lead levels as point of comparison, as lead represents a well-studied heavy metal that is known to accumulate in bone tissue. Overall, these data further elucidate the effect of elevated Mn exposure on bone tissue across the lifespan and establish the potential diagnostic benefits of using bone as a biomarker of Mn exposure.

In my second research chapter, we used the same rodent model of early childhood oral Mn exposure as mentioned above to determine whether Mn causes lasting disruption to the mPFC catecholaminergic systems, using 1) quantitative protein immunohistochemistry measures of tyrosine hydroxylase (TH), DAT, NET, DA D1 and D2 receptors, and the α_{2A} adrenergic receptor, 2) microdialysis for evoked DA and NE release, and 3) quantification of dendritic spine density on pyramidal mPFC neurons. We also determined whether changes in mPFC catecholaminergic systems were associated with heightened behavioral reactivity in an open field behavioral paradigm. To the best of my knowledge, this was additionally the first study to examine the impact of early postnatal Mn exposure on astrocyte reactivity in distinct astrocytic phenotypes (A1 vs A2 astrocytes). This was accomplished by assessing astrocyte reactivity based on protein levels of glial fibrillary acidic protein (GFAP), complement C3, and S100A10, the latter two used

as markers of reactive astrocytes an A1 proinflammatory or A2 anti-inflammatory phenotype, respectively.

Finally, this dissertation also includes some previously unpublished proteomics data from our group which used a cell model of DA-producing undifferentiated PC12 (rat pheochromocytoma) cells as an *in vitro* model to examine the potential mechanisms by which early life developmental Mn exposure may contribute to neurotoxicity in DAergic-like cells (Appendix, pg. 171). My contributions to the proteomics work involved expanding on the analysis of the protein-specific changes and conducting a literature review of the altered proteins to improve the functional understanding of how the Mn-altered proteins may contribute to neurological deficits in humans. Specifically, undifferentiated PC12 cells were exposed to 100 μM $MnCl_2$ for 24 hours (n=4-6 independent replicates), followed by assessment of cellular Mn levels, cytotoxicity (i.e., lactate dehydrogenase, ATP, isoprostanes), DA metabolites, and changes in protein expression identified using a 2-D differential in-gel electrophoresis (DIGE) method. Proteins that were significantly altered by Mn exposure were selected from the gel and analyzed by MALDI-mass spectrometry to determine their identity. Cellular Mn levels increased ~100-fold following Mn exposure, with no measurable change in cell viability, lactate dehydrogenase, ATP, or 8-isoprostanes. Levels of cellular DA were significantly reduced by 20% relative to controls, while the DA metabolites DOPAC and homovanillic acid (HVA) increased 45-145% over controls. There were no changes in cellular serotonin levels. A total of 46 proteins (out of ~600) showed changes in

expression as a result of Mn exposure, (25 increased, 21 decreased), and 30 of these proteins were positively identified. Multiple proteins involved in DA metabolism (catechol-O-methyltransferase 1, COMT-1), packaging (secretogranin-II), and protein degradation (ubiquitin C-terminal hydrolase L1, UCH-L1) were significantly altered by Mn exposure. COMT-1 increased by 47%, secretogranin-II (all 7 measured isoforms) decreased by 40%, but UCH-L1 increased by 90%, relative to controls. All protein changes were confirmed by Western blot analyses. Overall, these data suggest that elevated Mn exposure may result in reduced packaging and elevated turnover of cellular DA, and potentially alter DAergic function without causing overt cytotoxicity. The increased expression of UCH-L1 following Mn exposure may suggest a potential mechanism of Mn-related loss of DAergic cells in the context of Mn-induced Parkinsonism.

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CHAPTER 2: TARGET AND EXPOSURE BIOMARKERS: BLOOD, BRAIN, AND BONE MANGANESE ACROSS THE LIFESPAN

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Abstract

Studies have established associations between environmental and occupational manganese (Mn) exposure and executive and motor function deficits in children, adolescents, and adults. These health effect risks from elevated Mn exposure underscore the need for effective exposure biomarkers to improve exposure classification and help detect and diagnose Mn-related impairments. This is particularly true for children and adolescents, because the risk of elevated exposure and associated health effects changes over the lifespan. Here, neonate rats were orally exposed to 0, 25, or 50 mg Mn/kg/day during early life (PND 1-21) or lifelong through ~PND 500 to determine the relationship between oral Mn exposure and blood, brain, and bone Mn levels over the lifespan, whether Mn accumulates in bone, and whether elevated bone Mn altered the local atomic and mineral structure of bone using synchrotron-based X-ray analyses (XRD, XANES, and EXAFS), or its biomechanical properties using electro-servo-hydraulic testing. Additionally, we assessed bone Mn levels in aged humans (age 41-93; female, n=30; male, n=19) living in regions impacted by historic ferromanganese alloy plant activity. The animal

studies show that blood, brain, and bone Mn levels naturally decrease across the lifespan in the absence of elevated Mn exposure. With elevated exposure, bone Mn levels are strongly associated with blood Mn levels, are more sensitive/responsive to elevated exposures than either blood or brain Mn, and that Mn did not accumulate with lifelong elevated exposure in bone, brain, or blood. Further, elevated early life Mn exposure producing bone Mn levels as high as 166 μg/g caused some changes in mineral properties including altered local atomic structure of bone hydroxyapatite, along with some biomechanical changes in bone stiffness in weanlings or young adult animals. In aged humans, bone Mn levels were universally very low (ranging from 0.014 – 0.17 μg/g), and decrease with age, but do not vary based on sex or female parity history. In contrast with Pb, bone Mn showed no evidence of accumulation over the lifespan, and therefore may not be a biomarker of cumulative long-term exposure. Collectively, these findings indicate that bone may be a useful biomarker of recent ongoing oral Mn exposure in humans, and that bone may be a relatively minor target of elevated Mn exposure, based on the limited functional alterations reported here.

1.0. Introduction

Environmental manganese (Mn) exposure is a growing public health concern in the U.S. and other countries due to expanding evidence that children may be exposed to harmful levels of Mn exposure from multiple sources, including drinking water (Wasserman *et al.* 2006b; Bouchard *et al.* 2007; Bouchard *et al.* 2011), soil and

dust (Gunier *et al.* 2014a; Gunier *et al.* 2014b; Lucchini *et al.* 2012a), and their diet (Crinella 2012). Recent epidemiological studies have shown that elevated Mn exposure is associated with reductions in Full Scale IQ and verbal comprehension, along with impaired attention, impulse control, and fine motor function in children and adolescents (Mora *et al.* 2018; Oulhote *et al.* 2014; Ericson *et al.* 2007; Bouchard *et al.* 2011). Occupational exposures from mining, dry-cell battery production, and ferromanganese alloy plants have also been associated with adverse health outcomes in adults, including Mn-induced parkinsonism and other neurodegenerative conditions (Lucchini *et al.* 1999; Smith *et al.* 2007; Long *et al.* 2014a; Kwakye *et al.* 2015).

The improved understanding of the health risks from elevated Mn exposure have led to an increased need for effective exposure biomarkers to help detect and diagnose Mn-related impairments, especially given that children are more vulnerable to elevated exposures and the neurotoxic effects of Mn than adults, and exposure risk changes over the lifespan (Ljung and Vahter 2007; Erikson *et al.* 2007; Kern *et al.* 2010; Mora *et al.* 2015a). Reported biomarkers for Mn exposure have included blood, hair, saliva, urine, nails, and teeth (Gil *et al.* 2011; Arora *et al.* 2012b; Laohaudomchok *et al.* 2011; Ward *et al.* 2018; Claus Henn *et al.* 2010; Haynes *et al.* 2015; Butler *et al.* 2019; Mora *et al.* 2018; Lucchini *et al.* 2012a; Mora *et al.* 2015a; Oulhote *et al.* 2014). Blood and urine Mn levels appear to reflect only recent exposures over the span of several days to weeks (Järvisalo *et al.* 1992; Cowan *et al.* 2009; Smith *et al.* 2007), while hair and nail Mn levels have been reported to reflect

exposures on the scale of ~3-12 months (Eastman *et al.* 2013; Reiss *et al.* 2015; Jursa *et al.* 2018; Laohaudomchok *et al.* 2011; Ward *et al.* 2018). Teeth or skeletal Mn levels may reflect exposures over longer periods of months to years, based on recent studies (Claus Henn *et al.* 2018; Rolle-McFarland *et al.* 2018; Austin *et al.* 2017; Horton *et al.* 2018; Arora *et al.* 2012a).

The extent that candidate Mn exposure biomarkers are associated with adverse health outcomes, such as cognitive and behavioral deficits, is mixed (Haynes *et al.* 2015; Mora *et al.* 2015a; Lucchini *et al.* 2012a; Lucchini *et al.* 1999; Smith *et al.* 2007). For example, some studies have reported associations between Mn levels in hair, blood, and teeth with cognitive or behavioral impairments (Menezes-Filho *et al.* 2011; Haynes *et al.* 2015; Mora *et al.* 2015a), while others have reported no association between blood and urinary Mn with health outcomes (Lucchini *et al.* 2012b; Lucchini *et al.* 1999; Smith *et al.* 2007). In the case of tooth Mn levels, recent studies suggest that higher prenatal dentine Mn levels are associated with improved visual spatial abilities, impulse control and attentional function, whereas higher postnatal dentine Mn levels are associated with no, or adverse neurobehavioral effects, in children depending on age, sex, and outcome (Bauer *et al.* 2017; Horton *et al.* 2018; Claus Henn *et al.* 2018; Mora *et al.* 2015a). These differences across studies in the extent that the Mn exposure biomarker(s) are associated with adverse health effects may result in part from exposure misclassification, further underscoring the need for an improved understanding of Mn exposure and exposure biomarkers over the lifespan.

Bone Mn represents a potential biomarker of long term Mn exposure, as Mn levels have been shown to increase in bone during developmental periods, and Mn in bone has been estimated to account for roughly 40% of body Mn (Aschner and Aschner 2005; Andersen *et al.* 1999; O'Neal *et al.* 2014). The basis for Mn incorporation into bone mineral may be due in part to Mn^{2+} serving somewhat as a biologic analog to Ca^{2+} (Frausto da Silva and Williams 2001). This Mn^{2+} - Ca^{2+} relationship in mineralized tissues may be similar to the well-established relationship between $Pb^{2+}$and Ca^{2+}, which leads to the accumulation of lead in mineralized tissues and the utility of bone and tooth lead levels as biomarkers of cumulative lead exposure (Hu 1998; Smith *et al.* 1996; Téllez-Rojo *et al.* 2004; Specht *et al.* 2016; Arora *et al.* 2012b). Moreover, recent technological advances have led to the development of portable neutron activation systems for *in vivo* assessment of bone Mn levels in humans (Pejović-Milić *et al.* 2009; Liu *et al.* 2013; Rolle-McFarland *et al.* 2018), suggesting the emerging feasibility of assessing bone Mn levels as an exposure biomarker to complement other tissue measures currently in use. Additionally, the fact that Mn is an essential nutrient that plays a role in skeletal development and maintenance, while lead serves no essential biological function may inform differences in bone-lead vs. bone-Mn interactions (Andersen *et al.* 1999; O'Neal *et al.* 2014; Aschner and Aschner 2005). If Mn accumulates in bone with elevated exposure, similar to lead, bone Mn may prove to be an informative biomarker to assess Mn body burden over the lifespan.

Here, we investigated the relationship between oral Mn exposure and tissue Mn levels over the lifespan in a rodent model of early postnatal vs. lifelong Mn exposure. Specifically, we determined whether Mn accumulates in bone over the lifespan, the extent that bone Mn levels were associated with elevated Mn levels in the brain, and whether elevated bone Mn altered the mineral structure or physical properties of bone. We also assessed the levels of skeletal Mn in aged humans living in regions impacted by historic ferromanganese alloy plant activity to determine typical bone Mn levels in environmentally-exposed adults. Finally, regarding accumulation of Mn into bone over the lifespan and the utility of bone Mn as an integrative biomarker of Mn exposure, we define the term "accumulation" as a net increase in bone Mn levels over time with steady-state exposure. Collectively, these findings further elucidate the impact of Mn exposure on bone tissue across the lifespan and establish the potential benefits of using bone as biomarker of Mn exposure.

2.0. Materials and Methods

2.1. Rodent subjects

All subjects were born in-house from nulliparous timed-pregnant Long Evans rats (obtained from Charles River on gestational age 18 d). Twelve to 24 hours after parturition (designated PND 1, birth = PND 0), litters were sexed, weighed, and culled to eight pups per litter such that each litter was composed of five to six males and the remainder females. Only one male per litter was assigned to a particular Mn

treatment condition. Animals (dams and weaned pups) were fed Harlan Teklad rodent chow #2018 (reported by the manufacturer to contain 118 mg Mn/kg) and housed in polycarbonate cages at a constant temperature of 21 ± 2°C. At PND 22, all pups were weaned and pair-housed (two rats per cage) with an animal of the same Mn treatment group and maintained on a reversed 10:14 hr light/ dark cycle. Animals reported in the present study were littermates of animals that underwent behavioral testing for attentional, impulse control, and fine motor functions over ~PND 30 – 120 (animals sacrificed on PND 24 and 66), or were the behaviorally tested animals that were sacrificed following microdialysis measurement of brain neurotransmitter levels prior to sacrifice (median PND 490, range PND 292-889); findings from the behavioral and microdialysis studies are reported elsewhere (Beaudin *et al.* 2017a; Beaudin *et al.* 2017b; Beaudin *et al.* 2015; Beaudin *et al.* 2013; Lasley *et al.* 2020). Males were exclusively used because studies have suggested that males may be more sensitive than females to developmental Mn neurotoxicity (Kern et al. 2010; Lucchini et al. 2012; Takser et al. 2003), and attentional dysfunction is two to three times more prevalent in boys than girls (Feldman and Reiff 2014; Willcutt 2012). All animal care and treatments were approved by the institutional IACUC and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals.

Mn exposure:

Neonatal rats were orally exposed to Mn doses of 0, 25, or 50 mg Mn/kg/d starting on PND 1 through weaning on PND 21 (early postnatal Mn exposure), or throughout life until the end of the study. For dosing over PND 1–21, Mn was delivered once daily directly into the mouth of each pup (~20 μL/dose) via a micropipette fitted with a flexible polyethylene pipet tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received the vehicle solution. For the Mn dosing solution, a 225 mg Mn/mL stock solution of $MnCl_2$ was prepared by dissolving $MnCl_2 \cdot 4H2O$ with Milli-Q™ water; aliquots of the stock solution were diluted with a 2.5% (w/v) solution of the natural sweetener stevia to facilitate oral dosing of the pups. Oral Mn exposure post-weaning (PND 22 – end of study) occurred via the animals' drinking water. For this, a 42 mg Mn/mL stock Mn solution was prepared as above and diluted with tap water to a final concentration of 420 μg Mn/mL in a polycarbonate carboy. The stock solutions were made fresh weekly, and water bottles were refilled with fresh water two to three-times per week. Water bottle weights were recorded at refilling to determine water intake per cage, and daily Mn intake per kg body weight was estimated based on daily measured body weights of the two rats housed per cage. Drinking water Mn concentrations were adjusted weekly as needed to maintain target daily oral Mn intake levels of 25 or 50 mg/kg/d based on measured water intake rates. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both; pre-weaning exposure to 50 mg Mn/kg/d produces a relative increase in Mn intake that approximates the increase reported in infants and young children exposed to Mn-contaminated water or soy-

based formulas (or both) (Kern and Smith 2011; Beaudin *et al.* 2017a; Beaudin *et al.* 2015; Beaudin *et al.* 2013; Kern *et al.* 2010). Chronic oral exposure to the same daily Mn dose was maintained after weaning via drinking water to model the situation where children may continue to suffer chronic elevated Mn exposures from a variety of environmental sources (e.g., contaminated well water, dust, etc.) (Lucas *et al.* 2015; Bouchard *et al.* 2011; Oulhote *et al.* 2014).

2.2. Human subjects

Forty-nine subjects (30 female, 19 male) scheduled to undergo hip joint replacement due to hip osteoarthritis or femur head fracture (International Classification of Disease codes M16 and S72, respectively) consented to provide a bone and blood sample for metal analysis. The subjects resided in one of three geographically distinct sites within the province of Brescia, Italy: Vallecamonica, an area with historical ferromanganese alloy production for over a century that ended in 2001; Bagnolo Mella, an area with currently active ferromanganese alloy industrial activity since 1974; or Garda Lake, a tourist region with no history of ferromanganese alloy activity (Lucchini et al. 2007). The mean age of participants was 82.1 ± 9 years for females and 74.0 ± 10 years for males. Intact femoral head bone samples were collected from all 49 subjects, while 44 blood samples were collected several days before surgery (26 female, 18 male). In addition, parity history was obtained from all female subjects via questionnaire. The study was approved by the ethical committee of the responsible local health authority or hospital ("ASL Vallecamonica – Sebino,"

"Comitato Etico dell'Azienda Ospedaleria di Desenzano del Garda" and "Comitato Etico dell'Azienda Ospedaliera Spedali Civili di Brescia") and informed consent was obtained from each subject prior to sample collection.

2.3. Sample collection

Rat blood, brain, and bone tissues for Mn analyses were collected from PND 24, PND 66, and ~PND 490 rats (n = 10 – 16/treatment group and time point), as reported in Beaudin et al. (2013, 2015, 2017). Briefly, animals were euthanized via sodium pentobarbital overdose (75 mg/kg intraperitoneal injection) and exsanguination, and whole blood (2 – 3 mL) was collected from the left ventricle of the surgically-exposed heart and stored in EDTA Vacutainers at -20 °C for analyses. Whole brain was immediately removed, bisected into hemispheres, and the hind-brain regions of each hemisphere collected and stored at -80 °C for Mn concentration determinations (forebrain was dedicated to other outcome measures). The right and left femur was dissected free of the hindlimb and adherent soft tissue and periosteum removed with a stainless steel scalpel.

Human bone samples of the intact femoral head removed during hip arthroplasty were stored at at -20 °C in sterile polyethylene containers until processing for analyses. There was no visible sign of bone degeneration in any of the bone samples. Whole blood samples were collected with butterfly catheters into trace metal free vacutainers. Within a HEPA filtered-air laboratory, bone-core samples containing subchondral and trabecular regions were obtained by drilling through the

center of the femur head (anterior-posterior axis) using a custom-fabricated, hollow titanium alloy drill bit (5.4 mm internal diameter), as reported elsewhere (Smith *et al.* 1996).

2.4. Blood, brain, and bone tissues analyses for metal concentrations

Within a trace metal clean HEPA filtered-air laboratory, aliquots of rat or human whole blood were digested overnight at room temperature with 16N HNO_3 (Optima grade, Fisher Scientific), followed by addition of H_2O_2 and Milli-Q water. Digestates were centrifuged (13,000 x g for 15 min.) and the supernatant collected for Mn analysis. For rat brain, aliquots of homogenized hind-brain tissue (~200 mg wet weight) were dried to a constant weight at 65 °C then digested with hot 16 N HNO_3, evaporated and redissolved in 1 N HNO_3 for analyses. For rat bone, the right femur was bisected and any blood/bone marrow within the femur shaft was removed and the bone rinsed with ultrapure Milli-Q water. For human bone samples, ~1-2 mm thick sections of the femoral head bone cores were dissected from the cores using a stainless-steel scalpel to obtain analytical samples from within the bone core. Both rat and human bone samples were rinsed repeatedly with 1% quartz-distilled HNO_3 and ultrapure water, dried to a constant weight at 65 °C, then digested with hot 16 N HNO_3, evaporated and redissolved in 1 N HNO_3 for analyses. For Mn and Pb (human samples only) analyses, rhodium and thallium (human samples only) were added to sample aliquots as internal standards, and Mn and Pb levels determined using a Thermo Element XR inductively coupled plasma – mass spectrometer in low (Pb) or

medium (Mn) resolution, measuring masses ^{55}Mn, ^{208}Pb, ^{103}Rh, and ^{205}Tl (the latter two for internal standardization). External standardization for Mn and Pb used certified SPEX standards (Spex Industries, Inc., Edison, NJ). National Institutes of Standards and Technology SRM 1577b (bovine liver) and 1486 (bone meal) were used to evaluate procedural accuracy. The analytical detection limit for Mn in blood, brain, and bone was 0.018, 0.015, and 0.003 ng/mL, respectively, while the analytical detection limit for Pb in human bone was 0.005 ng/mL.

2.5. Synchrotron-based analyses using XRD, XANES, and EXAFS

Bone samples from rat femurs were analyzed at the Synchrotron Radiation Laboratory, Stanford University. X-ray diffraction (XRD) measurements were performed at an energy of 17600 eV on Beamline 7-2. Samples were pulverized and set into a 0.3 mm quartz capillary tube. XRD patterns were collected in $Q(A^{-1})$ space up to $Q=8\ A^{-1}$. X-ray absorption spectroscopy (XAS) experiments, including X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) were performed at beamline 11-2 in fluorescence mode with a 100-element Ge detector. Whole sections of the femur specimens were placed between two layers of Kapton tape and inside an aluminum holder. Samples were placed inside an environmental chamber with a continuous He gas flow to avoid scattering and absorption by air. Raw data treatment and quantitative analyses were performed using the Demeter platform (Ravel and Newville 2005).

2.6. Biomechanical testing of bone strength properties

Biomechanical testing was performed on PND 66 rat femurs, which were hydrated by soaking in 37°C Hanks' Balanced Salt Solution, HBSS; Sigma-Aldrich) for 12 h prior to testing. Each specimen was subjected to a three-point bending test; the bone was loaded such that the posterior surface was under tension and the anterior surface was under compression, using an MTS 831 electro-servo-hydraulic test machine (MTS Corp., Eden Prairie, MN). Each femur was loaded to failure at a displacement rate of 0.01 mm/s, and the load and displacement measured, the former using a calibrated 225 N load cell. After testing, a two-point average of the diameter and a six-point average of the cortical shell thickness were measured at the fracture site of each tibia using digital calipers with a 0.01 mm readout. The peak load (N) was recorded from the maximum load in each test. The corresponding yield and ultimate strengths of the central femurs (σ) were calculated, in units of Pa, from the standard equation for a beam in three-point bending:

$$\sigma = \frac{PLy}{4I}$$

where respectively, P is the load at yielding (i.e., at the onset of inelastic deformation) or the maximum load reached during the bending test; L is the major span between the loading support pins; y is the distance from the center of mass; and I is the moment of inertia of the cross-section. The stiffness was measured in terms of the initial elastic slope of the load-displacement curve. In addition, the toughness (work to failure, W_f) was calculated from the load–displacement curve as the work to fracture (energy absorption); specifically, W_f was defined (in units of kJ/m^2) as the

area under the load–displacement curve divided by twice the projected area of the fracture surface. All tests were done blinded to experimental treatment condition.

2.7. Statistical analysis and experimental design

Blood and brain Mn level data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's *post hoc* test for pairwise comparisons. Data were log10 transformed before analysis if necessary to achieve normal distribution and variance homogeneity. In all cases, the significance level was set at $p \leq 0.05$. Tissue biomarker correlations were evaluated by generating Pearson's correlations between blood, brain, and bone across all ages and treatment groups. The slope from each Pearson's correlation output was used as the measure for comparison.

3.0. Results

3.1. Tissue Mn decreases across the lifespan without elevated Mn exposure

In order to determine how natural changes in tissue Mn levels across the lifespan, in the absence of elevated Mn exposure, might affect tissue Mn levels in the presence of elevated exposure, we first assessed the concentrations of blood, brain, and bone Mn at PND 24, 66, and ~500 in control rats. Overall, Mn levels in all measured tissues decreased from early post-weaning life through adulthood, with reductions in tissue Mn most pronounced between PND 24 post-weaning and PND 66 young adulthood (Figure 1a). This was evidenced by a main effect of age on blood Mn [$F(2, 37) = 430$, $p < 0.0001$], reflecting significant differences between PND 24, 66, and ~500 groups (p's < 0.0001). The mean blood Mn concentration naturally

decreased ~60% from PND 24 (24.2 ± 0.79 ng/mL) to PND 66 (9.76 ± 0.28 ng/mL), and decreased an additional ~40% from PND 66 to PND ~500 (5.76 ± 0.28 ng/mL), for an overall reduction of 76% from PND 24 to ~500 (Figure 1a).

For brain Mn levels there was also a significant main effect of age [$F(2, 34) = 145$, $p < 0.0001$], reflecting a significant reduction of ~40% between PND 24 (3.61 ± 0.12 µg/g) and PND 66 (2.13 ± 0.031 µg/g) ($p < 0.0001$). Thereafter, there was a small nonsignificant decrease of ~8 % from PND 66 to PND 500 (1.95 ± 0.063 µg/g) ($p = 0.17$). Overall, mean brain Mn levels decreased by 46% from PND 24 to ~500 ($p < 0.0001$) (Figure 1b). Natural age-related changes in bone Mn levels showed a similar pattern as brain, with a significant main effect of age [$F(2, 44) = 63.2$, $p < 0.0001$] that was driven by a significant reduction of ~75% from PND 24 (2.66 ± 0.30 µg/g) to PND 66 (0.65 ± 0.062 µg/g) ($p < 0.0001$), but no further reduction from PND 66 to ~500 (0.59 ± 0.064 µg/g) ($p = 0.95$). Overall, bone Mn levels significantly declined by ~78% from PND 24 to PND ~500 ($p < 0.0001$) (Figure 1c).

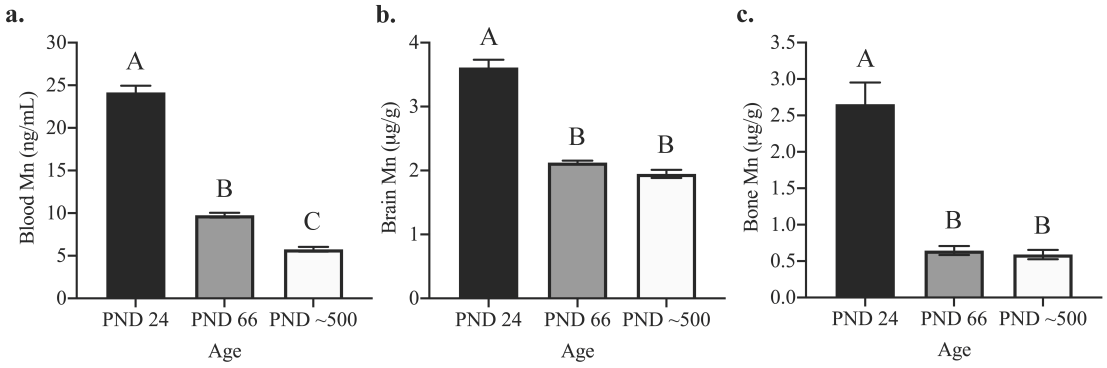


Figure 1. Tissue Mn naturally decreases across the lifespan without elevated Mn exposure. Bar chart shows tissue Mn levels for (**a**) blood

(ng/mL), (**b**) brain (μg/g dry weight), and (**c**) bone (μg/g dry weight) in control animals that received no elevated Mn exposure (n = 10-16 animals/treatment group). Data are least squares means ± SEM values generated from one-way ANOVA. Bars with different superscripts are statistically different ($p < 0.05$), based on Tukey's multiple comparisons test.

3.2. Tissue Mn levels do not accumulate across the lifespan in the presence of elevated Mn exposure

To determine how tissue Mn levels change, and in particular whether Mn accumulates across the lifespan in the presence of continued elevated oral exposure, we measured Mn concentrations in blood, brain, and bone in weanling (PND 24), young adults (PND 66), and aged adults (~PND 500) exposed to different levels of Mn (0, 25, or 50 mg Mn/kg/day) for different exposure durations (early life from PND 1-21, or lifelong from PND 1 through PND 66 or ~PND 500).

Comparison between Mn groups within age groups

First, to determine how Mn exposure restricted to the pre-weaning period or continuous throughout life affects tissue Mn levels at different life stages, we performed statistical analyses on tissue Mn levels within the post-weaning, young adult, and aged adult age groups. There was a significant main effect of oral Mn exposure to increase blood, brain, and bone Mn levels at each of the three lifestages (ANOVA results Supplement 1, Table 1). Specifically, at PND 24, blood, brain, and bone Mn levels in the early life 25 and 50 groups were significantly higher than controls (p's < 0.0001, Figure 2, lowercase superscripts). However, when comparing the differences between the 25 and 50 Mn dose groups at PND 24, only blood Mn

levels in the early 50 group were significantly higher than the early 25 ($p = 0.044$), while brain and bone levels in these two groups trended towards being significantly different ($p = 0.066$ and $p = 0.065$, respectively). In the PND 66 young adult animals, all four Mn exposure groups were significantly elevated vs. controls for blood (p's < 0.048) and bone (p's < 0.001), while for brain only the early life 50 and lifelong 25 and 50 groups were higher than controls (p's < 0.024; early life 25 vs controls, $p = 0.86$). At the ~PND 500 aged adult life stage, differences between the lifelong and early life exposure groups became apparent, relative to controls, with the lifelong 25 and 50 groups being significantly elevated over both controls and their early life exposure group counterparts for blood (p's < 0.012) and bone (p's < 0.047). For brain, only the lifelong 25 and 50 groups were significantly different than controls (p's <0.0001).

Tissue Mn levels decline from weaning to young adulthood, even in the presence of ongoing elevated Mn exposure

Comparison within tissues across ages

Next, we determined how exposure impacts tissue Mn levels in each tissue (blood, brain, and bone) *across* the lifespan. Initially, we performed ANOVA to assess how early life Mn exposure over PND 1- 21 impacts tissue Mn levels in PND 24 weanling versus PND 66 young adult animals. There was a significant main effect of age on tissue Mn levels for blood [$F(5, 81) = 279$, $p < 0.0001$], brain [$F(5, 80) =$

91.5, $p < 0.0001$], and bone [F(5, 81) = 154, $p < 0.0001$], largely reflecting the much higher tissue Mn levels in PND 24 weanling animals compared to their PND 66 counterparts (Figure 2, bars with *). The interaction of age x Mn exposure group was also significant for all three tissues (p's < 0.0001), reflecting that differences in tissue Mn levels between Mn exposure group was significant for the PND 24 versus PND 66 animals exposed over PND 1-21. For example, blood Mn levels in PND 66 animals exposed to 25 or 50 mg Mn/kg/day over early life (PND 1 – 21) were significantly lower than their PND 24 counterparts (p's < 0.0001). This same pattern was observed for brain and bone, which similarly showed significant reductions in tissue Mn concentrations in PND 66 versus PND 24 animals following early life Mn exposure (p's < 0.0001).

Subsequently, we performed ANOVA to determine how continued lifelong exposure affected tissue Mn levels in PND 66 young adults compared to their PND 24 counterparts. For this analyses, only the lifelong Mn exposure groups at PND 24 and 66 were included. There was again a significant main effect of age on tissue Mn levels for blood [F(5, 81) = 250, $p < 0.0001$], brain [F(5, 81) = 87.4, $p < 0.0001$], and bone [F(5, 81) = 145, $p < 0.0001$], as well as a significant age x Mn exposure group interaction (p's < 0.0001 for all three tissues). These results reflect that there were significant reductions in tissue Mn levels from PND 24 to PND 66, despite the continuous oral Mn exposure over this time (p's < 0.0001) (Figure 2, bars with *).

Tissue Mn levels decline from young to aged adulthood, even in the presence of ongoing elevated oral Mn exposure

Comparison within tissue across ages

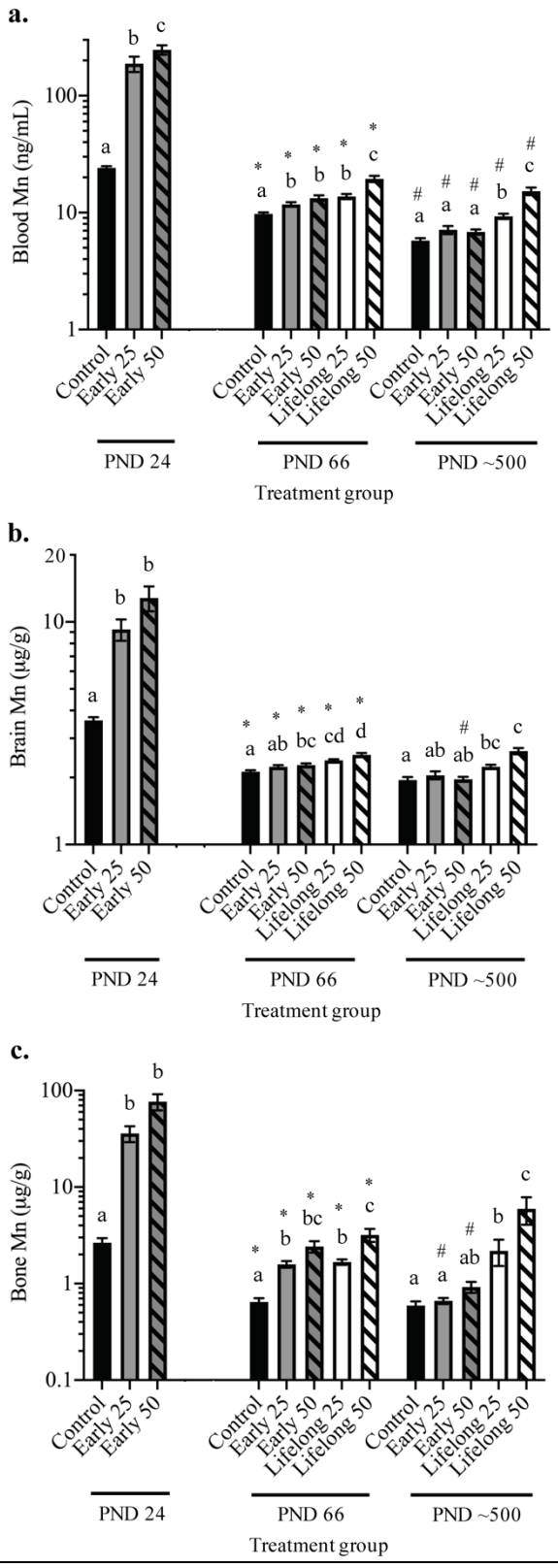
In light of the substantially greater impact of oral Mn exposure to increase tissue Mn levels in PND 24 weanlings versus their PND 66 counterparts, and specifically to determine whether prolonged oral Mn exposure resulted in the accumulation of higher tissue Mn levels in aged versus young adults, we performed subsequent analyses specifically comparing tissue Mn concentrations in the PND 66 young adult and ~PND 500 aged adult animals exposed to oral Mn throughout their lifespan. The main effects of age and Mn exposure were again significant for blood [$F(9, 157) = 13.8$, $p < 0.0001$], brain [$F(9, 143) = 12.7$, $p < 0.0001$], and bone [$F(9, 176) = 17.3$, $p < 0.0001$]. However, the age x Mn exposure interaction was only significant for brain ($p = 0.034$) and bone ($p = 0.0054$), but not blood ($p = 0.14$). The significant age x Mn interaction for brain and bone tissues reflect the fact that Mn levels in these tissues of one or both of the early life Mn exposure groups in the aged ~PND 500 animals were significantly lower than their PND 66 counterparts, while tissue Mn levels in the PND 66 and ~PND 500 continuous lifelong exposure groups were not measurably different. In contrast, the non-significant age x Mn exposure interaction for blood reflects that blood Mn levels measurably declined from PND 66 to ~PND 500 in both the early life and lifelong Mn treatment groups, even though the latter aged adult group continued to receive daily oral Mn exposure in their drinking

water (Figure 2, # indicating significantly different from PND 66 counterpart). In fact, when comparing tissue Mn concentrations in the continuous lifelong 25 and 50 Mn exposure groups at PND 66 versus ~500, tissue Mn levels in PND ~500 aged adult animals were either lower than (blood, p's < 0.014) or not statistically different from (brain, bone, p's > 0.72) their PND 66 counterparts (Figure 2, bars with # reflect PND 500 groups that are significantly lower than their PND 66 counterpart of the identical Mn exposure group). Collectively, these findings show that continuous lifelong oral Mn exposure does not result in Mn accumulation in these tissues.

Notably, modest reductions in tissue Mn are evident between PND 66 and ~500 in the early life Mn groups that were exposed over PND 1-21. For example, blood Mn levels in the PND ~500 control, early life 25, and early life 50 groups were significantly lower than their PND 66 counterparts (p = 0.041 for controls, p's < 0.009 for the Mn groups). In slight contrast, brain Mn levels in the early life 50 ~PND 500 aged adults were measurably lower than their PND 66 counterparts (p = 0.016), while there was no measurable reduction between the young and aged adults in the control and early 25 Mn groups (p's > 0.44). For bone, Mn levels in the ~PND 500 early life 25 and 50 groups were measurably lower than in their PND 66 counterparts (p's < 0.023), but there was no difference between controls (p = 0.99).

Figure 2. Tissue Mn does not accumulate in blood, brain, or bone across the lifespan in the presence of elevated oral Mn exposure. Bar charts show tissue Mn levels for (**a**) blood (ng/mL), (**b**) brain (µg/g dry weight), and (**c**) bone (µg/g) on a log10 scale, grouped by treatment group and age (n = 10-16 animals/treatment and age group). Data are least squares means ± SEM values generated from one-way ANOVA. Bars with different superscripts are statistically different (p < 0.05), based on Tukey's multiple comparisons test. Lowercase superscripts are comparisons within an age across treatment

groups. * represents comparison between groups for PND 66 that are statistically different from their PND 24 counterpart of the same exposure dose. # represents comparison between ages for PND ~500 that are statistically different from their PND 66 counterpart for the same exposure group.


a.
Blood Mn (ng/mL)
100
10
1
Control
Early 25
Early 50
Lifelong 25
Lifelong 50
PND 24
PND 66
PND ~500
Treatment group
b.
Brain Mn (µg/g)
20
10
1
Control
Early 25
Early 50
Lifelong 25
Lifelong 50
PND 24
PND 66
PND ~500
Treatment group
c.
Bone Mn (µg/g)
100
10
1
0.1
Control
Early 25
Early 50
Lifelong 25
Lifelong 50
PND 24
PND 66
PND ~500
Treatment group

There is no relationship between bone Mn levels and age in aged adult animals exposed to lifelong elevated oral Mn

In order to further address whether chronic oral Mn exposure results in the accumulation of Mn in bone tissue, we performed linear regression analyses between animal age and bone Mn level in the aged adult lifelong 25 and 50 Mn-exposed animals according to their age at sacrifice. The age range for the PND ~500 aged adult lifelong 25 (PND 388 – 617, median 490) and 50 (PND 273 – 624, median 486) Mn-exposed animals varied because animals were also used in microdialysis studies of brain neurotransmitter release that spanned these prolonged intervals, as noted above (Lasley *et al.* 2020). Results show that there is no relationship between age and bone Mn level in either the continuous lifelong 25 ($R = 0.091$, $p = 0.24$) or lifelong 50 ($R = 0.029$, $p = 0.46$) Mn exposure groups, and neither regression slope is significantly different from zero (p's > 0.23), further substantiating that there is no evidence of Mn accumulation in bone tissue with prolonged elevated oral Mn exposure (Supplement 1, Figure 1).

Overall, early pre-weaning life showed the greatest susceptibility to elevated oral Mn exposure, based on the highest tissue Mn levels at PND 24 for all tissues. Notably, the very elevated tissue Mn levels in PND 24 weanling animals decline significantly with age, especially between early post-weaning and young adulthood, even in the presence of continuous oral Mn exposure. However, there remains a significant relationship between elevated ongoing oral Mn dose and tissue Mn levels within the young adult and aged adult age classes, indicating that tissues Mn levels,

and most notably bone Mn in adult animals, are measurably affected by ongoing oral Mn exposure. Finally, there is clear evidence that Mn does not accumulate in brain or bone tissue with prolonged lifelong exposure from young adulthood into aged adulthood.

3.3. Sensitivity of tissue Mn increases to oral Mn exposure

Based on the dose-response relationship between continuous lifelong oral Mn exposure and tissue Mn levels across the lifespan, we explored the association between tissue Mn levels in order to determine which of the three tissues is the most sensitive biomarker of ongoing oral Mn exposure. For this, we used Pearson's correlations of best-fit regressions between bone and blood, brain and blood, and bone and brain Mn levels, and the resultant regression fit (Pearson's R and associated p-value) and the regression sensitivity (slope) for specific comparisons. In order to compare regression parameters across tissues with inherently different Mn concentration units (i.e., ng/mL for blood vs. μg/g for brain and bone), values within a tissue and age group for each individual animal were normalized to their respective average control group value (i.e., % control), and these normalized values were then used in the regression analyses.

3.3.1. Bone Mn is strongly associated with blood Mn levels

Among all pairwise correlations made for each pair of tissues and treatment groups across ages, all of the correlations were statistically significant (p's < 0.05),

except the relationship between bone and brain Mn for the lifelong exposure groups at PND 500 ($p = 0.38$) (Figure 3). When comparing regression slopes of brain versus blood, bone versus blood, and brain versus bone for all ages and treatment groups, a pattern was observed in which the slopes of the blood versus bone linear regressions were notably steeper than the blood versus brain and bone versus brain regressions. For example, while both the blood versus bone and blood versus brain correlations are statistically highly significant (p's < 0.0001), the slopes of the blood versus bone regressions (e.g., 3.18 for PND 66) are substantially steeper compared to the blood vs. brain regressions slope (e.g., 0.1 for PND 66) (Supplement 1, Figure 2a, and b, respectively), indicating a much greater relative increase in bone Mn for a given increase in blood Mn. These findings suggest that bone Mn levels may be a more sensitive biomarker of ongoing elevated oral Mn exposure than either blood or brain Mn.

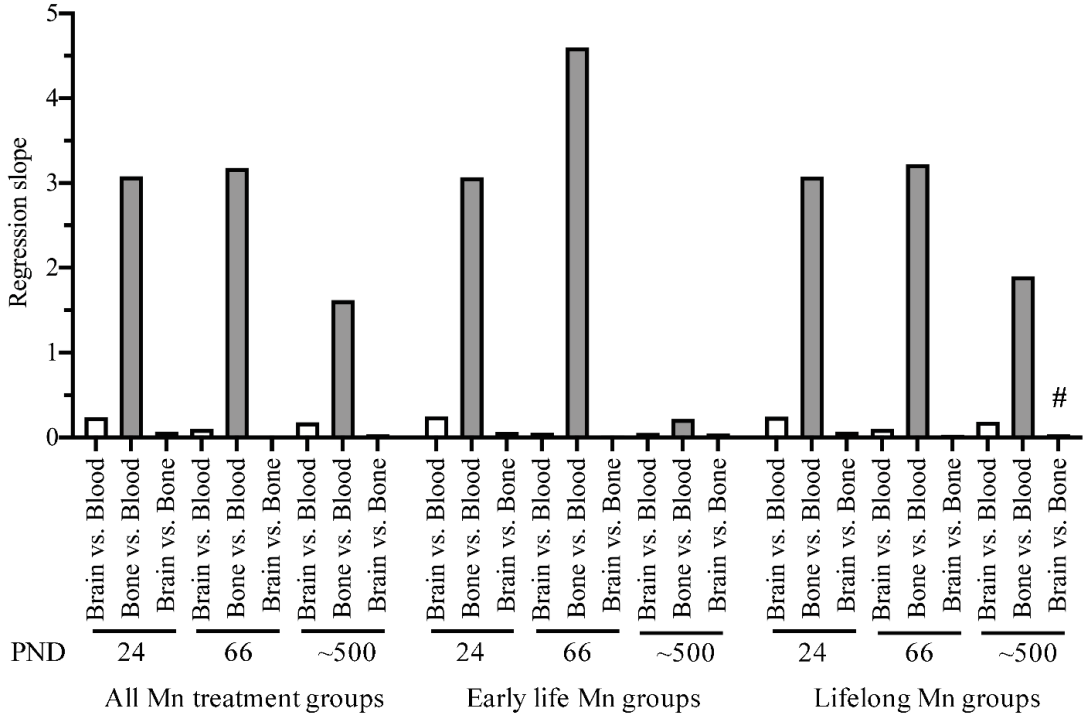


Figure 3. Bone Mn levels are highly sensitive to increases in blood Mn levels. Data are regression slopes (Y vs. X) from Pearson's correlation coefficient generated from tissue Mn values normalized to percentage of age matched controls separately for blood, brain, and bone tissues. All Pearson's correlations were significant (Pearson's R from 0.0101 to 0.508, with associated p-values, $p < 0.05$), with the exception of one indicated with # ($p = 0.38$).

3.4. Mn exposure and bone properties

3.4.1. Elevated bone Mn does not alter the gross crystalline structure of bone mineral

In light of the proportionally greater increase in bone Mn with elevated exposure compared to blood or brain Mn levels (i.e., in PND 24 animals an ~30-fold increase in bone Mn between the control and 50 mg Mn/kg/d exposure groups, whereas blood and brain Mn levels increased by ~10-fold and ~3.6-fold, respectively), and the very high levels of bone Mn attained in young weanling

animals (i.e., ~80 ug/g or higher in animals exposed to 50 mg Mn/kg/d over PND 1 – 21), the possibility exists that elevated Mn exposure may affect the properties of bone mineral during skeletal growth, as has been shown to occur with elevated lead exposures (Álvarez-Lloret *et al.* 2017; Beier *et al.* 2016; Monir *et al.* 2010). To explore this, we used X-ray diffraction (XRD) analysis to identify the gross crystalline structure properties of femur bone mineral in PND 24 rats. For analysis, a single femur sample was selected from each of the control, 25 and 50 mg Mn/kg/day groups with measured bone Mn levels in the alternate femur from the same animal of 2.4, 58, 166 μg/g, respectively. All three samples yielded XRD spectra comparable to the hydroxyapatite standard, indicating no significant alteration in mineral particle size and gross crystalline structure of the bone mineral in the Mn-exposed animals (Supplement 1, Figure 3).

3.4.2. At elevated levels, Mn in bone exists as Mn^{2+}

While Mn is known to exist in the Mn^{2+} and Mn^{3+} valence states within vertebrate organisms, the vast majority of cellular Mn is present as Mn^{2+} (Reaney *et al.* 2006; Reaney *et al.* 2002; Gunter *et al.* 2006). Mn^{2+} is also known to serve somewhat as a biologic analog to Ca^{2+} (Frausto da Silva and Williams 2001). Thus, bone Mn may be expected to exist largely in the Mn^{2+} valence state, though it is not known whether elevated Mn exposure may lead to incorporation of other valence states of Mn in bone, with possible implications for altered bone mineral structure. To address this, we used X-ray absorption near edge structure (XANES) analysis to

determine whether Mn exposure altered the valence state of Mn in bone mineral in the same 25 and 50 mg Mn/kg/d bone mineral samples used for XRD analyses above. Results show that in control (2.4 μg Mn/g) and elevated bone Mn samples, Mn exists in the Mn^{2+} valence state, with no detectable Mn^{3+} or Mn^{4+} (Supplement 1, Figure 4).

3.4.3. Elevated bone Mn alters the local atomic structure of the hydroxyapatite matrix

Mn plays a role in many cellular processes throughout the body, and has the ability to form complexes with a variety of local atomic structures in multiple cell types (Geszvain *et al.* 2012; Aschner and Aschner 2005). Given this, we explored whether elevated Mn in bone tissue may alter the local atomic structure of hydroxyapatite bone mineral, using extended X-ray absorption fine structure (EXAFS) analysis for the same elevated Mn bone sample (166 μg/g) used for the XRD and XANES analyses reported above. While the bulk X-ray diffraction analyses indicate no co-precipitation of Mn phases into the bone structure, analysis of the local structure through EXAFS indicates that Mn is substituting for Ca^{2+}, as evidence by the Mn^{2+}-Ca^{2+} coordination into the hydroxyapatite structure (Supplement 1, Table 2). The contraction of the distance between Ca^{2+} and hydroxyapatite is due to the smaller atomic size of Mn^{2+} relative to Ca^{2+} (Supplement 1, Figure 5).

3.4.4. Elevated bone Mn may alter the physical properties of bone

Based on the X-ray-based analyses above, it is possible that due to the incorporation of Mn into the bone matrix, elevated Mn exposure may alter the mechanical properties of bone resulting from potential effects on other aspects of bone metabolism, such as bone cell metabolism, organic matrix synthesis, etc. To address this, we conducted biomechanical analyses using an electro-servo-hydraulic test instrument to measure the mechanical properties of femurs in PND 66 rats from all five Mn exposure groups (n = 13-16/group) (femurs from PND 24 rats were not fully mineralized and were unsuitable for biomechanical analysis). These analyses generated outcomes of stiffness, yield strength, ultimate strength, and work of fracture (Conti *et al.* 2012; Beier *et al.* 2016). Overall, there was a significant main effect of postnatal Mn exposure on femur stiffness [$F(4, 69) = 4.91$, $p = 0.0016$]. Specifically, there was a significant increase in femur stiffness in the early life 25 and 50 Mn groups, compared to controls (2283 and 2199 MPa vs. 1489 MPa, respectively; p's < 0.0035). In contrast, lifelong Mn exposure caused a trending, but statistically non-significant increase in femur stiffness in the lifelong 50 group (1988 MPa), relative to controls ($p = 0.093$), while there was a smaller non-significant increase in the lifelong 25 group (1975 MPa, $p = 0.107$). There were no measurable differences in femur stiffness between the early and lifelong 25 and 50 Mn exposure groups in the PND 66 animals (p's > 0.52) (Figure 4).

In addition to femur stiffness, we also evaluated other physical properties of bone, including yield strength, ultimate strength, and work of fracture, to determine whether they were altered by elevated bone Mn levels. There was no significant main

effect of Mn exposure on yield strength [F(4, 67) = 0.43, $p = 0.78$], ultimate strength [F(4, 66) = 1.84, $p = 0.13$], or work of fracture [F(4, 67) = 0.78, $p = 0.54$] (data not shown).

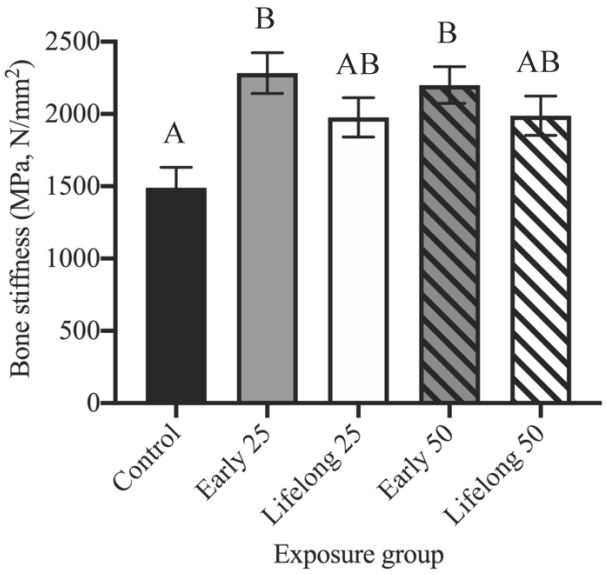


Figure 4. Postnatal Mn exposure increases bone stiffness. Bar chart shows femur stiffness in PND 66 rats (MPa, N/mm^2, n = 13-16 animals/treatment group). Data are least squares means ± SEM values generated from the ANOVA statistical model with all five treatment groups. Bars with different superscripts are statistically different ($p < 0.05$), based on Tukey's multiple comparisons test.

3.5. Bone Mn and lead levels in aged humans

3.5.1. Bone Mn levels do not differ by sex, but tend to decrease with age

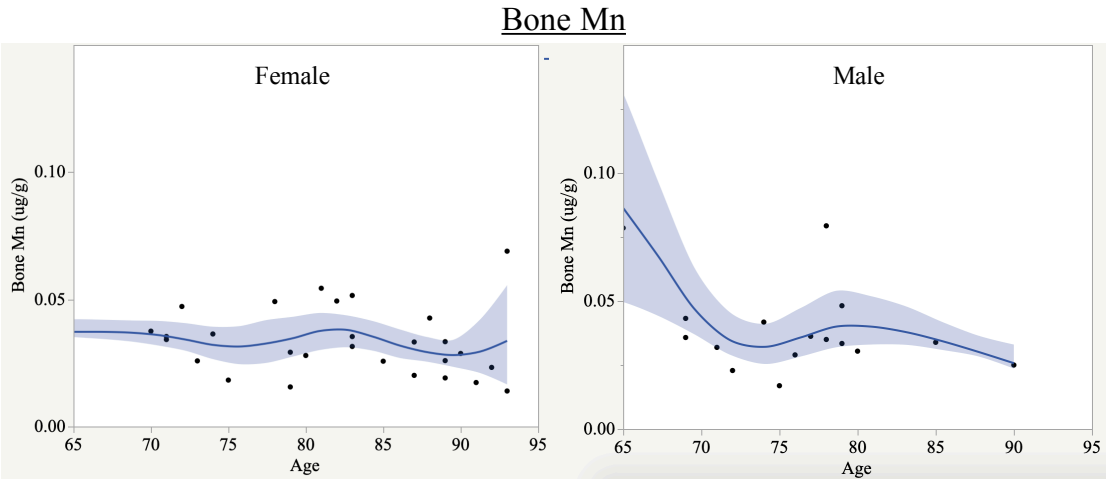
We measured bone and blood Mn and lead (Pb) levels in environmentally-exposed aged adults, since this life stage is under-represented in environmental exposure studies, and because they may further inform whether Mn accumulates in the human skeleton and hence the potential utility of bone as a biomarker of cumulative Mn exposure. Bone samples were obtained from 30 female and 19 male

subjects, age 41 – 95, undergoing hip arthroplasty surgery; blood samples were collected from 44 of these subjects (26 female, 18 male). Subjects were lifelong residents of the Province of Brescia, Italy, which contains varying amounts of ferromanganese industrial activity. Blood Mn levels were 9.76 ± 3.88 ng/mL in females (mean ± SD, range 6.03 – 23.5 ng/mL), and 9.49 ± 3.12 ng/mL in males (range 5.42 – 15.3 ng/mL). Bone Mn levels in these aged subjects were universally low, ranging from 0.014 – 0.17 μg/g (mean 0.038 μg/g ± 0.024 SD, n=49). Bone Mn levels in males (mean 0.046 μg/g ± 0.035 SD, median 0.036 μg/g, range 0.017 – 0.17 μg/g, n = 19) trended towards being slightly higher compared to females (mean 0.034 μg/g ± 0.013 SD, median 0.033 μg/g, range 0.014 – 0.069 μg/g, n=30) [$F(1, 48) = 3.231$, $p = 0.079$]. Notably, one male subject who had a history of occupational metal exposure had a relatively high bone Mn concentration of 0.17 μg/g. When this individual was excluded from the analysis, the statistically trending effect of higher bone Mn in males was no longer present [$F(1, 47) = 1.628$, $p = 0.208$]. While there was no effect of sex on bone Mn, there was a trending, but statistically non-significant effect of age ($p = 0.073$), suggesting that bone Mn levels decrease by ~1% per year over the age range of our study group (Figure 5a).

Blood lead levels in these subjects were 38.2 ± 23.2 ng/mL in females (mean ± SD, range 9.17 - 107 ng/mL), and 81.6 ± 57.8 ng/mL in males (range 25.1 – 241 ng/mL). Using an ANCOVA-like linear model, we also assessed the effect of sex and age on bone lead levels. Sex was a significant predictor of bone lead levels in humans ($\beta = 1.49$, $p < 0.0001$), with males (mean 5.96 μg/g ± 4.23 SD, median 5.04 μg/g,

range 1.16 – 17.5 µg/g, n = 19) having higher lead concentrations than females (mean 1.93 µg/g ± 1.74 SD, median 1.33 µg/g, range 0.28 – 7.15 µg/g, n = 30). The male with the highest bone Mn (0.17 µg/g) had a bone Pb level (5.76 µg/g) that was near the mean for males. Age was also a significant predictor of bone lead (β= 0.031, p = 0.027). The overall model fit was $R^2 = 0.45$ [$F(1, 45) = 18.57$, $p < 0.0001$], showing that bone lead levels in males are ~3.5-times higher than in females, and that overall bone lead levels increase ~3.3% per year (Figure 5b).

a.



b.

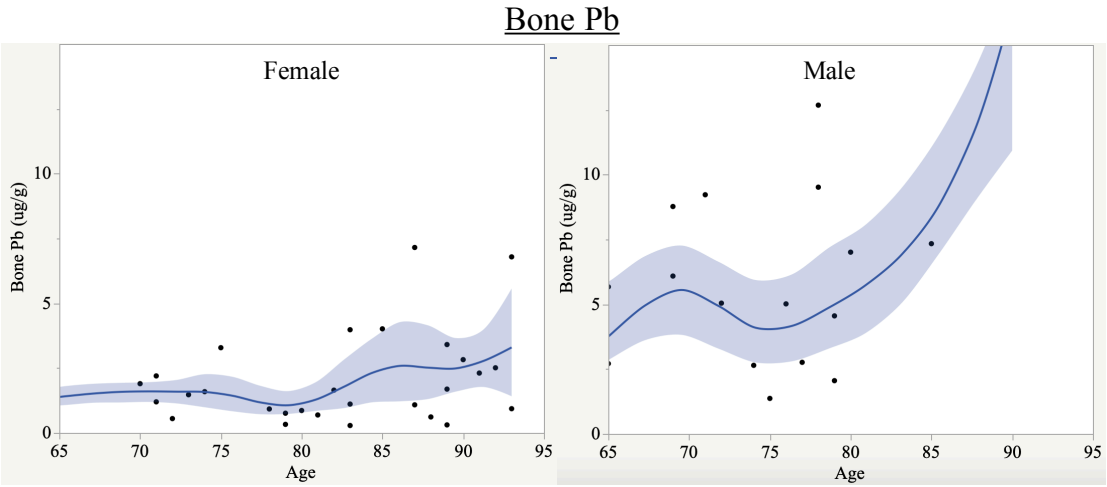


Figure 5. In aged humans, bone Mn trends towards a decrease with age, while bone Pb levels increase with age. Data are individual human bone concentrations of (**a**) Mn and (**b**) lead in µg/g, as a function of age for males (n=19) and females (n=30). Blue line is fit based on a generalized non-linear model, shaded region is 95% confidence interval.

3.5.2. Parity history is not associated with altered bone Mn or bone Pb levels

Prior studies have established a relationship between bone lead levels and pregnancy/parity history in women (Gulson *et al.* 1997; Hernandez-Avila *et al.* 2000). Furthermore, maternal skeletal lead is known to be remobilized during pregnancy and lactation (Franklin *et al.* 1997; Manton *et al.* 2003; Gulson *et al.* 2004; Gulson *et al.* 1997). In light of this, we explored the relationship between parity history and bone Mn levels in aged females with zero (n = 3), one (n = 5), two (n = 6), three (n = 7), or more than three (n = 4) children (parity information was unavailable for five female subjects) using linear regression analysis. Parity history had a non-significant, but statistically trending main effect on bone Mn levels (β= 0.092, p = 0.054), that was largely influenced by a single female with seven children. Removing this individual

resulted in a clearly non-significant regression coefficient (β= 0.098, $p = 0.24$). Similarly, there was no relationship between parity history and bone lead levels in females when analyzed assuming log-linearity, similar to Mn above (β = 0.12, $p = 0.31$).

4.0. Discussion

Our animal model findings demonstrate that Mn levels in blood, brain, and bone decline naturally with age in the absence of elevated exposure, and do not accumulate in the presence of prolonged elevated oral Mn exposure. Among these tissues, bone Mn is the most responsive biomarker of ongoing oral Mn exposure. While X-ray-based analyses of bone samples show that with elevated exposure Mn^{2+} can replace Ca^{2+} in the hydroxyapatite mineral, the gross physical structure of hydroxyapatite bone mineral is not measurably altered. However, elevated Mn exposure does alter bone stiffness, suggesting that elevated exposure may cause alterations in the physical properties of bone not captured in the X-ray-based analyses. Data from our animal model are complemented by bone Mn analyses in aged humans, showing that bone Mn decreases with age, with no effect of sex, or parity history in females. Collectively, these findings indicate that bone may be a useful biomarker of recent ongoing oral Mn exposure in humans, and that bone may be a relatively minor target of elevated Mn exposure, based on the limited functional alterations reported here.

4.1. Tissue Mn naturally declines with age in the absence of elevated Mn exposure

Results in our rodent model show that, in the absence of elevated exposure, blood, brain, and bone Mn levels naturally decline with age across the lifespan (Figure 1). The reduction in tissue Mn levels from early life to late adulthood likely reflects a decline in the absorption/retention of Mn with age, presumably reflecting a lower biological need for Mn in adulthood relative to early developmental life. The primary evidence for this comes from studies in animal models and adult humans. For example, Davidsson et al. (1989) reported Mn absorption rates in adult humans of 8.2%, 2.4%, and 0.7% for human milk, cow's milk, and soy formula, respectively. These human findings are corroborated by rodent studies showing that adult rats absorb < 5% of ingested Mn (Ballatori *et al.* 1987; Mena 1974). In contrast, young rats <15 days of age absorb over 40% of ingested Mn (Keen *et al.* 1986; Miller *et al.* 1975a; Pappas *et al.* 1997). Our data show that in control animals blood, brain, and bone Mn levels decrease substantially from weaning (PND 24) to young adulthood (PND 66), and blood Mn levels (but not brain or bone Mn) decline further from young adulthood to aged adulthood (PND ~500) (Figure 1). This pattern is consistent with the greater control of intestinal and hepatic regulation of oral Mn intake on blood Mn levels in adults, and may also reflect the relatively shorter residence time of Mn in blood compared to brain and bone (Crossgrove and Zheng 2004; O'Neal and Zheng 2015; Järvisalo *et al.* 1992; Smith *et al.* 2007; Cowan *et al.* 2009; O'Neal *et al.* 2014).

4.2. Tissue Mn levels do not accumulate across the lifespan in the presence of elevated Mn exposure, but instead reflect ongoing recent exposure

The question of whether Mn accumulates in tissues with elevated exposures over the lifespan is of tremendous interest in identifying exposure biomarkers that may reflect temporally integrated or cumulative exposures, and for identifying potential target tissues of toxicological effects. Here, we define "accumulation" of Mn as a net increase in tissue Mn levels over time with steady-state elevated exposure. Based on this definition, our results show that continuous oral Mn exposure in rodents throughout life did not lead to the accumulation of Mn in blood, brain, or bone (Figures 2 and 3). In fact, tissue Mn levels decline over the lifespan from post-weaning to young adulthood for all tissues, and blood Mn continues to decline from young adulthood into aged adulthood, even in the presence of ongoing elevated oral Mn exposure (Figure 2a, lifelong 25 and 50 groups at PND ~500). The lack of Mn accumulation in bone with prolonged ongoing oral exposure is further illustrated when considering only the aged adult animals continuously exposed to oral 25 or 50 mg Mn/kg/d since birth; these animals were sacrificed at ages spanning PND 292 – 889 (median PND 490) due to experimental constraints in brain microdialysis measurements (Lasley *et al.* 2020). Regression analyses of bone Mn level versus age yields non-significant regression fits (p's ≥ 0.24), with slopes that do not measurably differ from zero (Figure 3 and Supplement 1, Figure 1). Together, these data underscore that age-related regulation of oral Mn uptake and elimination plays a

significant role in modulating susceptibility to elevated oral Mn exposure, in that the early developmental life stage where oral Mn exposure produces the highest tissue Mn levels is also of the life stage of greatest susceptibility to Mn neurotoxicity (Conley *et al.* 2020; Beaudin *et al.* 2013; Beaudin *et al.* 2017a; Beaudin *et al.* 2017b; Oulhote *et al.* 2014; Mora *et al.* 2015a; Claus Henn *et al.* 2018).

It is noteworthy that blood Mn levels measurably declined from young adulthood (PND 66) to aged adulthood (~PND 500) in the presence of ongoing elevated oral Mn exposure (Figure 2a, see lifelong 25 and 50 groups), while brain and bone Mn levels did not (Figure 2 b, c). This may suggest that for tissues with longer Mn residence times compared to blood (i.e., brain and bone), continuous elevated oral Mn exposure may offset the relative influence of natural age-based reductions in tissue Mn levels. This suggestion is supported by that fact that the ~PND 500 aged animals continuously exposed to elevated oral Mn exposure had significantly higher blood, brain, and bone Mn levels compared to controls, whereas the early life Mn exposed groups did not differ from controls (Figure 2).

Our findings in rodents showing that bone Mn levels reflect on-going oral exposures, but do not accumulate Mn with elevated exposures spanning birth through aged adulthood have important implications for studies of environmentally and occupationally exposed humans. In particular, these results suggest that bone Mn levels may not be a good biomarker of cumulative oral Mn exposure integrated over prolonged durations, as has been shown for bone lead levels (Hu *et al.* 1998; Hu 1998; Aufderheide *et al.* 1981). This conclusion contrasts somewhat the findings of

Rolle-McFarland et al. (2018), who reported that Mn accumulated in bone tissue in a population of 60 Chinese industrial workers. That study used neutron activation to measure bone Mn *in vivo*, and characterized occupational Mn exposure using a Cumulative Exposure Index (CEI) that relied partly on worker questionnaire responses to classify occupational exposure into qualitative exposure rankings of high, medium, or low. While the CEI approach is commonly used in occupational studies where significant challenges exist in performing comprehensive exposure assessments spanning months to years, it may not have been able to accurately distinguish whether higher bone Mn levels were reflecting Mn accumulation from prolonged exposure (e.g., years to decades) from the influence of more recent higher exposures over shorter durations proximal to the bone Mn measurements. It is also noteworthy that the exposure pathway in that study was inhalation, whereas in the present study it was oral, and there is some evidence that Mn exposures may follow different toxicokinetic profiles following oral versus inhalation exposures (Roels *et al.* 1997).

4.3. Bone Mn levels appear to be a more sensitive biomarker of ongoing oral Mn exposure than either blood or brain Mn

Although we found no evidence of Mn accumulation in bone in aged adult rats with continuous oral exposure, our biomarker sensitivity analyses show that bone is a more sensitive biomarker to ongoing oral Mn exposure than blood or brain (Figure 3). This interpretation is supported by comparison of tissue Mn levels among

controls and the high dose Mn group (50 mg Mn/kg/day) across tissues at PND 24, the age group where the continuous oral Mn exposures produced the highest tissue Mn levels. For example, blood Mn levels increased ~10-fold (from 24 ng/mL in controls to 247 ng/mL in animals exposed to 50 mg Mn/kg/d over PND 1 – 21), and brain Mn increased ~3.6-fold (from 3.6 to 12.8 μg/g), whereas bone Mn increased ~29-fold (from 2.7 to 77 μg/g). The greater relative increase of bone Mn levels with ongoing elevated exposure, compared to relative increase in blood or brain Mn, is also evidenced by the slopes of the bivariate Pearson's regressions of normalized tissue Mn levels (Figure 3). The bone vs blood (Y vs. X) regression slopes in the PND 24, 66, and ~500 lifelong Mn exposure groups range from 1.86 to 3.28 (mean 2.72 ± 0.76 SD), showing there is a several-fold greater relative increase in bone Mn for a given increase in blood Mn. Moreover, the bone vs. blood regression slopes are >10-fold steeper than the slopes of the brain vs. blood regressions (0.12 – 0.27 for the three age groups), showing that a given increase in blood Mn produces a much greater increase in bone Mn than brain Mn levels. Collectively, these findings suggest that bone Mn levels may be a more sensitive biomarker of ongoing oral Mn exposure than levels of Mn in other tissues. While Mn does not accumulate in bone per se, bone Mn levels do appear to reflect an intermediate exposure duration of weeks to months, i.e., longer than the duration for blood, consistent with the estimated 143 days (~4.7 months) half-life of Mn in bone reported by O'Neal et al. (2014).

4.4. Elevated Mn exposure caused some changes in bone mineral properties, accompanied by changes in the physical properties of bone

The oral Mn exposure conditions that produced elevated bone Mn levels in adult animals did not result in changes to the gross mineral structure of hydroxyapatite in bone (Supplement 1, Figure 3). However, EXAFS analysis revealed that elevated Mn exposure did lead to changes in the local atomic structure of hydroxyapatite mineral due to the substitution of Mn^{2+} for Ca^{2+} in the coordination of the hydroxyapatite mineral structure (Supplement 1, Figure 5). Not unexpectedly, the substituted Mn was in the Mn^{2+} valence state, consistent with prior evidence showing that the predominant Mn valence state in eukaryotic cells is Mn^{2+} (Reaney and Smith 2005; Reaney *et al.* 2002; Aschner and Aschner 2005).

The finding that elevated bone Mn altered the local atomic structure, but not the gross crystalline structure of hydroxyapatite mineral is noteworthy, in light of the changes in the physical properties of bone determined through our biomechanical testing. We evaluated a number of physical properties of femurs from PND 66 rats, including stiffness, yield strength, ultimate strength, and work, but only femur stiffness was measurably increased with Mn exposure, and only in the early life 25 and 50 Mn exposure groups compared to controls (bone stiffness trended higher in the lifelong 25 and 50 Mn groups, but did not reach significance) (Figure 4). This finding of minimal biomechanical alterations to bone containing elevated Mn levels suggests that bone may be a target organ for Mn effects, though likely not as sensitive and impacted as other organ systems such as the brain, particularly when considering

the elevated bone Mn levels observed here versus levels reported in environmentally or occupationally exposed humans. For example, bone Mn levels in the PND 66 young adult animals exposed to the highest Mn dose (i.e., 50 mg Mn/kg/d over PND 1 – 21 or lifelong) averaged ~2.4 – 3.2 μg/g, though levels were much higher in the younger PND 24 weanlings exposed over PND 1 – 21 (mean 77 μg/g, Figure 2). By comparison, bone Mn levels of 0.89 to 2.6 μg/g have been reported in occupationally exposed adults using neutron activation analysis (Wells *et al.* 2018; Rolle-McFarland *et al.* 2018).

Other toxic metals such as lead, which is also known to substitute for Ca^{2+} in the hydroxyapatite mineral matrix, and to some extent cadmium, have been shown to significantly reduce bone mineral density and strength in mice (Monir *et al.* 2010), along with decreasing trabecular bone surface area and increasing risk of fracture in rats (Álvarez-Lloret *et al.* 2017). Alterations to bone metabolism following lead exposure have been reported in epidemiological studies, including a recent study from Ravibabu and colleagues, in which long-term occupational lead exposure in a group of 176 male lead-battery workers was positively associated with significantly higher levels of biomarkers of bone formation (i.e., serum bone-specific alkaline phosphatase) and bone resorption (i.e., serum pyridinoline, tartarate-resistant acid phosphatase-5b, urinary hydroxyproline), along with a negative association with serum osteocalcin (Ravibabu *et al.* 2020). Similarly, Akbal et al. found a negative association between occupational lead exposure and vertebral bone density in middle-aged male lead-battery workers in Turkey (Akbal *et al.* 2014). Long-term exposures

to other metals, such as cadmium, are also associated with harmful effects to the human skeleton, including reduced bone mineral density, higher incidence of fractures, and higher risk of osteoporosis (Engström *et al.* 2012; Wallin *et al.* 2016).

4.5. Bone Mn levels in aged humans decrease with age, and do not very with sex or parity history

In order to determine bone Mn and lead levels in environmentally exposed aged humans (mean age of 82 and 74 years for females and males, respectively), and whether bone Mn levels varied with age, sex and parity, we obtained intact femoral head bone samples from female (n = 30) and male human (n = 19) subjects from the province of Brescia, Italy, undergoing hip joint replacement due to osteoarthritis. Our findings show that bone Mn levels are generally very low in these aged adults (mean 0.038 μg/g), and that there was a trending ~1% per year reduction in bone Mn levels with age (Figure 5). We did not find a significant relationship between bone Mn and sex, which may reflect a similar rate of osteoarthritis and osteoporosis in these male and female subjects who underwent joint arthroplasty. Prior research has shown that osteoarthritis and osteoporosis are more prevalent in women, and that both conditions lead to loss of bone mineral over time (Maleki-Fischbach and Jordan 2010; Zhang and Jordan 2010; Alswat 2017). We also found that there was no relationship between bone Mn levels and parity history in females. We may have expected a decrease in bone Mn levels with parity history, given that a number of studies have reported that maternal blood Mn levels increase from ~7 ng/mL to ~17-25 ng/mL over the course

of pregnancy and into the postnatal lactation period (Yamamoto *et al.* 2019; Mora *et al.* 2015b), suggesting that there may be increased mobilization of maternal bone Mn with mobilization of bone mineral over pregnancy and lactation, as has been shown with bone lead (Manton *et al.* 2003; Téllez-Rojo *et al.* 2004; Gulson *et al.* 2004; Gulson *et al.* 1997).

Bone lead levels represent a valuable point of comparison relative to Mn since both metals are able to substitute with Ca^{2+} in hydroxyapatite mineral, and elevated lead exposures are similarly associated with industrial activity (Fleming *et al.* 1997; Hernberg 2000; Kaufman *et al.* 1994; Rudolph *et al.* 1990; Grimsley and Adams-Mount 1994), and there is a substantial body of evidence on lead in the skeleton (Marcus 1985; O'Flaherty 1993; Rabinowitz *et al.* 1973; Rădulescu and Lundgren 2019). For example, studies have shown that lead accumulates in the skeleton with age, that the skeleton can contribute 40-70% of circulating blood lead in environmentally exposed adults (Smith *et al.* 1996; Gulson *et al.* 1997), and that there is significant remobilization of bone lead with bone mineral mobilization over pregnancy and lactation (Manton *et al.* 2003; Téllez-Rojo *et al.* 2004; Gulson *et al.* 2004; Gulson *et al.* 1997). Here we show that bone lead levels in these aged adults (mean 3.49 μg/g, median 2.20 μg/g) were ~100-fold higher than bone Mn levels, and that bone lead levels were associated with both age and sex. Specifically, bone lead levels increased with age by approximately 3.3% per year over the age range of these subjects (41 – 95 years), and males have ~3.5-times higher bone lead levels compared to females (Figure 5). These findings are consistent with multiple studies that show

males tend to have higher tissue lead levels relative to females, in part due to presumably higher likelihood of elevated environmental and/or occupational exposure (Hu *et al.* 1991; Smith *et al.* 2002; Hu 1998). Additionally, the lack of a relationship between bone lead and parity history in females was somewhat unexpected as lead has been reported to remobilize during pregnancy and lactation as bone mineral turnover increases (Manton *et al.* 2003; Téllez-Rojo *et al.* 2004; Gulson *et al.* 2004; Gulson *et al.* 1997).

4.6. Study strengths and limitations

There are several strengths and limitations of this study that may provide context for interpreting our findings. Study strengths include the use of a prolonged animal exposure design to evaluate both early life and lifelong Mn exposure paradigms into aged adulthood, along with a controlled and well-characterized Mn exposure regimen that is relevant to human environmental exposures (see Beaudin *et al.* 2017a for a detailed exposure rationale). Additional strengths are the comprehensive use of advanced analytical methods (i.e., XRD, XANES, EXAFS) to assess whether Mn accumulates in bone tissue, and whether Mn exposure affected the mineral and atomic structure of bone relative to the physical biomechanical properties. Finally, the evaluation in aged humans to determine the relationship between age, sex, and parity history on bone Mn relative to bone Pb levels is an additional strength of this study. There are also several noteworthy limitations of this study, including using only femurs, which are predominantly cortical bone, to assess

bone Mn levels in the animal studies. Others may find different results using bones that are primarily trabecular in composition. Regarding our human subjects, the diagnosis of osteoarthritis and/or osteoporosis in this group may have influenced the levels of bone Mn measured in our samples, and may not necessarily be generalized to a healthier population.

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CHAPTER 3: EARLY POSTNATAL MANGANESE EXPOSURE CAUSES AROUSAL DYSREGULATION AND LASTING HYPOFUNCTIONING OF THE PREFRONTAL CATECHOLAMINERGIC SYSTEMS

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Abstract

Studies have reported associations between environmental manganese (Mn) exposure and impaired cognition, attention, impulse control, and fine motor function in children. Our recent rodent studies established that elevated Mn exposure causes these impairments. Here, rats were exposed orally to 0, 25, or 50 mg Mn/kg/day during early postnatal life (PND 1-21) or lifelong to determine whether early life Mn exposure causes heightened behavioral reactivity in the open field, lasting changes in the catecholaminergic systems in the medial prefrontal cortex (mPFC), altered dendritic spine density, and whether lifelong exposure exacerbates these effects. We also assessed astrocyte reactivity (glial fibrillary acidic protein, GFAP), and astrocyte complement C3 and S100A10 protein levels as markers of A1 proinflammatory or A2 anti-inflammatory reactive astrocytes. Postnatal Mn exposure caused heightened behavioral reactivity during the first 5 – 10 minute intervals of daily open field test sessions, consistent with impairments in arousal regulation. Mn exposure reduced the evoked release of norepinephrine (NE) and caused decreased protein levels of

tyrosine hydroxylase (TH), dopamine (DA) and NE transporters, and DA D1 receptors, along with increased DA D2 receptors. Mn also caused a lasting increase in reactive astrocytes (GFAP) exhibiting increased A1 and A2 phenotypes, with a greater induction of the A1 proinflammatory phenotype. These results demonstrate that early life Mn exposure causes broad lasting hypofunctioning of the mPFC catecholaminergic systems, consistent with the impaired arousal regulation, attention, impulse control, and fine motor function reported in these animals, suggesting that mPFC catecholaminergic dysfunction may underlie similar impairments reported in Mn-exposed children.

1.0. Introduction

Elevated environmental Mn exposure is emerging as a substantial public health problem in the U.S. and elsewhere, where vulnerable children may be exposed to elevated levels of Mn from drinking water (Wasserman *et al.* 2006a; Bouchard *et al.* 2007; Bouchard *et al.* 2011; Ljung and Vahter 2007), soil and dust (Gunier *et al.* 2014a; Gunier *et al.* 2014b; Lucas *et al.* 2015; Lucchini *et al.* 2012a), and dietary sources (Crinella 2003; Crinella 2012). Epidemiological studies have reported associations between environmental Mn exposure and impaired cognition, attention, impulse control, and fine motor function in children and adolescents (Oulhote *et al.* 2014; Ericson *et al.* 2007; Bouchard *et al.* 2011). Our recent rodent studies recapitulated these functional impairments in children, demonstrating that developmental Mn exposure causes highly specific and lasting impairments in

selective and focused attention, impulse control, arousal regulation, and fine motor function (Beaudin *et al.* 2017a; Beaudin *et al.* 2013; Beaudin *et al.* 2015; Beaudin *et al.* 2017b). These impacts of Mn on attention and impulse control are particularly concerning, considering that impairments in these areas of functioning, including attention deficit hyperactivity disorder (ADHD), are among the most prevalent neurodevelopmental disorders in children (Willcutt 2012; Feldman and Reiff 2014; Xu *et al.* 2018).

The pattern of neurobehavioral dysfunction caused by developmental Mn exposure implicates disrupted function of the medial prefrontal cortex (mPFC), given that the mPFC plays an important role in mediating arousal regulation and executive function, including attentional function and cognitive flexibility, among others (Maddux and Holland 2011; Bissonette *et al.* 2008). The catecholaminergic systems within the mPFC has been shown to be a critical mediator for regulating executive function, in which the dopamine (DA) D1 and α_{2A} adrenergic receptors are especially important (Arnsten and Dudley 2005; Arnsten and Pliszka 2011). However, the neurobiological mechanisms underlying these Mn impairments are poorly understood. Converging evidence suggests that altered catecholaminergic activity in the mesocorticolimbic circuit due to elevated Mn may play an important underlying role (Kern *et al.* 2010; Kern and Smith 2011; McDougall *et al.* 2008; Reichel *et al.* 2006), consistent with the important role of the catecholaminergic systems in regulating mPFC function (Arnsten and Pliszka 2011; Arnsten 2009a). Studies in mammals have shown that developmental Mn exposure reduces striatal DA release

(Reichel *et al.* 2006; McDougall *et al.* 2008), as well as DA D1 receptor and DA transporter (DAT) protein levels in the striatum and nucleus accumbens (McDougall *et al.* 2008; Reichel *et al.* 2006; Kern *et al.* 2010; Kern and Smith 2011), but relatively few studies have examined the effects of developmental Mn exposure on the catecholaminergic systems in the mPFC. Limited available evidence has shown that developmental Mn exposure increases D2 receptor levels in the mPFC of weanling rodents that may last into adulthood (Kern *et al.* 2010; Kern and Smith 2011). Our recent studies have revealed lasting reductions in evoked DA and norepinephrine (NE) release in the mPFC in adulthood in animals exposed only during early postnatal development (Beaudin *et al.* 2015; Lasley *et al.* 2020), with lasting changes in PFC DA D2 protein levels in adults (Kern and Smith 2011). However, the extent to which developmental and lifelong Mn exposure impacts the mPFC catecholaminergic systems in adulthood is not well known.

Developmental Mn exposure has also been shown to cause neuroinflammation, manifesting in reactive astrocytes, increased expression of glial fibrillary acidic protein (GFAP), and increased release of proinflammatory cytokines (Moreno et al., 2009; Kern and Smith, 2011; Popichak et al., 2018). Astrocytes and microglia are important mediators of neuroinflammation (Prinz and Priller 2014; Liddelow and Barres 2017), and astrocytes in particular play an important role in the dynamic restructuring of synapses during neurodevelopment (Dallérac *et al.* 2018; Farhy-Tselnicker and Allen 2018). Recent evidence has demonstrated that environmental toxicants can induce a proinflammatory A1 phenotype in astrocytes

and lead to altered synaptic function (Liddelow *et al.* 2017). However, little is known about whether developmental Mn exposure induces a proinflammatory A1 phenotype in mPFC astrocytes, and whether this effect is associated with Mn-induced changes in the mPFC catecholaminergic systems.

Here we used our rodent model of early childhood oral Mn exposure to systematically investigate whether Mn causes enduring disruption in the catecholaminergic system in the mPFC, using (1) quantitative protein immunohistochemistry measures of tyrosine hydroxylase (TH), DAT and NE transporter (NET), DA D1 and D2 receptors, and the α_{2A} adrenergic receptor, (2) microdialysis for DA and NE release, and (3) quantification of dendritic spine density on pyramidal mPFC neurons. We also assessed whether changes in the mPFC catecholaminergic systems were associated with heightened behavioral reactivity in an open field behavioral paradigm. Finally, we assessed astrocyte reactivity based on protein levels of GFAP, complement C3, and S100A10, the latter two as markers of reactive astrocytes expressing an A1 proinflammatory or A2 anti-inflammatory phenotype, respectively. Given our recent findings that lifelong oral Mn exposure into adulthood did not worsen the enduring attentional and fine motor deficits of exposure restricted to early postnatal life (Beaudin *et al.* 2017a; Beaudin *et al.* 2017b), we also tested whether continued oral Mn exposure throughout postnatal life exacerbated the catecholaminergic systems and astrocyte reactivity effects of the early postnatal exposure.

2.0. Materials and Methods

2.1. Subjects

The data reported here arose from three different, but identically treated, cohorts of male Long-Evans rats. Cohorts were generated, treated, and assessed in sequential fashion. The open field data are generated from cohorts 1 and 2. The immunohistochemistry (IHC), Mn biomarker, and spine density data are from cohort 2 littermates to the behaviorally tested (open field) animals, whereas the neurochemistry data are from cohort 3 (Figure 1). All subjects were born in-house from nulliparous timed-pregnant Long Evans rats (obtained from Charles River on gestational day 18, RRID: RGD_2308852). Twelve to 24 hours after parturition (designated PND 1, birth = PND 0), litters were sexed, weighed, and culled to eight pups per litter such that each litter was composed of five to six males and the remainder females. Only one male per litter was assigned to a particular Mn treatment condition. Animals (dams and weaned pups) were fed Harlan Teklad rodent chow #2920 (reported by the manufacturer to contain 80 mg Mn/kg) and housed in polycarbonate cages at a constant temperature of 21 ± 2°C. At PND 22, all pups were weaned and pair-housed with an animal of the same Mn treatment group and maintained on a reversed 10:14 hr light/ dark cycle. All aspects of behavioral testing and feeding were carried out during the active (dark) phase of the animals' diurnal cycle. Males were exclusively used because attentional dysfunction is two to three times more prevalent in boys than girls (Feldman and Reiff 2014; Willcutt 2012), and because our prior studies have established that early postnatal Mn exposure causes

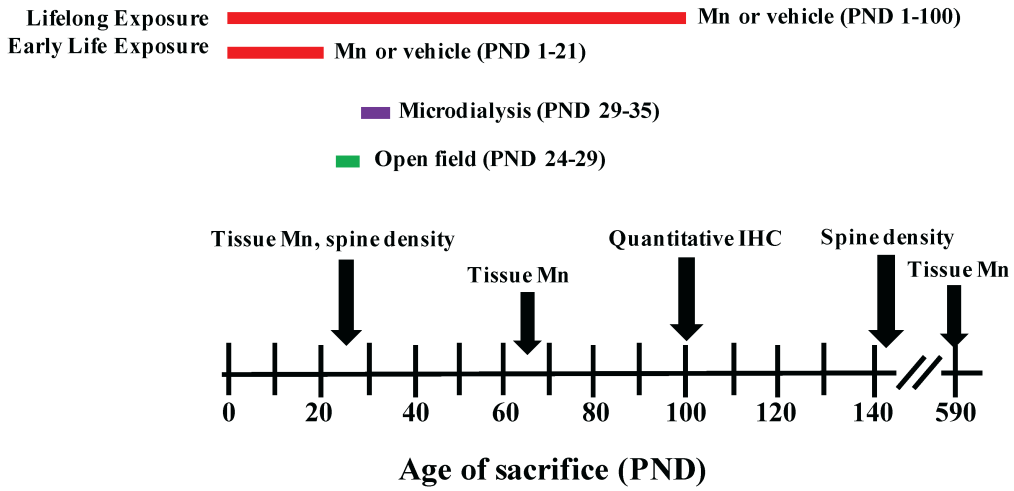

Figure 1. Time-line of experimental procedures and outcome measures, indicating timing of early life (PND 1-21, top red bar), and lifelong (PND 1 – end of study for particular outcome) Mn exposure relative to the outcome measures. Sample sizes per experimental procedure were: open field, n = 21-23 animals/treatment group; tissue Mn concentrations, n = 5-12 animals/group and timepoint; microdialysis of DA, NE, n = 8-12 animals/group; quantitative IHC of catecholamine systems and astrocyte proteins, n = 5-6 animals/group; spine density, n = 5-10 animals/group. No animals were excluded from the study based on exclusion criteria of poor health (see Materials and Methods).

lasting impairments in learning, attention, and fine motor function in male rats (Kern *et al.* 2010; Beaudin *et al.* 2017a; Beaudin *et al.* 2017b; Beaudin *et al.* 2015; Beaudin *et al.* 2013). All animal procedures were approved by the institutional IACUC (protocols Smitd0912 and 234193) and adhered to National Institutes of Health guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*. Criteria for exclusion of animals from the study were based on overt signs of poor animal health, including loss of body weight, absence of grooming, impaired function, and death; no animals were excluded from the study based on these criteria. This study was not pre-registered.

2.2. Manganese exposure protocol

Neonatal rats were orally exposed to Mn doses of 0, 25, or 50 mg Mn/kg/day starting on PND 1 through weaning on PND 21 (early postnatal Mn exposure), or throughout life until PND 100 (Figure 1). The early window of postnatal Mn exposure in rats corresponds to important frontal-cortical-striatal developmental events, including formation of DA and NE projections to the PFC, that are comparable to the gestational third trimester through adolescence in humans, whereas the continued lifelong exposure window additionally coincides with late adolescence to early adulthood in humans (Workman *et al.* 2013; Clancy *et al.* 2007; Nagarajan and Jonkman 2013; Posner and Rothbart 1998; Ruff and Rothbart 2010). For dosing over PND 1–21, Mn was delivered once daily directly into the mouth of each pup (~20 μL/dose) via a micropipette fitted with a flexible polyethylene pipet tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received the vehicle solution. For this, a 225 mg Mn/mL stock solution of $MnCl_2$ was prepared by dissolving $MnCl_2 \cdot 4H_2O$ with Milli-Q™ water; aliquots of the stock solution were diluted with a 2.5% (wt/vol) solution of the natural sweetener stevia to facilitate oral dosing of the pups. Oral Mn exposure post-weaning (PND 22 – end of study) occurred via the animals' drinking water. For this, a 42 mg Mn/mL stock Mn solution was prepared fresh weekly as above and diluted with tap water to a final concentration of 420 μg Mn/mL in a polycarbonate carboy. The stock solutions were made fresh weekly, and water bottles were refilled with fresh water two to three-times per week. Water bottle

weights were recorded at refilling to determine water intake per cage, and daily Mn intake per kg body weight was estimated based on daily measured body weights of the two rats housed per cage. Drinking water Mn concentrations were adjusted weekly as needed to maintain target daily oral Mn intake levels of 25 or 50 mg/kg/day based on measured water consumption. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both; pre-weaning exposure to 50 mg Mn/kg/day produces a relative increase in Mn intake that approximates the increase reported in infants and young children exposed to Mn-contaminated water or soy-based formulas (Beaudin *et al.* 2017a; Beaudin *et al.* 2015; Beaudin *et al.* 2013; Kern *et al.* 2010; Kern and Smith 2011). For lifelong Mn exposure groups, oral exposure to the same daily Mn dose was maintained after weaning via drinking water to model the situation where children may continue to suffer chronic elevated Mn exposures from a variety of environmental sources (e.g., contaminated well water, dust, etc.) (Bouchard *et al.* 2011; Lucas *et al.* 2015; Oulhote *et al.* 2014).

2.3. Open field testing

The animals' spontaneous exploration in a novel open field environment was tested 30 minutes each day for 5 consecutive days beginning on PND 24 (n = 21-23 animals/treatment group, 111 animals total). This age of testing corresponds to a critical developmental period of the mesocortical system involved in mediating open field locomotor activity in rodents (Kalsbeek *et al.* 1989). The open field apparatus

consisted of enclosed arenas made of white opaque polypropylene plastic and measuring 60 cm x 60 cm x 30 cm. Each arena was featureless and placed in a darkened testing room. A ceiling-mounted digital video camera recorded the locomotor activity of individual rats under infrared light. For each daily session the animals were gently lowered and placed in the same corner of the arena facing the walls. Digital video recordings were analyzed using an automated video tracking system (SMART System, San Diego Instruments, San Diego USA). Individual activity tracks were analyzed for two-dimensional distance and time traveling with the SMART software. Open field testing was carried out at the same time of day, in the same open arena apparatus, and by the same experimenter each day for each rat. Experimenters were blinded to the animals' Mn treatment condition and groups of animals tested by each experimenter were balanced by treatment.

2.4. Microdialysis measurement of extracellular brain DA and NE

Brain extracellular DA, NE, and DA metabolites were measured in the PFC of a separate cohort of PND 29 - 35 male Long-Evans rats by microdialysis (n = 8-12 animals/treatment group, 30 animals total). The average age of testing for each treatment group was 30.2 ± 1.1 days for control animals (mean ± SD), and 31.5 ± 1.5 days and 31.6 ± 1.9 days for the 25 and 50 mg Mn/kg/day groups, respectively. Animals were orally exposed to 0, 25, or 50 mg Mn/kg/day over PND 1 – 21 as described above. The 30 test subjects were obtained from 11 litters, with only one animal per litter assigned to a particular Mn treatment group.

Intracerebral dialysis

Rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (2 mg/kg, intraperitoneal injection, i.p.) supplemented with 2% (vol/vol) isoflurane in O2, mounted in a stereotaxic frame with flat skull surface, and plastic guide cannulae implanted into the right mPFC (from bregma, AP +2.5 mm, ML +1.4 mm, and DV -2.0 mm to the skull surface at a 12° angle to the vertical plane, lateral to medial approach; Paxinos and Watson, 1998). Cannulae were secured with dental acrylic and machine screws. Meloxicam (2 mg/kg, subcutaneous injection) was administered for post-operative pain. All anesthesia protocols were based on established standards for rodent surgery and were IACUC approved.

CMA12 dialysis probes (CMA Microdialysis, Kista, Sweden) with 3 mm active lengths of polyarylethersulfone membrane (concentric tube design, OD = 0.5 mm, MW cutoff = 20 kDa) were inserted into each cannula 3–4 days later, and the awake animal immediately placed into a plexiglass chamber which allowed freedom of movement. This probe location resulted in an area of dialyzed tissue that essentially comprised the dorsal to ventral extent of the mPFC region. The probe inlet was connected by fluorinated ethylene propylene (FEP) tubing to a syringe pump through a liquid switch and dual channel quartz-lined liquid swivel (Instech Labs, Plymouth Meeting, Pennsylvania). The probe outlet was connected to the swivel by the same tubing and to a collection vial in a fraction collector maintained at 4°C

(CMA170, CMA Microdialysis). Between test sessions the dialysis system was flushed extensively with high purity water.

Microdialysis experimental design

A modified Ringer's solution (in mM: Na^+ 145, K^+ 4.0, Ca^{2+} 1.3, Cl^- 152) was perfused through the probes during baseline sample collection. Two and one-half hours after probe insertion baseline extracellular fluid concentrations of analytes were assessed by two 40-min collections prior to switching for 100 min to modified Ringer's with 120 mM K^+ (K^+ replaced Na^+ to maintain isotonicity). Flow was maintained at 2.0 μL/min, and sample fractions were collected in tubes containing 5 μL of 0.1 M HCl and stored at -20°C until analysis. Desipramine (100 μM) was added to the perfusion medium in test sessions to inhibit reuptake of NE and DA and produce measurable amounts of the transmitters. Under these flow conditions, extraction efficiency of the perfusate from the dialysis probe is approximately 15%, indicating that the maximal extracellular fluid K+ concentration produced *in vivo* is in the range of 18 mM. Employing a reuptake inhibitor in the perfusion medium in order to elicit a quantifiable effect of K^+ stimulation on extracellular NE/DA in PFC has been utilized by others (e.g., Bymaster *et al.* 2002; Higashino *et al.* 2014). During the period of elevated extracellular fluid transmitter concentrations, 40-min sample collections were made followed by one additional 40-min collection for the return to baseline.

Catecholamine analysis

For determination of NE and DA, collected samples were loaded into an autosampler maintained at 4°C (Waters 717 Plus, Waters Chromatography Division, Milford, Massachusetts) and analyzed by isocratic liquid chromatography with electrochemical detection (LC-4C Amperometric Detector, BASi, West Lafayette, Indiana) at an oxidation potential of +700mV. Flow rate was 1.6 mL/min. The mobile phase consisted of 0.15 M monochloroacetate, pH 2.95, containing 1.3% acetonitrile (vol/vol), 1.7% tetrahydrofuran (vol/vol) (including 250 ppm butylated hydroxytoluene as an inhibitor), 0.86 mM sodium octylsulfate, and 0.18 mM EDTA, and was pumped through a 250 x 4.6mm, 5 μm Biophase ODS analytical column (PerkinElmer, Waltham, Massachusetts). Chromatographic peaks were quantified with EZChrom Elite software (Agilent Technologies, Pleasanton, California).

2.5. Quantitative immunohistochemistry

Immunostaining

For immunohistochemical analyses, male littermates of the behaviorally tested animals were exposed to Mn as described above and sacrificed at PND 100 (n = 5-6/animals treatment group, 30 animals total). At sacrifice, animals were deeply anesthetized with sodium pentobarbital and perfused intracardially with ice cold 0.9% (wt/vol) saline, followed by perfusion with ice cold 4% (wt/vol) paraformaldehyde (PFA). Whole brains were extracted, bisected into hemispheres, and cryopreserved as described elsewhere (Kern and Smith 2011). The PFA-fixed right hemisphere was

sectioned (Leica CM3050 S) into 20 µm coronal sections at -20°C in preparation for protein immunostaining for TH, DAT and NET, DA D1 and D2 receptors, adrenergic α_{2A} receptor, GFAP, and complement 3 (C3) and S100A10; the latter two were used as protein markers of reactive A1 or A2 astrocyte phenotype (Liddelow *et al.* 2017; Liddelow and Barres 2017). Brain sections were stored in 30% (wt/vol) sucrose in 0.01 M phosphate buffered saline (PBS) cryoprotectant solution at -20°C until immunostaining. From each brain, 2-3 sections within the mPFC were selected for staining, ranging from bregma AP +2.16-1.56 mm (Paxinos and Watson 2007). Prior to immunostaining, brain sections underwent antigen retrieval for 15 minutes in a 10 mM sodium citrate buffer solution composed of sodium citrate dihydrate in MilliQ water, and heated in a hot water bath at 80 °C. Following antigen retrieval, sections were washed three times in 0.01 M PBS for 10 min each.

Brain sections for the catecholaminergic and GFAP proteins were double-stained for two of the proteins per section, while labeling of A1 and A2 astrocyte phenotypes used a triple stain for C3, S100A10, and GFAP. For staining, brain sections were free-floated in a blocking solution containing 1% (vol/vol) normal donkey serum (Jackson Immunoresearch), 0.3% (vol/vol) Triton X-100 (Sigma Aldrich), and 1% (vol/vol) bovine serum albumin (Sigma Aldrich), in 0.01 M PBS for one hour. Primary antibody incubation was overnight at 4 °C using the following primary antibodies and dilutions in 0.5% (vol/vol) Triton X-100 in 0.01 M PBS: sheep polyclonal anti-TH, 1:1000 (Pel Freez Biologicals, P60101, RRID: AB_461070); rabbit polyclonal anti-DAT, 1:500 (EMD Millipore, AB1591P); mouse

monoclonal anti-NET, 1:500 (MAB Technologies, NET05-2; RRID: AB_2571639); rabbit polyclonal anti-D1 receptor, 1:50 (Alomone Labs, ADR-001, RRID: AB_2039826); rabbit polyclonal anti-D2 receptor, 1:250 (EMD Millipore, AB5084P); goat polyclonal anti-α_{2A} receptor, 1:200 (Santa Cruz Biotech, sc-1478); mouse monoclonal anti-GFAP, 1:1000 (EMD Millipore, MAB360); rabbit polyclonal anti-C3, 1:120 (MyBioSource, MBS2005172); chicken polyclonal anti-S100A10, 1:50 (Abcam, ab50737, RRID: AB_881813). Next, brain sections were washed three times and incubated in a secondary antibody solution composed of 10% (vol/vol) normal donkey serum and corresponding secondary antibodies in 0.5% (vol/vol) Triton X-100 in 0.01 M PBS for 2 hours at room temperature. Secondary antibodies were: donkey anti-sheep Alexa Fluor 488, 1:1000 (Abcam, ab150177, RRID: AB_2801320); donkey anti-rabbit Alexa Fluor 488, 1:1000 (Molecular Probes, ab150073); donkey anti-mouse Alexa Fluor 594, 1:1000 (ThermoFisher, A-21203, RRID: AB_2535789); donkey anti-rabbit Alexa Fluor 594, 1:1000 (ThermoFisher, A-21207, RRID: AB_141637); donkey anti-goat Alexa Fluor 594, 1:1000 (Abcam, 150132); donkey anti-chicken CF-350 (Millipore Sigma, SAB4600219). Brain sections were then washed three more times in 0.01 M PBS and incubated for 10 min in a 1:1000 DAPI stain in 0.01 M PBS to label cell nuclei. Finally, sections were washed three times in 0.01 M PBS and mounted on slides and coverslipped with Fluoromount G mounting media (Southern Biotech) in preparation for fluorescence microscopy. Prior to imaging, 5-6 sections were mounted per slide, balanced by Mn treatment condition.

Fluorescence microscopy and image quantification

Images were collected within the mPFC at 40x magnification for catecholaminergic proteins and GFAP, while C3 and S100A10 images were collected at 63x. To avoid bias in image acquisition, a box was drawn in both subregions of interest using the Zeiss ZEN imaging software, and a pseudorandomization tool determined three non-overlapping fields of view for collection. This yielded a total of six images per brain section and 12-18 images per animal across proteins, with the exception of C3 and S100A10, for which only four images per section were collected. All images per protein were captured under identical microscopy imaging settings, including exposure time and gain, using a Zeiss AxioImager microscope. Each image was collected in a z-stack format at 0.5 μm intervals between each z-focal plane over a total imaging range of 13 μm in the 20 μm brain slice. Following image collection, the number of z-plane images were reduced to 20 (10 μm z-plane distance) to remove out-of-focus z-planes, and then deconvolved using AutoQuant X3 software (version 3.1). Deconvolved images were imported into Imaris (software version 9.2) for fluorescence intensity quantification.

Fluorescence quantification was performed using the Imaris "Surfaces" tool, with unique quantification algorithms applied to each fluorescence channel and protein. Algorithms were customized to each protein, first by applying automated thresholds for absolute fluorescence intensity to determine whether quantified surfaces matched the amount of fluorescent objects in the image based on visual

inspection. If the automated thresholding appeared incongruous with the staining pattern, the "Background Subtraction" tool was used to improve specificity of the algorithm. The primary quantification outcomes used for analysis were the sum total fluorescence per three dimensional object summed across all objects per image, the total number of objects per image, and the total volume of all objects per image.

For astrocyte A1 and A2 phenotype analysis, a colocalization algorithm was developed in Imaris using GFAP as an identifier for reactive astrocytes, and then fluorescence intensity of GFAP-colocalized C3 (A1 phenotype) and S100A10 (A2 phenotype)-positive astrocytes was determined. For this, imaging channels for C3 and S100A10 were separately colocalized with GFAP, first by generating a GFAP surface algorithm, followed by a "Spots" algorithm for the more punctate staining patterns of C3 and S100A10 proteins. A "Distance to Surfaces" transformation tool was used to separately isolate C3 and S100A10 objects that were less than 0.2 μm from a GFAP surface object to include for quantification. Primary outcomes for quantification data were total fluorescence per image of all GFAP-colocalized C3 and S100A10 objects, respectively. In all cases of image acquisition and quantification, the experimenter was blinded to the treatment condition.

2.6. Dendritic spine density analysis

Spine density on PFC layer III pyramidal cell dendrites was quantified in separate cohorts of PND 24 or PND 145 animals treated with 0 or 50 mg Mn/kg/day over PND 1 – 21 (n = 10 cells/animal, 5-10 animals/treatment group and age, 28

animals total), as described previously (Beaudin *et al.* 2015). Briefly, rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% (wt/vol) saline. The brains were extracted, rinsed with Milli-Q water, and then prepared for Golgi–Cox staining using the rapid Golgi stain kit (FD Neuroethologies, Inc., Ellicott City, MD). For this, the brains were placed in a Golgi–Cox solution provided by the manufacturer and stored at room temperature in the dark for 14 days followed by 3 days in a 30% sucrose solution (wt/vol). Brains were cut into 250 μm coronal sections using a vibratome, mounted on gelatin-coated slides, and allowed to dry naturally at room temperature before staining within the next 12–24 hours. To be included in the analysis of spine density, the dendritic branch of a given neuron had to be well-impregnated and free of stain precipitations, blood vessels, and astrocytes. The mPFC brain region of interest was identified at 10x magnification using a Leica DM5500B widefield microscope fitted with a motorized stage and multi-point image acquisition. In the mPFC, five layer III pyramidal cells were selected from each hemisphere in the cortical areas (from bregma, AP + 2.16-1.56 mm, ML +0.2-1.5 mm, DV +0.9-3.6 mm; n =10 cells total) (Paxinos and Watson 2007). For the PND 24 animals, dendritic spines were counted live at 100x magnification from a ~30-50 μm segment of a single basal dendrite, and individual second-order and terminal tip (third-order) apical dendrites were defined from each pyramidal cell, with the condition that the entire dendritic segment was within the focal plane of the microscope. Similarly, second-order apical dendrites on 10 mPFC layer III pyramidal cells were counted in the PND 145 animals. The exact length of counted dendrite was determined using the length

measurement tool function of the microscope. Spine density was calculated as the number of spines per 10 μm of dendrite length. All neuronal cell selection and spine counting were done by individuals blind to the treatment conditions of the rats.

2.7. Blood and brain Mn levels, and blood hematocrit

Blood and brain Mn concentrations were determined in littermates of the immunostaining study animals that were retained specifically for tissue Mn analyses (PND 24 and PND 66) or that were sacrificed following behavioral testing and evoked neurotransmitter measurement by microdialysis (~PND 590; n = 6 – 12/treatment group and time point, 104 animals total), as reported in Beaudin et al. (2017a; 2015; 2013) and Lasley et al. (2020). Animals were heavily anesthetized with sodium pentobarbital overdose (75 mg/kg intraperitoneal injection), and whole blood (2 – 3 mL) was collected from the left ventricle of the surgically-exposed heart and stored in EDTA vacutainers at -20 °C for analyses. Whole brain was immediately removed, bisected into hemispheres, and hind-brain regions of each hemisphere were collected and stored at -80 °C for Mn concentration determinations (forebrain was dedicated to other outcome measures). Aliquots of whole blood were digested overnight at room temperature with 16 M HNO_3 (Optima grade, Fisher Scientific), followed by addition of H_2O_2 and Milli-Q water. Digestates were centrifuged (13,000 x g for 15 min.) and the supernatant collected for Mn analysis. For brain, aliquots of homogenized hind-brain tissue (~200 mg wet weight) were dried and digested with hot 16 M HNO_3, evaporated and redissolved in 1 M HNO_3 for analyses. Rhodium

was added to sample aliquots as an internal standard. Manganese levels were determined using a Thermo Element XR inductively coupled plasma – mass spectrometer, measuring masses ^{55}Mn and ^{103}Rh (the latter for internal standardization). External standardization for Mn used certified SPEX standards (Spex Industries, Inc., Edison, NJ). National Institutes of Standards and Technology SRM 1577b (bovine liver) was used to evaluate procedural accuracy. The analytical detection limit for Mn in blood and brain was 0.04 and 0.015 ng/mL, respectively. Finally, whole blood hematocrit was measured at PND 24 and 66 in cohorts 1 and 2 to assess whether Mn exposure overtly impacted body iron status.

2.8. Statistical analyses

The open field activity data were modeled by way of structured covariance mixed models. Fixed effects included in the model were Mn treatment as a between-subjects factor (five levels corresponding to the five treatment groups), and test day (five levels corresponding to the 5 days of testing) and test interval (six levels corresponding to the six 5-minute intervals per 30 minute test session) as within-subjects factors. In all models, animal was included as a random effect to account for correlations within observations from the same animal. Statistical tests used a Satterthwaite correction. Plots of residuals by experimental condition were used to examine the assumption of homogeneity of variance. The distribution of the random effect was inspected for approximate normality and presence of influential outliers. Significant main effects or interaction effects were followed by single-degree of freedom contrasts in order to clarify the nature of the interactions, using the Student's

t-test for pairwise comparisons of least squared means. Immunohistochemistry data were analyzed using a standard least squares mixed model analysis of variance that included Mn treatment group (five levels) as the between subjects factor and animal as a random effect. Pair-wise treatment group comparisons were performed using Tukey's *post hoc* test. Tukey outlier box plots were used to identify possible outliers, and frequency distribution plots were used to assess data normality. The DA and NE microdialysis data were analyzed by ANOVA for each neurotransmitter with Mn exposure group as the between subjects factor (three levels) and time after K^+ stimulation as the within subjects factor. Concentrations determined in individual animals were averaged at each baseline time point, then collapsed across time to yield a single group baseline value. Significant main effects or Mn x time interactions after K^+ initiation for neurotransmitter concentrations were followed by Tukey's *post hoc* test to make pair-wise comparisons between individual exposure groups. The ROUT and Grubb's test were used to identify outliers in the microdialysis data, and the most conservative outcome was accepted. Spine density data were analyzed similarly, with Mn treatment (two levels) as the main effect and animal as a random effect; data for the three dendrite locations (i.e., second-order, terminal tip, basal) and two ages of animals were analyzed separately. Data for blood and brain Mn levels were analyzed using the Wilcoxon/Kruskal-Wallis test. Data were log transformed before analysis if necessary to achieve normal distribution and homogeneity of variance. In all cases, the significance level was set at $p \leq 0.05$. Power analyses were applied to determine the sample size for open field behavioral outcomes (n = 21-23 rats in each of the five

treatment groups), based on the ability to detect small differences in group behavioral performance (Beaudin *et al.* 2013; Beaudin *et al.* 2015; Beaudin *et al.* 2017a; Beaudin *et al.* 2017b; Kern *et al.* 2010; Kern and Smith 2011). For the IHC (n = 5-6 animals/treatment), Mn biomarker (n = 5-12 animals/treatment group), and spine density (n = 5-10 animals/treatment group) outcomes, the group sizes were based on power analyses indicating the number of animals per group needed to statistically detect differences between treatments, taking into account plausible effect sizes and group variances reported in our published studies (Kern and Smith 2011; Beaudin *et al.* 2013; Beaudin *et al.* 2015; Beaudin *et al.* 2017a; Beaudin *et al.* 2017b). Analyses were conducted using SAS (version 9.4) for Windows on a mainframe computer (open field data), GraphPad Prism v.6.04 for Windows (GraphPad Software, San Diego, CA, microdialysis data), or JMP (version 13.0; SAS Institute, Inc. for all other data).

3.0. Results

Overall, early postnatal Mn exposure led to increased behavioral reactivity in the novel open field environment, and produced lasting changes in the catecholaminergic systems of the mPFC. Specifically, in the open field, Mn exposure increased total distance traveled, but only in the initial minutes of each daily test session. With respect to the mPFC catecholaminergic systems, Mn exposure produced lasting alterations in the synaptic proteins TH, D1, D2, DAT, and NET, and reductions in evoked outflow of extracellular NE. In contrast, protein levels of the

α_{2A} adrenergic receptor were unchanged by Mn. These catecholaminergic systems changes were accompanied by a lasting increase in astrocyte GFAP protein levels, and a predominantly proinflammatory A1 astrocyte phenotype, but no measurable change in mPFC layer III pyramidal cell dendritic spine density was observed. These findings are detailed below.

3.1. Early postnatal Mn exposure caused increased behavioral reactivity in the open field

To determine the effect of early and lifelong postnatal Mn exposure on behavioral reactivity and habituation to a novel environment, the distance traveled in an open field was measured in daily 30 minute test sessions over 5 consecutive days, starting on PND 24. The main effect of Mn exposure was not significant [$F(4, 102) = 1.13$, $p = 0.34$], nor was the interaction of Mn exposure x test session day [$F(16, 776) = 1.27$, $p = 0.21$]. The three-way interaction of Mn x test session day x within-session time interval [$F(80, 2724) = 0.82$, $p = 0.86$] was also not significant. However, the effects of test session day [$F(4, 776) = 12.36$, $p < 0.0001$] and within-session time interval [$F(5, 975) = 988.13$, $p < 0.0001$] were significant, as was the interaction of Mn exposure x within-session time interval [$F(20, 974) = 2.03$, $p = 0.0047$] (Figure 2). Specifically, early postnatal Mn exposure over PND 1 – 21 increased the total distance travelled within the first 5-minute interval of the daily 30-minute testing session across the 5 days of testing in both the early 25 ($p = 0.0042$) and early 50 Mn groups ($p = 0.0022$), relative to controls (Figure 2a). Lifelong Mn exposure (PND 1 –

29) also caused a significant increase in total distance travelled over the first two 5-minute intervals for the lifelong 25 ($p = 0.038$) and lifelong 50 Mn ($p = 0.041$) groups, as well as a trending increase for both treatment groups during the third within-session interval ($p = 0.078$ and 0.076 for the lifelong 25 and 50 groups, respectively), relative to controls (Figure 2b). A direct comparison of the lifelong and early life exposure groups showed that the longer exposure did not exacerbate the Mn effect (contrasts between the lifelong vs. early life Mn groups for each within-session time interval yielded p's > 0.3 and p's > 0.18 for the 25 and 50 Mn groups, respectively). This latter result is not surprising, given that the lifelong Mn groups

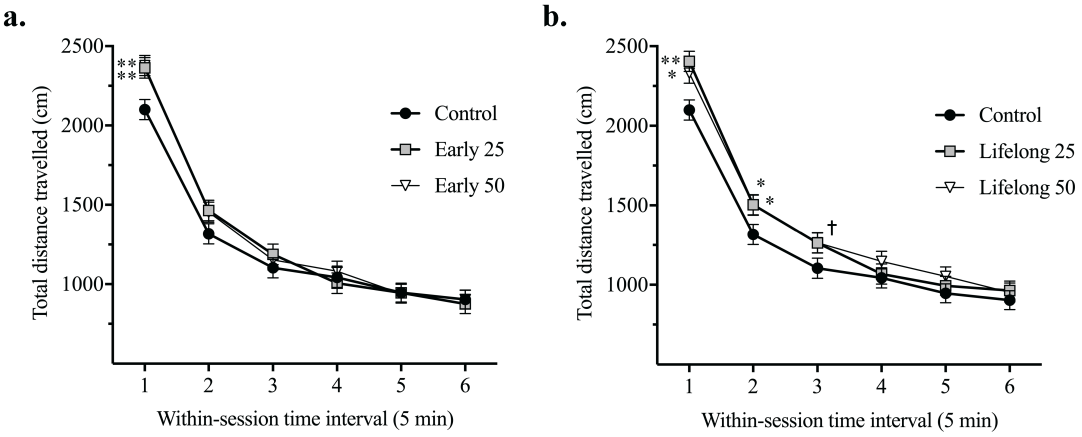

Figure 2. Early postnatal Mn exposure caused increased behavioral reactivity in the open field. Total distance (cm) for the early (**a**) and lifelong (**b**) postnatal Mn exposure groups as a function of time in the open field, shown in 5 minute within-session intervals in a daily 30-min open field test sessions conducted over 5 consecutive days; data are collapsed across the 5 daily test sessions because there was no main effect or interaction involving test session day. * and ** indicate $p < 0.05$ and $p < 0.01$ versus controls, respectively. † indicates $0.05 \leq p < 0.1$ versus controls. Data are least squares means ± SEM (n = 21-23 animals/group).

received only one additional week of exposure versus the early life Mn groups during open field testing over PND 24 – 29.

3.2. Early postnatal Mn exposure caused dose-dependent reductions in evoked NE outflow in the mPFC

In order to determine the effect of early postnatal Mn exposure on catecholaminergic systems function and to elucidate the alterations that may underlie the Mn-induced impairments in attention, impulse control, and arousal regulation reported previously (Beaudin *et al.* 2013; Beaudin *et al.* 2015; Beaudin *et al.* 2017a; Beaudin *et al.* 2017b), we assessed the impact of early postnatal Mn exposure over PND 1 – 21 on the evoked release of DA and NE in the mPFC of PND 29-35 animals. For extracellular NE there was a significant main effect of exposure [$F(2, 27) = 5.10$, $p = 0.0133$] as well as a significant Mn exposure x time interaction [$F(14, 189) = 1.91$, $p = 0.028$]. These effects were observed as statistically significant decreases from control values at the 40 minute time point after initiation of K^+ stimulation in both Mn groups ($p < 0.01$ and $p < 0.0001$ for the early 25 and 50 Mn groups, respectively; Figure 3a). Moreover, NE in the early 50 Mn group at this time point was found to be significantly less than the early 25 Mn group ($p < 0.05$), establishing a dose dependence. Analogous effects of early life Mn exposure on extracellular DA were not apparent – neither a main effect of Mn exposure [$F(2, 27) = 1.76$, $p = 0.191$] nor an exposure x time interaction [$F(14, 189) = 1.37$, $p = 0.173$] were present, even though a dose-dependent ordering of NE responses was noted (Figure 3b).

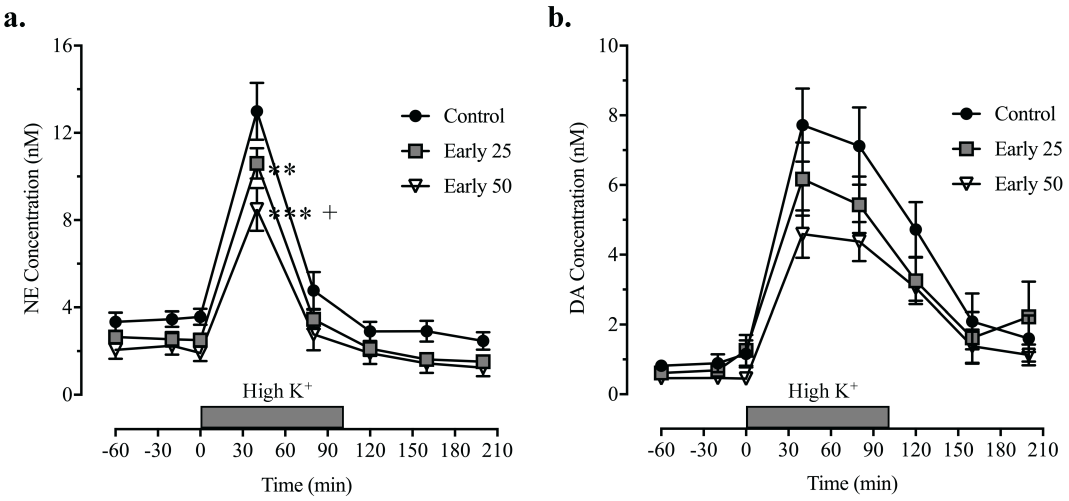

Figure 3. Mn exposure caused a significant reduction in the stimulated release of NE in the mPFC of young adolescent animals. Concentrations of **(a)** NE and **(b)** DA in nM as a function of time after administration of a high K^+ stimulus. Statistical analysis reflects differences in NE at 40 min after initiation of high K^+ perfusion based on Tukey's multiple comparison test. ** and *** indicate $p < 0.01$ and $p < 0.0001$ versus controls, respectively. $^+$ indicates $p < 0.05$ vs. the early 25 Mn dose. Data are means ± SEM (n = 8-12 animals/group).

3.3. Quantitative immunohistochemistry of PFC catecholaminergic synaptic proteins

To further determine the impact of early and lifelong postnatal Mn exposure on the neuronal synaptic environment, we measured protein levels of the catecholaminergic systems proteins TH, DAT, NET, DA D1 and D2 receptors, and the adrenergic α_{2A} receptor in the mPFC of PND 100 animals. Many of these proteins have been shown to play a role in neurobehavioral functions mediated by the mPFC, including arousal regulation, attention, and impulse control functions (Arnsten and Dudley 2005; Arnsten 2009b; Arnsten and Pliszka 2011; Schmeichel and Berridge 2013; Logue and Gould 2014) and have been shown to be disrupted by early postnatal Mn exposure (Kern *et al.* 2010; Kern and Smith 2011; Mcdougall *et al.* 2011;

McDougall *et al.* 2008; Anderson *et al.* 2009). Notably, based on DAPI-labeling there was no effect of Mn exposure on total cell numbers collected in the immunofluorescence images [$F(4, 22.53) = 1.209$, $p = 0.33$; overall mean number of DAPI-labeled cells/image = 185 ± 1.0 SEM, n = 540 images from 30 animals x 18 images/animal; the range across images = 125 – 236 cells/image].

3.3.1. Early postnatal Mn exposure caused lasting reductions in presynaptic catecholaminergic protein levels

The impact of early and lifelong Mn exposure on TH protein levels was assessed to determine whether the reduction in evoked NE outflow in PND 29 – 35 animals was consistent with changes in TH levels in mPFC neurons in PND 100 animals. Overall, postnatal Mn exposure led to a significant reduction in mPFC TH levels [$F(4, 24.79) = 88.85$, $p < 0.0001$]. Specifically, early postnatal Mn exposure to the higher 50 mg Mn/kg/day dose caused a significant reduction in TH protein levels to ~58% of controls ($p = 0.019$), while no measurable change was found in the lower dose early 25 group ($p = 0.77$). By comparison, lifelong Mn exposure over PND 1 – 100 caused a significant decrease in TH levels for both the lifelong 25 and lifelong 50 groups to ~54% and ~45% of controls, respectively (p's <0.0001 for both). Moreover, TH protein levels in the both the lifelong 25 and 50 exposure groups were significantly lower than their early life counterparts ($p < 0.0001$ and $p = 0.019$, respectively), indicating that lifelong Mn exposure further exacerbated the impacts of early life Mn exposure on TH protein levels in the mPFC (Figure 4a).

DAT and NET protein levels were measured in the mPFC of PND 100 animals to determine whether catecholamine reuptake transporters were impacted by postnatal Mn exposure in a manner consistent with the effect of Mn exposure to reduce TH protein levels and evoked release of NE. Levels of DAT were significantly reduced by Mn exposure [$F(4, 24.64) = 20.72$, $p < 0.0001$], with the early 50 Mn group reduced to ~23% of controls ($p < 0.0001$), while the early 25 group trended towards a decrease to ~60% of controls ($p = 0.075$). Similarly, lifelong exposure to the 25 and 50 Mn doses caused a significant reduction in DAT to ~40% ($p = 0.0008$) and ~22% ($p < 0.0001$) of controls, respectively. However, the lifelong Mn exposure groups were not different from their early life counterparts (p's > 0.34), indicating that lifelong Mn exposure did not worsen the effects of early life exposure on DAT protein levels in the mPFC (Figure 4b).

Similarly, NET protein levels were also significantly reduced by Mn exposure [$F(4, 25.16) = 42.47$, $p < 0.0001$], with the early life 25 and 50 exposures reducing NET levels to ~57% and ~36% of controls, respectively ($p < 0.0001$ for both). Lifelong Mn exposure caused similar reductions in mPFC NET levels to ~63% ($p = 0.0002$) and ~47% ($p < 0.0001$) of controls for the lifelong 25 and 50 Mn groups, respectively (Figure 4c). As with DAT protein above, lifelong Mn exposure did not worsen the effects of early life Mn on NET protein levels (p's > 0.74 for the lifelong vs. early life Mn group contrasts).

To assess whether the effect(s) of Mn exposure on catecholaminergic protein levels based on immunofluorescence intensity reported above could be accounted for

by changes in the number or volume of Imaris-rendered immunofluorescent objects, we determined the average number and volume of Imaris-rendered objects in the collected images. With this, we assumed that changes in average object number and volume reflects changes in the number and size of spatially separate puncta of the expressed protein. Results show that both TH and NET, but not DAT object number and volume, were affected by Mn exposure. Specifically, early postnatal Mn exposure caused reductions in both TH total object number [$F(4, 25) = 108.30$, $p < 0.0001$; Supplement 2, Figure 1a] and TH object volume [$F(4, 25.09) = 39.82$, $p < 0.0001$; Supplement 2, Figure 1b], whereas for NET, only the object volume was significantly reduced from controls across all Mn groups [$F(4, 23.63) = 8.98$, $p = 0.0001$; p's < 0.008 for all specific contrasts relative to control; Supplement 2, Figures 1f, 1e]. By comparison, Mn had no effect on DAT object number [$F(4, 24.84) = 1.64$, $p = 0.19$; Supplement 2, Figure 1c] or object volume [$F(4, 25.02) = 1.14$, $p = 0.36$; Supplement 2, Figure 1d].

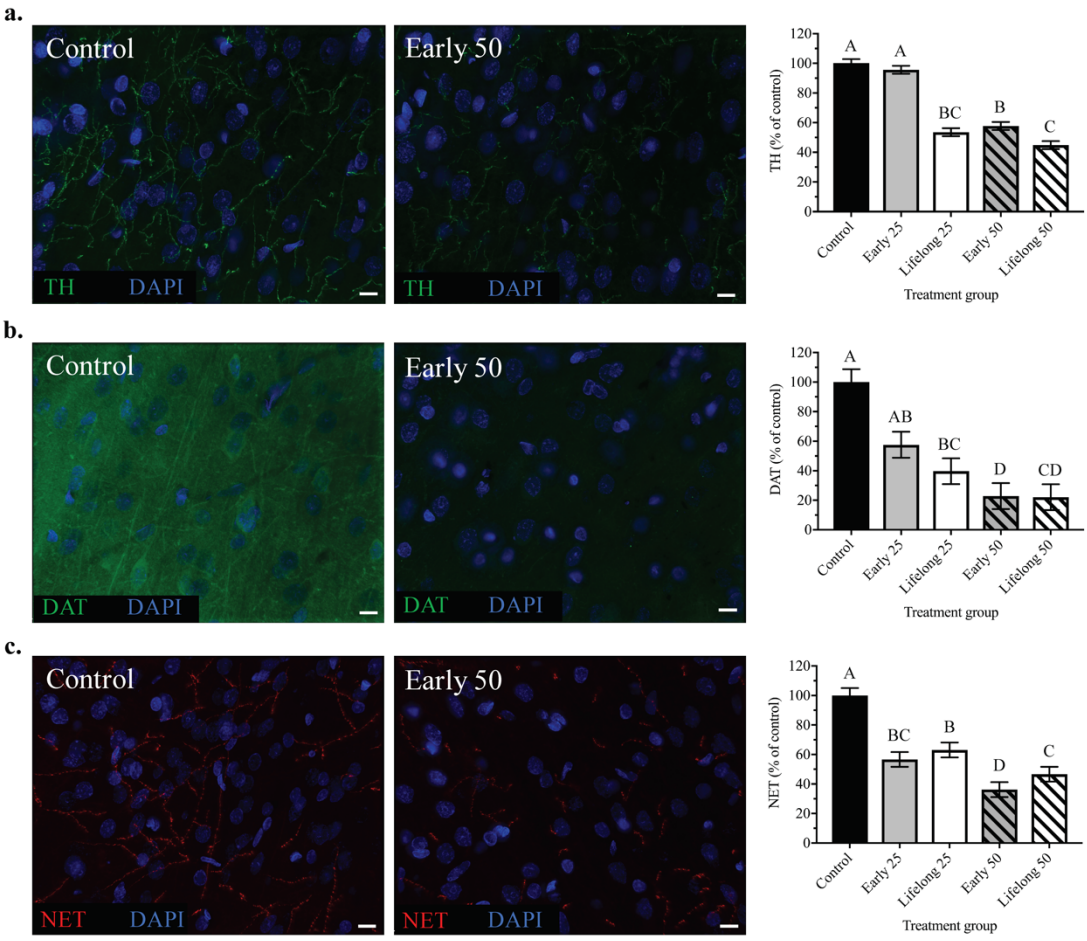

Figure 4. Postnatal Mn exposure caused lasting reductions in PFC TH, DAT, and NET protein levels. Representative immunohistochemistry images of (**a**) TH, (**b**) DAT and (**c**) NET staining from mPFC brain sections of control and early life 50 Mn treatment groups. Representative images at 63x magnification for clarity (scale bars, 10 µm). Bar charts show quantified fluorescence intensity reflecting 12 images/animal (at 40x magnification) and n = 6 animals/treatment group; data are least squares means ± SEM, shown as percent of control values generated from the statistical model that included all five treatment groups. Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test.

3.3.2. Postnatal Mn exposure caused lasting alterations in dopaminergic, but not α_{2A}, receptor protein levels

To determine whether the behavioral changes reported here and in our previous studies (Beaudin *et al.* 2017a; Beaudin *et al.* 2017b; Beaudin *et al.* 2015;

Beaudin *et al.* 2013; Kern and Smith 2011; Kern *et al.* 2010) reflected alterations in the abundance of catecholaminergic neurotransmitter receptors, we quantified the protein levels of DA D1 and D2 receptors, along with adrenergic α_{2A} receptors in the mPFC of PND 100 animals. Notably, the presence and direction of effects of postnatal Mn exposure were different for the dopaminergic versus α_{2A} adrenergic receptors, and for the direction of the effect on the excitatory D1 (decreased) versus inhibitory D2 (increased) receptors (Figure 5). Specifically, D1 receptor levels were reduced by Mn exposure [$F(4, 25.47) = 117.96$, $p < 0.0001$], with a measurable effect of both Mn exposure dose and duration. Early postnatal exposure to the 25 and 50 mg/kg/day Mn doses caused significant reductions in D1 protein levels to ~78% and ~47% of controls, respectively ($p < 0.0001$ for both). Lifelong Mn exposure caused similar reductions in D1 to ~70% and ~38% of controls for the lifelong 25 and 50 groups, respectively ($p < 0.0001$ for both). Moreover, lifelong exposure to the higher 50 Mn dose worsened the effects of Mn exposure restricted to early postnatal life on mPFC D1 protein levels ($p = 0.026$), while lifelong exposure to the lower 25 Mn dose led to a trending reduction compared to early life exposure ($p = 0.085$) (Figure 5a).

Postnatal Mn exposure also led to significant lasting changes in mPFC D2 receptor levels, but the effects were directionally opposite to those observed for D1 receptor protein. Specifically, early life exposure to the higher 50 Mn dose caused a significant increase in D2 protein levels to ~240% of controls ($p < 0.0001$), while the lower early life 25 dose had no measurable effect ($p = 0.54$) (Figure 5b). By comparison, both lifelong Mn exposure doses significantly increased mPFC D2

protein levels to ~115% ($p = 0.011$) and ~247% ($p < 0.0001$) of controls for the lifelong 25 and 50 doses, respectively. However, there were no measurable differences in D2 protein levels between the lifelong Mn groups and their early life Mn counterparts (p's >0.28 for group contrasts), indicating that lifelong Mn exposure did not worsen the effects from Mn exposure restricted to early postnatal life (Figure 5b).

In contrast to the Mn effects reported above, there was no measurable effect of Mn on α_{2A} receptor levels [$F(4, 23.94) = 0.119$, $p = 0.97$] (Figure 5c). In addition, regarding assessment of Imaris-rendered object number and volume for D1, D2, and α_{2A}, only D1 object number was significantly affected by Mn exposure [$F(4, 25.16) = 3.40$, $p = 0.024$], reflecting a lower number of D1 objects in the lifelong 50 Mn group versus controls ($p = 0.013$; Supplement 2, Figure 1g). Mn exposure also caused a trending reduction in D1 object volume ($p = 0.072$; Supplement 2, Figure 1h), but had no effect on object number or volume for D2 and α_{2A} (p's > 0.153; Supplement 2, Figures 1i – 1k).

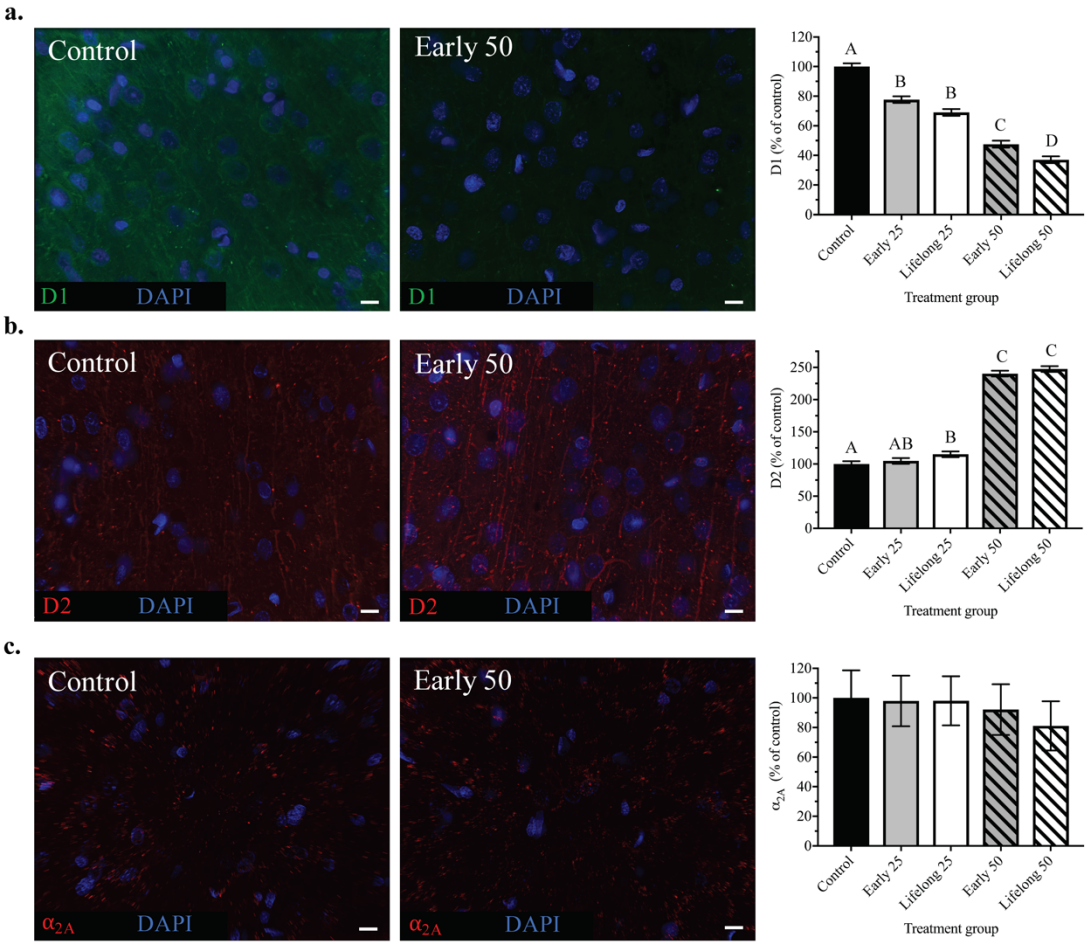

Figure 5. D1 and D2 protein levels, but not α_{2A}, were altered by early postnatal Mn exposure. Representative immunohistochemistry images of (**a**) D1, (**b**) D2 and (**c**) α_{2A} receptor staining from mPFC brain sections of control and early life 50 Mn treatment groups. Bar charts show quantified fluorescence intensity reflecting 12-18 images/animal (40x magnification), where n = 6 animals/treatment group. Representative images at 63x magnification for clarity (scale bars, 10 μm). Data are least squares means ± SEM shown as percent of control values generated from the statistical model that included all five treatment groups. Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test.

3.3.3. Early postnatal Mn exposure caused lasting increases in astrocyte reactivity

We measured astrocyte GFAP protein levels in order to determine whether postnatal Mn exposure led to heightened astrocyte reactivity as an indicator of

neuroinflammation, which could alter the synaptic environment and possibly contribute to changes in catecholaminergic synaptic proteins within the mPFC. Postnatal Mn exposure caused lasting increases in astrocyte GFAP levels [$F(4, 25.09) = 40.93$, $p < 0.0001$], with increases of ~242% and ~215% of controls in the early life and lifelong 50 mg/kg/day Mn dose groups, respectively (p's < 0.0001 for both; Figure 6). In contrast, astrocyte GFAP levels in the early life and lifelong 25 mg/kg/day Mn dose groups were not different from controls (p's >0.70). Moreover, astrocyte GFAP levels were not measurably different between the lifelong versus early life 50 groups, indicating that lifelong Mn exposure did not alter the lasting effects caused by Mn exposure restricted to early life ($p = 0.67$). Finally, there was no effect of Mn exposure on GFAP object number in the mPFC ($p = 0.78$; Supplement 2, Figure 1l), though there was an effect of Mn on GFAP total object volume [$F(4, 28.97) = 17.05$, $p < 0.0001$; Supplement 2, Figure 1m], with an increase in GFAP object volume specifically in the early life 50 group versus controls ($p = 0.0004$; Supplement 2, Figure 1m).

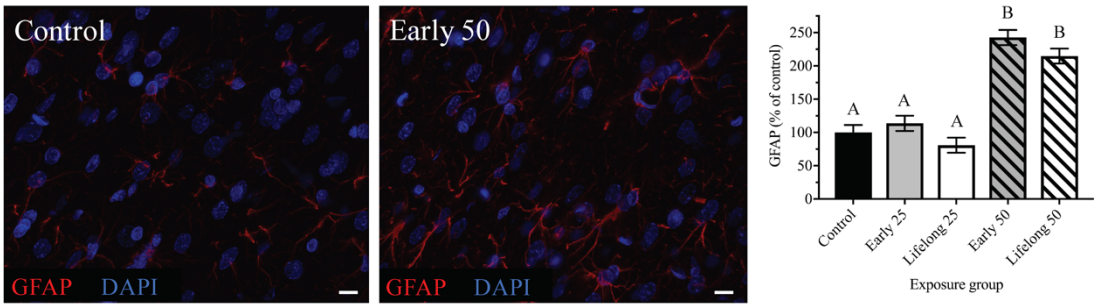

Figure 6. GFAP protein levels varied in a dose-dependent manner in response to early postnatal Mn exposure. Representative immunohistochemistry images of GFAP staining from mPFC brain sections of control and early life 50 Mn treatment groups. Bar charts show quantified

fluorescence intensity reflecting 12 images/animal (40x magnification), where n = 6 animals/treatment group. Representative images at 63x magnification for clarity (scale bars, 10 μm). Data are least squares means ± SEM shown as percent of control values generated from the statistical model that included all five treatment groups. Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test.

3.3.4. A1 reactive astrocytes were induced in greater proportions than A2 astrocytes by early postnatal Mn exposure

In order to further investigate the inflammatory phenotype of the reactive astrocytes in the mPFC following Mn exposure, we co-immunostained mPFC brain sections with GFAP and complement C3 or S100A10 protein-specific antibodies – the latter two as markers for proinflammatory A1 and anti-inflammatory A2 astrocyte phenotypes, respectively (Liddelow *et al.* 2017; Liddelow and Barres 2017). Given that astrocyte reactivity (i.e., increased GFAP) in PND 100 animals was most evident in the early life 50 Mn group, we restricted analyses to the early life 25 and 50 Mn groups versus controls. Results show that early life Mn exposure caused significant increases in both GFAP co-localized C3 [$F(2, 12) = 23.73$, $p < 0.0001$], and S100A10 [$F(2, 12) = 45.91$, $p < 0.0001$]. Specifically, early life exposure to the higher 50 mg/kg/day Mn dose increased GFAP co-localized C3 to ~570% of controls ($p < 0.0001$), and increased GFAP co-localized S100A10 levels to 200% of controls ($p < 0.0001$) (Figures 7c, d). In contrast, there was no effect of the lower early life 25 mg/kg/day Mn dose on either GFAP co-localized C3 or S100A10 (p's ≥ 0.90 for both).

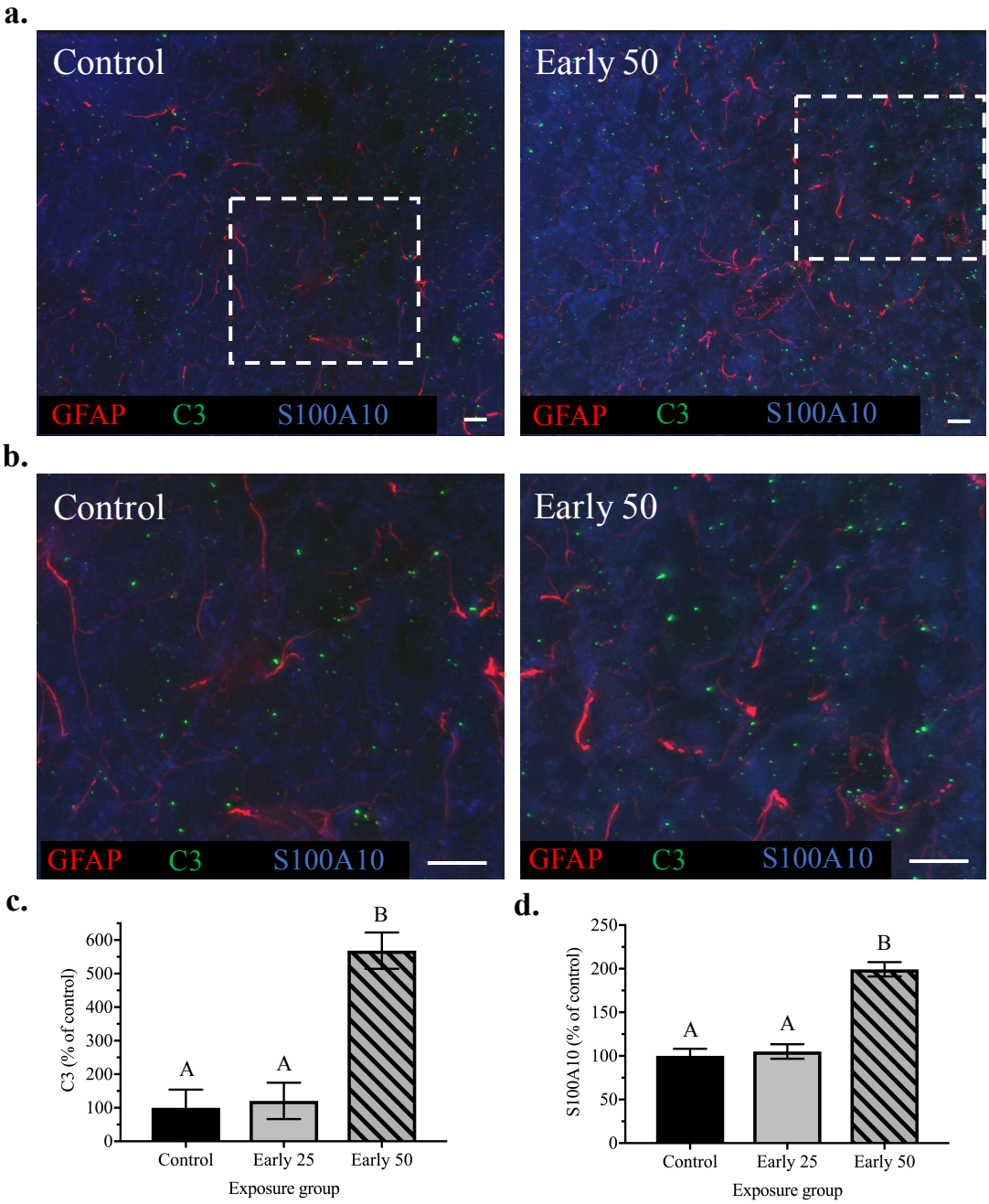

Figure 7. C3 and S100A10 protein levels are increased in response to early postnatal Mn exposure. Representative immunohistochemistry images of (**a)** GFAP, C3, and S100A10 staining from mPFC brain sections of control and early life 50 Mn treatment groups at 63x magnification; white boxes are enlarged (**b**) for clarity (scale bars, 10 μm). (**c, d**) Bar charts show quantified fluorescence intensity of GFAP-colocalized puncta for C3 and S100A10, respectively; data reflect 4 images/animal, where n = 5 animals/treatment

group. Data are least squares means ± SEM shown as percent of control values generated from the statistical model that included three treatment groups. Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test.

3.4. Dendritic Spine Density

Early postnatal Mn exposure did not alter mPFC dendritic spine density

To determine whether the lasting changes in catecholaminergic protein levels and increased inflammatory reactive astrocytes were accompanied by changes in dendritic spine density, we quantified spine density on mPFC layer III pyramidal cell dendrites (# spines/10 μm dendrite length) in PND 24 and PND 145 animals exposed over early postnatal life to the higher 50 mg/kg/day Mn dose. In postweaned PND 24 animals, there was no effect of early life Mn exposure on spine density on apical (2^{nd} order + terminal tip) dendrites [$F(1, 7.76) = 0.73$, $p = 0.42$], or on basal dendrites [$F(1, 6.91) = 1.04$, $p = 0.34$] of mPFC layer III pyramidal neurons. Similarly, there was no effect of early life Mn on spine density on apical 2^{nd} order dendrites in PND 145 animals [$F(1, 16.39) = 2.74$, $p = 0.12$] (Figure 8).

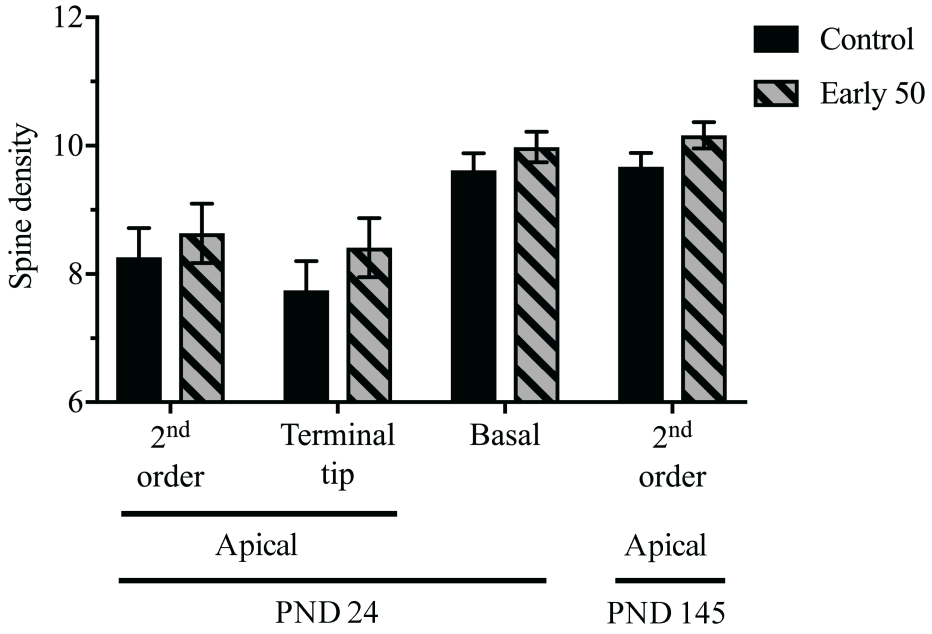

Figure 8. Dendritic spine density was not measurably altered in the mPFC following Mn exposure. Data are least squares mean spine density (spines/10 µm dendrite length ± SEM) on mPFC layer III pyramidal neuron dendrites in the control and early 50 mg/kg/day Mn-exposed groups across dendrite type and animal age (n = 10 neurons/animal, 5-10 animals/treatment group).

3.5. Tissue Mn and blood hematocrit levels

Mn exposure resulted in body Mn levels consistent with environmental exposures

Early postnatal Mn exposure led to a dose-dependent increase in blood and brain Mn levels across age groups at PND 24, 66, and ~590, though levels were significantly higher in the PND 24 weanlings compared to their older adolescent and adult counterparts. Notably, tissue Mn levels in the latter two age groups were very comparable to each other across Mn treatment condition, and within the adolescent and adult ages tissue Mn levels in the Mn exposed groups were only slightly higher than their age-matched controls (Table 1). Finally, there were no measurable

differences in blood hematocrit levels between Mn exposure groups at age PND 24 (hematocrit range 39.4 – 41.0 % between treatment groups, $F(2, 15) = 1.49$, $p = 0.26$) or PND 66 (range 45.6 – 46.4 % between treatment groups, $F(4, 35) = 0.18$, $p = 0.95$).

Table 1. Blood and brain Mn concentrations of animals at PND 24, 66, and ~590.

	Age (PND)	Control	25 mg Mn/kg/day Early life	25 mg Mn/kg/day Lifelong	50 mg Mn/kg/day Early life	50 mg Mn/kg/day Lifelong
Blood	24	24.6 ± 1.21 (6)$^{A, a}$	NA	131 ± 22.7 (7)$^{B, a}$	NA	231 ± 33 (7)$^{C, a}$
	66	9.3 ± 0.59 (7)$^{A, b}$	12.2 ± 0.69 (8)^{B}	12.9 ± 0.86 (9)$^{B, b}$	11.5 ± 0.57 (8)^{B}	18.6 ± 1.9 (7)$^{C, b}$
	590	5.66 ± 0.57 (5)$^{A, c}$	6.98 ± 0.95 (11)^{A}	21.9 ± 8.66 (9)$^{B, c}$	8.28 ± 1.32 (8)^{AB}	16.8 ± 1.83 (10)$^{C, b}$
Brain	24	3.62 ± 0.13 (6)$^{A, a}$	NA	6.49 ± 0.56 (7)$^{AB, a}$	NA	11.5 ± 2.57 (6)$^{B, a}$
	66	2.12 ± 0.060 (7)$^{A, b}$	2.19 ± 0.031 (8)^{A}	2.41 ± 0.045 (9)$^{BC, b}$	2.26 ± 0.062 (8)^{AB}	2.51 ± 0.068 (7)$^{C, b}$
	590	1.82 ± 0.11 (6)$^{A, b}$	2.20 ± 0.17 (12)^{A}	2.19 ± 0.049 (10)$^{AB, b}$	2.00 ± 0.086 (7)^{A}	2.68 ± 0.11 (11)$^{C, b}$

Data are mean ± SEM with group sizes in parentheses; blood Mn in ng/mL, brain Mn in µg/g dry weight. Uppercase superscripts: within an age group and tissue, treatment groups with different capital letters are statistically different from one another ($p < 0.05$), based on Wilcoxon / Kruskal-Wallis test. Lowercase superscripts: within a treatment group and tissue, values across ages with different lowercase superscripts are statistically different from one another. PND, postnatal day.

4.0. Discussion

Our findings show that early postnatal Mn exposure causes heightened behavioral reactivity, reductions in evoked NE outflow, lasting alterations to catecholaminergic systems protein levels within the mPFC, and lasting heightened astrocyte reactivity that is dominated by a proinflammatory A1 astrocyte phenotype.

In general, neither the behavioral nor catecholaminergic effects were exacerbated by continued Mn exposure following weaning. Given that behavioral and cognitive function are among the most important public health outcomes, understanding how developmental exposure to environmental toxicants such as Mn impacts neurobehavioral function is key in devising effective treatment strategies (Developmental Toxicology 2000; Landrigan *et al.* 2002). These findings and their implications for humans exposed to elevated Mn are discussed below.

4.1. Postnatal Mn exposure caused broad lasting alterations in mPFC catecholaminergic systems

Early postnatal Mn exposure over PND 1-21 caused lasting hypofunctioning of the catecholaminergic systems in the mPFC, as evidenced by the significant Mn-dose related reductions in TH, DAT, NET, and D1 receptors, and increased D2 receptor protein levels in PND 100 young adults. These effects were generally not worsened by continued exposure following weaning through PND 100. Notably, blood and brain tissue Mn levels in the early life Mn-exposed groups were comparable to control levels well before PND 100, indicating that the catecholaminergic systems disruptions were due to elevated Mn during the early postnatal life exposure period, rather than elevated Mn levels in the PND 100 young adults (Table 1). Whereas lifelong Mn exposure generally did not significantly worsen the effects of early postnatal exposure, the dose-response for effects on the catecholaminergic systems qualitatively appears to correspond to the increasing

degree of exposure insult (i.e., control < early 25 Mn < lifelong 25 Mn < early 50 Mn < lifelong 50 Mn) (Figure 9). The Mn effect on the mPFC catecholaminergic systems, while broad in scope, was also somewhat specific, as there was no measurable effect of Mn on α_{2A} receptor protein levels (Figures 5c and 9). Finally, these catecholaminergic protein changes, most notably the reduction in TH (Figure 4a and 9), are consistent with the lasting reductions in the evoked release of NE in young weanling (Figure 3a, b) and adult (Beaudin *et al.* 2015; Lasley *et al.* 2020) animals exposed to the same oral Mn doses.

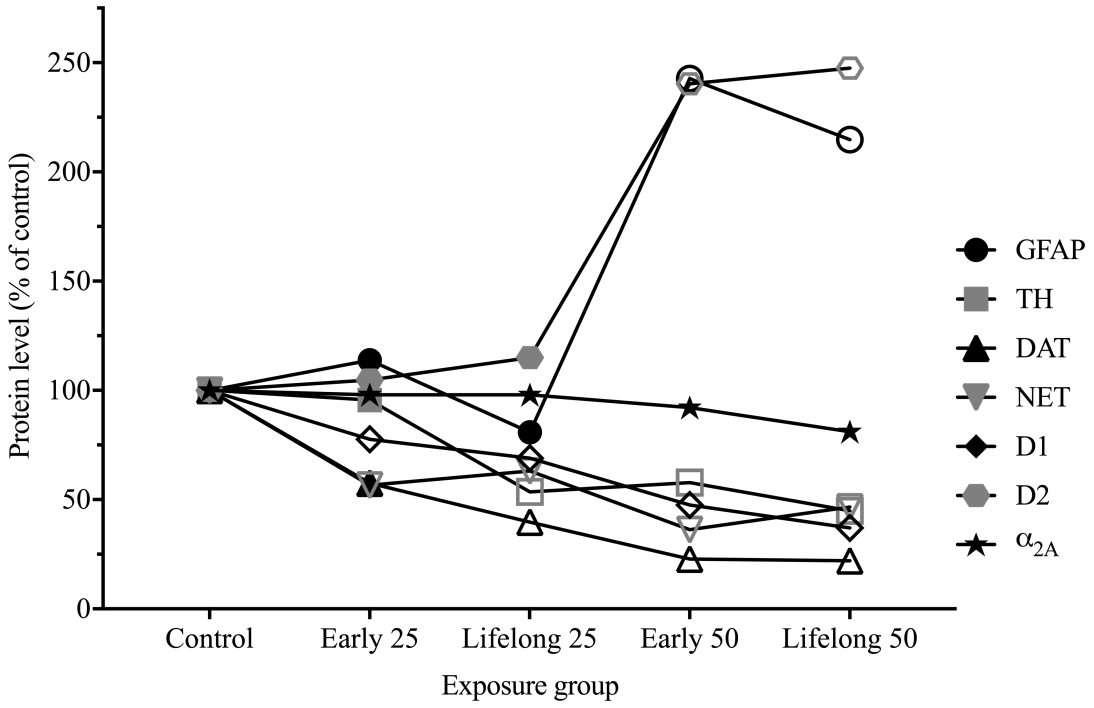

Figure 9. Mean levels of mPFC proteins across treatment groups, with levels of each protein normalized to its respective control group (error bars omitted for clarity). Symbol shape indicates the protein, and open symbols indicate significantly different from respective control ($p < 0.05$), based on statistical models that included all five treatment groups.

The mechanism(s) through which early life Mn exposure causes these lasting effects on the catecholaminergic systems is not well understood. One possibility is they may be driven by a Mn-induced reduction in TH expression that results in reduced DA and NE synthesis, leading to compensatory reductions in NET, DAT, and D1 expression, along with heightened D2 levels that may represent additional compensatory changes following lower synaptic catecholamine levels. Alternatively, or in addition, these lasting protein level changes may be mediated via epigenetic mechanisms, given that expression of several of them (e.g., TH, D2, DAT, and NET) are in part epigenetically regulated via DNA methylation (Hillemacher *et al.* 2009; Archer *et al.* 2011; Day *et al.* 2013; Groleau *et al.* 2014). This latter suggestion is supported by several studies in human neuroblastoma SH-SY5Y cells showing that Mn exposure led to hypermethylated TH promoter and downregulated TH gene transcription (Tarale *et al.* 2016; Gandhi *et al.* 2018). Recent evidence has also shown that TH expression can be mediated in part by the activity of c-RET kinase, and that human neuroblastoma cells exposed to 30 μM Mn experienced a c-RET mediated reduction in TH (Kumasaka *et al.* 2017). Finally, we believe that the reductions in TH, DAT, NET, and D1 immunofluorescence reflect reduced protein levels within Imaris-rendered objects, rather than fewer DAT and/or NET-positive cells/nerve terminals, since there was no Mn effect on total cell number or on the number of Imaris-rendered DAT or NET objects, relative to controls; DAT and NET are widely used as a markers for catecholaminergic nerve terminals (Miller *et al.* 1997; Stephenson *et al.* 2007; Moron *et al.* 2002).

Prior studies from our group have reported similar reductions in DAT and D1 within the striatum and nucleus accumbens, along with increased D2 in the mPFC of PND 24 rats exposed to the same 50 mg/kg/day Mn dose over PND 1-21 (Kern and Smith 2011; Kern *et al.* 2010). In those studies the increased mPFC D2 levels persisted into adulthood (PND 107), while the reduced DAT and D1 levels were normalized to control levels in adults. Together, these changes are consistent with our prior results showing reduced evoked release of DA and NE in the mPFC of adult rats following lifelong Mn exposure (Beaudin *et al.* 2015; Lasley *et al.* 2020). Others have reported similar, albeit more limited, effects of early life Mn exposure on brain catecholaminergic systems. For example, McDougall et al. (2008) showed that oral exposure to 750 μg Mn/day over PND 1-21 reduced DAT protein expression and [^{3}H]DA uptake in the striatum and nucleus accumbens, along with reduced striatal DA efflux in PND 90 rats. Subsequently, McDougall et al. (2011) showed that the same Mn exposure paradigm decreased the abundance of D2 binding sites in the PFC, but increased D2 binding sites and protein levels in the dorsal striatum of PND 90 rats. Anderson et al. (2009) reported that weanling rats exposed orally to 1 mg Mn/mL via drinking water for 6 weeks post-weaning exhibited reductions in evoked release of striatal NE levels, and reduced NET and α2 receptor protein levels in the striatum. Regarding our observed reduction in TH protein levels, Peres et al. (2016) similarly found that rats exposed to 20 mg Mn/kg/day via i.p. injection over PND 8-12 exhibited a significant reduction in striatal TH protein levels at PND 70, when tissue Mn concentrations were no longer elevated, and the reduced TH protein levels

correlated with TH phosphorylation at serine 40 and 19. Other rodent studies in adults treated with Mn via i.p. injection have similarly reported increased D2 binding sites and protein levels in the dorsal striatum (Seth *et al.* 1981; Nam and Kim 2008b). Interestingly, our findings may help explain the Mn-induced effects of another protein involved in the catecholaminergic system, DARPP-32, a DA-regulated phosphoprotein that is known to play a role in DAergic protein kinase A-dependent signaling (Scheggi *et al.* 2018). Given that D1 receptor activation is known to stimulate phosphorylation of DARPP-32 at threonine 34, and the fact that Cordova et al. (2013) recently reported that neonatal exposure to 20 mg Mn/kg/day, i.p, over PND 8-27 caused a decrease in DARPP-32 phosphorylation at threonine 34, their finding may reflect the synaptic alterations between D1 and D2 receptor levels reported here.

4.2. Changes in the mPFC catecholaminergic systems were associated with heightened behavioral reactivity and attentional impairments

All groups of animals were significantly more active during the initial 5-10 minutes of each daily test session relative to later in each of the sessions, reflecting heightened arousal engendered by the handling, novelty, and stress of being in an open field testing environment (Prut and Belzung 2003). However, this transient period of heightened arousal was significantly greater for all four Mn exposure groups relative to controls (Figure 2a, b). This pattern of effects suggests a transient increase in arousal state in the Mn animals (relative to controls) that dissipated over

the course of the daily test session. These findings suggest that Mn caused either a heightened emotional response to handling and the novel, inherently stressful environment, and/or an impaired ability to regulate this heightened arousal. This interpretation is consistent with our prior findings that exposure to 25 mg Mn/kg/day over PND 1-21 impaired arousal regulation in adulthood in a 5-Choice Serial Reaction Time task, in which animals displayed a transient impairment of response accuracy for non-distraction trials in a visual discrimination and attention test (Beaudin *et al.* 2017a).

These findings of impaired emotion regulation reported here further elucidate the behavioral phenotype produced by early postnatal Mn exposure. Prior animal studies have reported that oral Mn exposure caused lasting deficits in spatial learning and memory (Golub *et al.* 2005; Kern *et al.* 2010), as well as lasting impairments in executive functioning (e.g., deficits in focused and selective attention, impulse control, and fine motor function), consistent with the mPFC catecholamine systems changes reported here (Beaudin *et al.* 2017b; Beaudin *et al.* 2017a; Beaudin *et al.* 2015; Beaudin *et al.* 2013). Together, these findings have important implications for the environmental etiology of neurobehavioral disorders, such as ADHD, and their underlying neurobiology in children (Arnsten *et al.* 2015; Arnsten and Pliszka 2011). This is underscored by the fact that attention and impulse control dysfunctions, including ADHD, are the most prevalent neurodevelopmental disorders in children, affecting ~6-11% of all U.S. children age 6-17 years (Feldman and Reiff 2014; Kaiser *et al.* 2015; Willcutt 2012).

4.3. Early postnatal Mn exposure caused a proportionally greater lasting induction of A1 proinflammatory versus A2 neuroprotective astrocytes

The above lasting changes in mPFC catecholaminergic proteins due to early postnatal Mn exposure were accompanied by a lasting heightened reactivity of GFAP-positive astrocytes in the early postnatal and lifelong 50 mg Mn/kg/day groups to ~250% of controls. Moreover, in the GFAP-positive astrocytes, the relative increase in the A1 proinflammatory astrocyte marker C3 (to ~568% of controls) was ~2.8-fold greater than the anti-inflammatory A2 marker S100A10 (200% of controls), suggesting an overall proinflammatory neuroenvironment in the mPFC; this interpretation assumes that the proportional increase in C3 versus S100A10 protein levels in GFAP-positive astrocytes directly reflects the relative increase in A1 and A2 cell phenotypes. Prior *in vivo* and *in vitro* studies have previously shown that Mn exposure may promote the reactivity of astrocytes and microglia to contribute to neuroinflammation, as measured by increased proinflammatory gene and protein expression, along with higher levels of proinflammatory cytokines (Kern and Smith 2011; Liu *et al.* 2006; Zhao *et al.* 2009; Popichak *et al.* 2018; Jin *et al.* 2019). Further, Liddelow et al. (2017) recently demonstrated that reactive astrocytes exhibiting an A1 proinflammatory phenotype led to loss of synaptic function, increased synaptic pruning, impaired endocytosis of extracellular debris, and an increased risk for neurodegeneration. Given the evidence that C3 and S100A10 are distinct, non-overlapping markers of A1 and A2 phenotypic astrocytes (Liddelow and Barres 2017;

Liddelow *et al.* 2017), our findings suggest that multiple sub-populations of GFAP-positive astrocytes are induced by early postnatal Mn exposure, with a lasting net shift towards an A1 proinflammatory phenotype. These results are consistent with other studies of neurotoxic insults. For example, Zamanian et al. (2012) showed that lipopolysaccharide via i.p. injection in mice induced a heterogeneous astrocyte response, with cortical reactive astrocytes exhibiting phenotypes similar to the A1 and A2 phenotypes described by Liddelow et al. (2017).

While our findings show evidence of lasting astrocyte reactivity skewed towards the proinflammatory phenotype, it is not known whether the heightened astrocyte reactivity is involved in the catecholaminergic protein and neurotransmitter changes reported above. For example, the fact that early postnatal exposure to the lower 25 mg/kg/day Mn dose caused lasting reductions in DAT and NET protein levels, but did not increase astrocyte reactivity (GFAP) or markers of A1 or A2 astrocyte phenotypes (Figure 7c, d), does not necessarily reflect an absence of astrocyte-related effects on the mPFC catecholaminergic system, since our analyses were limited to changes in astrocyte protein expression and not more detailed tests of astrocyte function. Additionally, the lack of an effect of early life 50 mg/kg/day Mn exposure on mPFC pyramidal neuron spine density (Figure 8) suggests that the neuroinflammatory environment present in the Mn-exposed animals did not lead to neurobiological changes that altered dendritic spine density in the mPFC.

Collectively, the lasting changes in the rat mPFC catecholaminergic systems caused by early postnatal Mn exposure reported here are consistent with, and may

well underlie, the lasting impairments in arousal regulation, selective and focused attention, impulse control, and fine motor function that we have reported in Mn-exposed animals (Beaudin *et al.* 2017a; Beaudin *et al.* 2015; Beaudin *et al.* 2013). These findings have significant implications for human Mn exposure by suggesting that the attentional and executive function impairments associated with Mn exposure in children may be due to hypofunctioning of the PFC catecholaminergic systems (Wasserman *et al.* 2006a; Oulhote *et al.* 2014; Haynes *et al.* 2015).

5.0. Acknowledgements and conflict of interest disclosure

The authors would like to thank T. Jursa for analytical assistance, J. Alvarado, A. Chen, A. Cruz, J. Fee, M. Fung, R. Garcia, K. Goetz, S. Greenberg, C. Horton, I. Jing, K. Kekkonen, M. Kern, G. Kouklis, J. Sabile, T. Lau, S. Lee, L. Loh, A. Luo, C. Matysiak, D. Michue, H. Monday, M. Ngo, L. Nguyen, S. Nisam, M. Quail, C. Rew, M. Richter, K. Riffel, J. Shen, A. Smith, A. Spock, D. Tsang, R. Turk, A. Watson, F. Wu, K. Younes, S. Young, and S. Zhong for their assistance with behavioral testing. We also thank B. Abrams for assistance with microscopy and Imaris quantification, R. Jaramillo and D. McDonald for assistance in image processing, and R. Eastman, R. Cathey, E. Hiolski, C. Moyer, M. Camps, S. Mukhopadhyay, and Y. Zuo for their contributions to the study. The authors have no competing or potential financial conflicts of interest to declare.

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CHAPTER 4: CONCLUSIONS

Environmental Mn exposure continues to pose a risk to public health, both because of its ability to act as a developmental neurotoxicant in early lifestages (Wasserman *et al.* 2006a; Mora *et al.* 2018; Oulhote *et al.* 2014; Beaudin *et al.* 2017a; Beaudin *et al.* 2013; Beaudin *et al.* 2015), and its established harmful neurological impacts in occupationally exposed adults (Lucchini *et al.* 1999; Smith *et al.* 2007; Long *et al.* 2014b; Kwakye *et al.* 2015). Throughout my dissertation research, I have expanded on these issues using a combination of rodent models and human tissue-based experiments to address knowledge gaps relating to the specific catecholaminergic system alterations following early postnatal Mn exposure that are associated with attentional dysfunction in children, in addition to the impact of lifelong Mn exposure on bone Mn levels and the potential for using bone as a long-term biomarker of Mn exposure.

For my first research chapter, I focused on biomarkers of environmental Mn exposure to assess the viability of using bone tissue as a marker of long-term Mn exposure, including whether Mn accumulates in bone following continued lifelong exposure. Using our animal model of early life vs. lifelong Mn exposure, our findings demonstrate that Mn levels in blood, brain, and bone decline naturally with age in the absence of elevated exposure, and do not accumulate in the presence of prolonged elevated oral Mn exposure. Among the examined tissue types, bone was the most responsive biomarker to ongoing oral Mn exposure. X-ray-based analyses of bone mineral showed that Mn^{2+} can replace Ca^{2+} in the hydroxyapatite mineral under

conditions of elevated Mn exposure, while the gross physical structure of the hydroxyapatite mineral was not measurably altered. Alternatively, elevated Mn exposure did increase bone stiffness for a subset of Mn-exposed animals, suggesting that elevated exposure may alter physical properties of bone in a manner not detected by the X-ray-based analyses. Analyses in aged humans accompanied our animal model studies and showed that bone Mn decreases with age, but does not differ measurably based on sex, or parity history in females. These data stand in stark contrast to bone lead, which unlike Mn, does accumulate with age. Overall, this evidence indicates that bone may be a valuable biomarker of recent ongoing oral Mn exposure in humans, and bone may be a relatively minor target of elevated Mn exposure compared to other tissues, based on the limited functional changes identified here. These findings, coupled with developing technologies to measure bone Mn in vivo (i.e., portable neutron activation systems), will enable future opportunities to perform more comprehensive exposure assessments and reduce exposure mis-classification, a current limitation of environmental epidemiological studies (Pejović-Milić *et al.* 2009; Liu *et al.* 2013; Rolle-McFarland *et al.* 2018), that may prove useful for occupationally-exposed individuals.

My second and final research chapter focused on the impacts of early postnatal Mn exposure on the catecholaminergic systems that are essential for regulating attentional function, we identified multiple neurobiological and behavioral alterations caused by Mn exposure in our rodent model. Specifically, our findings show that early postnatal Mn exposure caused heightened behavioral reactivity in the

first 5-10 minute interval of daily open field testing sessions in a manner consistent with arousal dysregulation. These behavioral changes were accompanied by lasting alterations in several catecholaminergic protein levels in the mPFC, including significant Mn-dose related reductions in TH, DAT, NET, and D1 receptors, along with an increase in D2 receptor protein levels in PND 100 young adult animals. Among astrocyte cell types, early postnatal Mn exposure induced significant astrocyte activation, predominantly towards the proinflammatory A1 phenotype, and with less induction of the A2 neuroprotective phenotype. This broad hypofunctioning of the mPFC catecholaminergic system and markers of neuroinflammation significantly extends prior studies from our group and others that suggest catecholaminergic dysfunction may underlie the executive function deficits in Mn-exposed children (Beaudin *et al.* 2017a; Beaudin *et al.* 2015; Kern and Smith 2011; Kern *et al.* 2010; McDougall *et al.* 2008; Reichel *et al.* 2006; Conley *et al.* 2020). Since the precise mechanism of these Mn-induced childhood impairments are still not fully understood, further research is warranted to test the viability of pharmacological therapies that target regions of the catecholaminergic system identified here.

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APPENDICES

Appendix A.

Aspects of these experiments and data analyses were contributed by Steve Reaney PhD, Stephanie Whitman, and Ted Holman, PhD.

Proteomics Dataset in Undifferentiated PC12 Cells Exposed to 100 μM $MnCl_2$ for 24 hours

Materials and Methods

Reagents

Materials were obtained from the following vendors: Undifferentiated PC12 (rat pheochromocytoma) cells, American Type Culture Collection (ATCC, Manassas, VA); fetal bovine serum (FBS), ATCC; heat-inactivated horse serum and fungizone (250 units/ml), Life Technologies, Inc.; RPMI 1640 Medium, ATCC; HPLC grade solvents (methanol and acetonitrile), and optima grade nitric acid (HNO_3), Fisher Scientific. Collagen coated plates, BD glassware, penicillin (10,000 units/ml) /streptomycin (100 mg/ml), and all other reagents were from Sigma Aldrich.

Cell culture

Undifferentiated PC12 cells, a clonal catecholaminergic cell line derived from rat pheochromocytoma were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 unit/ml fungizone. Cells were cultured on collagen coated plates in a humidified environment at 37°C with 5% CO_2. All experiments were restricted to cell passages 4 through 7.

For manganese exposures, cells were exposed to medium (RPMI + 5% (v/v) horse serum/fetal bovine serum) not supplemented with manganese (control) or supplemented with 100 μM Mn(II)-chloride for 24 hours. The Mn(II)-chloride solution used for the cell exposures was produced by dissolving Mn-chloride-hexahydrate in sterile ultra-pure water, as described elsewhere (Reaney and Smith, 2004). Aliquots of Mn solution was diluted in RPMI cell culture medium and incubated for 5-10 minutes prior to exposing the cells. Treatments were conducted with n=4-6 independent replicates per experiment, as indicated, and each experiment was repeated twice. Controls for the Mn(II)-chloride were exposed to vehicle ultra-pure (Milli-Q) water. Following exposure, the cells were harvested, pelleted (200 x g for 10 minutes), and the cell pellet washed twice with phosphate buffered saline (PBS). Cell pellets for total manganese accumulation measurements were washed once with 5 mM ethylenediaminetetraacetic acid (EDTA) followed by two PBS washes to remove surficial (non-accumulated) manganese from the intact cell sample. Cell pellets were frozen at -70˚C for later analyses.

Cellular manganese accumulation

Cellular manganese levels were determined by graphite furnace atomic absorption spectrometry using a Perkin Elmer 4100ZL Zeeman, using a modified procedure of (Witholt *et al.* 2000). The cell pellet was lysed via hypo-osmotic shock and sonication in ultra pure water. An aliquot of the sample was taken for protein determination, and the remaining sample was acid digested for manganese concentration analyses.

LDH and cell viability

Overt cytotoxicity of the manganese treatments was determined by measuring the lactate dehydrogenase (LDH) activity of the culture medium following exposure, and by Trypan Blue staining to determine cell viability. LDH activity was determined by monitoring the disappearance of NADH at 340 nm on a Beckman UV/Vis spectrophotometer equipped with a multi-cell reader. LDH activity was corrected for inherent medium activity and expressed as a function of cellular protein content. All protein concentrations were determined using the BioRad assay (BioRad Labs).

ATP

For assessment of the cellular ATP concentration, PC12 cells were cultured on 96-well plates and exposed to manganese for 24 hours as outlined above. Following exposure, the medium was removed and the cells washed once with warmed Hank's balanced salt solution (HBSS), and total cellular ATP determined using a commercially available luciferase based assay (Bio Whittaker). The ATP induced bioluminescence produced by luciferase was monitored at 560 nm on a multi-well bioluminescence/fluorescence plate reader (Varian, Cary Eclipse fluorescence spectrophotometer).

Neurotransmitters (dopamine and metabolites, and serotonin)

Following manganese exposures, cell pellets were resuspended in Hepes (10 mM, pH 7), and aliquots of cell suspension were sonicated on ice in a degassed solution containing 400 mM perchlorate, 2 mM Na_2EDTA, and 3,4-dihydroxybenzylamine (DHBA) as internal standard. External standards were diluted with the perchlorate solution in the same proportion as the samples. Supernatant fractions were separated via centrifugation for 5 minutes at 16,000 x g at 4°C. The sample was injected onto an HPLC (Beckman with Gold Software) fitted with a C_{18} 3-µm reversed-phase column (10 cm) for separation of dopamine and metabolites, and measured using electrochemical detection (BAS), according to procedures outlined by Freeman et al. (1993). Quantified compounds included dopamine (DA) and its metabolites (3,4-dyhydroxyphenylacetic acid, DOPAC, and homovanillic acid, HVA), and serotonin (5-hydroxytryptophan, 5-HT). Quantification was performed using DHBA spiked external standards prepared during sample processing. Data were expressed relative to protein concentration.

Gel electrophoresis and western blotting

Cellular abundance of COMT-1, secretogranin, UCH-L1, and tubulin proteins were determined with gel electrophoresis and western blotting, using previously described methods (Gwiazda *et al.* 2002). Briefly, aliquots of the cell pellet resuspended in PBS were lysed on ice in lysis buffer (PBS containing 1% Igepal-640 (v/v), 0.5 % (w/v) sodium deoxycholate, 0.1% (w/v) sodium lauryl sulfate, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, 10 µg/ml pepstatin, and 10 µg/ml leupeptin), centrifuged at 16,000 x g for 10 min, and the supernatant mixed with 4X NuPAGE sample loading buffer (Invitrogen) and stored at -70°C until analysis. Prior to gel loading, lysates in NuPAGE sample loading buffer were reduced by adding 10% (v/v) 0.75M dithiothreitol, and the sample loaded onto NuPAGE 12% Bis-Tris gels. Following immunoblotting with the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies), the protein bands were visualized using an ECL PLUS detection system (Amersham Biosciences). Band intensity was imaged and quantified using a Typhoon scanner and Image Quant software (Amersham Biosciences).

Isoprostanes

Isoprostanes are a family of non-enzymatic origin eicosanoids that result from the oxidation of lipids by ROS, and as such have been used as a marker of cellular oxidative stress. Cells were cultured in 96 well plates (~70,000 cells/well) and exposed to manganese in serum-free medium for 24 hours. Isoprostane concentrations were measured in culture medium following exposure using a competitive enzyme immunoassay for 8-isoprostane (Cayman Chemical Company), and a multi-well plate reader at a wavelength 405nm. Serum-free medium was used for these exposures in order to reduce the background 8-isoprostane generation that occurs with Mn(III) exposure in medium containing serum.

Statistics

Treatment comparisons for all outcomes except the 2-D DIGE analyses were performed using t-tests using appropriate adjustments for equality of variances. P-values less than 0.05 were considered statistically significant for all tests. All analyses were conducted using SYSTAT (SPSS Inc., 10th edition, 2000).

Sample preparation for 2-D DIGE analyses

The frozen cell pellets were resuspended in 200 µL of lysis buffer containing 30mM Tris, 7M urea, 2M thiourea, 4% CHAPS, and 0.5% Triton X-100 pH 8.5 and sonicated 3-4 times each (1 sec bursts and 20 sec rest intervals) at 50Hz. After sonication two different aliquots of sample (each in duplicate) were quantitated with the 2-D Quant Kit (Amersham Biosciences) while the remainder of the sample was treated with the 2-D Clean Up Kit (Amersham Biosciences) to remove any interfering substances. Samples were eventually brought up in lysis buffer to 10 mg/ml, based on results from 2-D Quant Kit. All kits were used according to manufacturer's recommendations.

Sample labeling

CyDyes (Amersham Biosciences) were reconstituted in 25 µL DMF according to manufacturer's specifications. An equal aliquot of every sample to be used in the experiment was mixed together to make an internal standard for each gel. All gels run had 50 µg of internal standard labeled with Cy 3 and 50 µg of either untreated or treated sample labeled with Cy 5. The 50 µg samples were then brought up to a pH of 8.5 by addition of small amounts of 0.1 M NaOH. The labeling of the proteins was carried out by amine reactive cyanine dyes (CyDyes) and in the absence of dithiothreitol (DTT), pharmalytes, primary amines or thiols that could interfere with the labeling. The fluorescent dyes, (Cy 3 and Cy 5) are of similar size (~500 Daltons) and charge but have different excitation/emission wavelengths. The dyes are added to the proteins so that the dyes are the limiting factor in the reaction and therefore each protein is minimally labeled (approximately 1-2% of each protein's lysine residues are labeled). The 50 µg sample for each gel was labeled separately with 400 pmoles of CyDye except for the internal standard which for all 6 samples were labeled simultaneously. The reaction proceeded on ice, in the dark, for 30 minutes. The labeling reaction was stopped by the addition of 1 µL of 10mM lysine and then left for 10 minutes on ice. Sample and internal standard were mixed together prior to rehydration of IEF strips.

Rehydration and running of IEF strips

Samples were mixed with rehydration buffer (8M urea, 4% CHAPS, 13mM DTT(Fisher Biotech), 2% IPG Buffer pH4-7 (Amersham Biosciences), 1%(w/v) bromophenol blue) to a volume of 450 µL. Immobilized pH Gradient (IPG) strips, pH 4-7, 24 cm (Amersham Biosciences) were rehydrated with each 450 µL sample overnight in the dark at room temperature according to manufacturer's recommendations. Isoeletric focusing was done with a Multiphor II apparatus (Amersham Biosciences) with the following protocol: 1hr at 5OOV/2mA/5W, 1hr at 1000V/2mA/5W, 1hr at 1500V/2mA/5W, 1hr at 2000V/2mA/5W, 1hr at 2500V/2mA/5W, and finally 18-20 hrs at 3000V/2mA/5W. The cathode electrode pad was soaked in 13mM DTT prior to focusing.

Equilibration of strips and running of PAGE gel

After focusing the strips were equilibrated with SDS Equilibration Buffer (50mM TrisCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, trace amounts of bromophenol blue) and 0.5% DTT for 10 mins and then SDS equilibration buffer with 4.5% (w/v) iodoacetamide for an additional 10mins. The 10% polyacrylamide gels were poured according to the manufacturer's specifications (using either the Ettan DALT six or DALT twelve casting apparatus, Amersham Biosciences). The plates used were low-fluorescent glass and the gels were bonded to the non-spacer plate with a bind-silane solution (8 mL ethanol, 200 µL acetic acid, 5 µL gamma-methacryloxypropyltrimethoxysilane (Sigma), 1.795 mL water) 1-4 hrs prior to pouring the gels. After equilibration of the strips they were washed in SDS Running Buffer (25 mM Tris, 192 mM glycine, 0.2% SDS) and then gently pushed into the

agarose solution (0.5% low-melt agarose, trace amounts of bromophenol blue, and 100 mL of SDS Running Buffer) that was layered at the top of the polyacrylamide gels. Gels were run on the Ettan DALT six at 2.5W/gel for the first 30 mins and then 100W until the bromophenol blue dye migrated off of the gel. Gels were run on the Ettan DALT twelve at 5W/gel for the first 30 mins and then 180W until the dye ran off of the gels.

Scanning of gels

The gels were fixed with 30% ethanol/10% acetic acid overnight in the dark. The different images of the gels were scanned in at a resolution of 100 microns with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences). The Cy3 images were scanned in with a 532nm laser and an emission filter of 580 nm BP (band pass) 30. The Cy5 image was scanned with a 633nm laser and an emission filter of 670 nm BP 30. The PMT (photo multiplier tube) setting for all gels was 525. The images were cropped of extraneous parts of the gel for analysis with ImageQuant v 5.2 (Amersham Biosciences).

Pick gels

In addition to the gels described above, two preparative "pick" gels were also run for every experiment because the amount of protein on an analytical gel would not be enough for identifying the proteins. The "pick" gel was 500 ug of the internal standard protein mix that was unlabeled. The running of the gel was as described above. After fixing, the pick gels were stained with Sypro Ruby Dye (Molecular Probes, Eugene,OR) overnight. The gels were destained 2 times with 10% methanol/ 7% acetic acid for 30 mins each. The images were then scanned in with a 532 nm laser and an emission filter of 610nm BP 30. The image was cropped identically to the other gels in the experiment and spots were detected with the Differential In-gel Analysis software (Decyder software v4.0, Amersham Biosciences). The spot maps were then entered into the Biological Variation Analysis software with all of the other gels and the spots matched to the other gels. A pick list from the pick gel was made inside the BVA module. Gels were then stained overnight with coomassie blue stain (50% methanol, 10% acetic acid, .05% brilliant blue R-250) and destained with 10% MeOH/7% acetic acid. Spots were excised from the gel with the Ettan Spot Picker (Amersham Biosciences) and put in a 96 well plate in 200 µL of water at -20°C until the spots were digested.

Digestion of spots

The gel plugs were washed 3 times with 100 µL of a 50 mM ammonium bicarbonate (Mallincrodt)/50% methanol solution for 20 minutes each. This was followed by a wash of 100 µL of 100% acetonitrile (EM Science) and then the complete drying of the plugs in a 96 well plate drier (Turbo Vap 96, Zymark) at 25°C. Sequencing grade trypsin (Promega) was reconstituted to a concentration of 40 ng/µL in 1 mM hydrochloric acid (EM Science). The trypsin was activated by mixing 5 µL of the above solution with 20 µL of 20 mM ammonium bicarbonate (per plug).

The trypsin was then layered on top of each plug and left at room temperature overnight with Costar Thermowell sealing tape (Corning, Inc.) to prevent evaporation of the liquid. The next day 3 μL of 1% trifluoroacetic acid (TFA, J.T. Baker) was added and the liquid from the plugs was then removed to a new 96 well plate. The plugs were washed 3 times for 20 minutes each with 20 μL of 75% Acetonitrile/0.5% TFA and all supernatants were removed to the new plate. The liquid was then evaporated off in the plate drier as before.

Mass spectrometry analysis

The proteins were analyzed using a Ettan MALDI-ToF/Pro (Amersham Biosciences). The digested proteins were dissolved in 5 μL of 50% Acetonitrile/0.5% TFA and 0.4 μL of the sample was spotted on the target. Once the liquid had evaporated the target was spotted with 0.4 μL of a saturated solution of α-cyano-4-hydroxycinnaminic acid (4-HCCA, Aldrich). The saturated solution of 4-HCCA was made up by dissolving 10 mg of the matrix in 50% Acetonitrile/0.5% TFA and vortexing. The solution was then spun down and 500 μL of the solution was removed and mixed with 500 μL more of 50% Acetonitrile/0.5% TFA. After evaporation of the liquid, the spots were run in reflectron mode. The MALDI was calibrated before beginning the runs with a mixture of angiotensin III (MW 897, Sigma) and adrenocorticotropic hormone fragment 18-39 (MW 2465.7, Sigma). The proteins were identified by peptide mass fingerprinting with Ettan MALDI-ToF Pro software 1.11 (Amersham Biosciences) and the results compared with Prospector for confirmation.

Results tables and figures

Table A1. MALDI-mass spectrometry results output for PC12 cells following exposure to 100 μM $MnCl_2$ for 24 hours. Each protein listed shows protein name, molecular weight (MW), method of analysis (MALDI/MS), percent change in protein expression, and P-value associated with protein change.

8/4 Expt	Protein Name	pI	MW (kDa)	ID method	Expression Change (%)	P-value of change	Peptide matches	% Coverage	P-Value of ID
374	dnaK-molecular chaperone grp 75 precursor	5.9	73.73	maldi	2.38	<0.001	12/15	21.3	0.002
	75 kDa glucose regulated protein								
	GRP 75								
	Peptide-binding protein 74								
	PBP74								
	MTHSP70								
	Mortalin								
407	secretogranin II	4.7	71.07	maldi	-1.41	<0.001	12/14	28.85	0.001
410	secretogranin II	4.7	71.07	maldi	-1.385	<0.001	13/16	25.2	0.002
415	secretogranin II	4.7	71.07		-1.465	<0.001	16/21	34.9	0.000
416	secretogranin II	4.7	71.07	maldi	-1.335	<0.001	14/20	25.45	0.004
423	secretogranin II	4.7	71.07	maldi	-1.465	<0.001	9/10	18.75	0.008
424	secretogranin II	4.7	71.07	maldi	-1.41	<0.001	9/12	20.75	0.026
429	secretogranin II	4.7	71.07	maldi	-1.465	<0.001	11/12	20.45	0.005
	Chromogranin C								
564	60 kDa heat shock protein, mitochondrial [Precursor]	5.3	57.91	maldi	1.815	<0.001	9/13	26.9	0.005
	Hsp60								
	60 kDa chaperonin								
	CPN60								
	Heat shock protein 60								
	HSP-60								
	Mitochondrial matrix protein P1								
	HSP-65								
850	ribonuclease/angiogenin inhibitor	4.6	49.89		-1.44	0.002	9/16	26.3	0.000
	Placental ribonuclease inhibitor								
	RAI								
	RNase inhibitor								
	RI								
864	ribonuclease/angiogenin inhibitor	4.6	49.89	maldi	-1.59	<0.001	12/17	38.5	0.003
865	ribonuclease/angiogenin inhibitor	4.6	49.89	maldi	1.4	<0.001	11/18	29.3	0.001
909	Gamma enolase (2-phospho-D-Glycerate hydro-lyase)	5	47.12	maldi	1.245	0.022	12/17	40.55	0.002
	Phosphopyruvate hydratase								
	2-phospho-D-glycerate hydro-lyase								
	Neural enolase								
	NSE								
	Enolase 2								
1021	isovaleryl coenzyme A dehydrogenase	8.5	46.42		-1.41	0.015	6/11	15.6	0.014
	Isovaleryl dehydrogenase [Precursor]								
	Isovaleryl CoA dehydrogenase								
1090	actin	5.3	41.73	maldi	1.725	<0.001	11/29	32.25	0.1075
1095	actin	5.3	41.72		-1.245	0.021	14/32	49.6	0.009
1096	actin	5.3	41.72		-1.2	0.065	7/14	26.4	0.056
1119	isovaleryl coenzyme A dehydrogenase	8.5	46.42	maldi	-1.28	0.013	8/18	22.3	0.051
	Isovaleryl dehydrogenase [Precursor]								
	Isovaleryl CoA dehydrogenase								
1973	ubiquitin carboxyterminal hydrolase L1/ubiquitin thiolesterase	5.1	24.76	maldi	2.415	<0.001	9/15	49.3	0.000
	Ubiquitinyl hydrolase 1								
	UCH-L1								
	Ubiquitin thiolesterase L1								
	Neuron cytoplasmic protein 9.5								
	PGP 9.5								
	PGP9.5								
2076	catecholamine-O-methyltransferase	5.4	29.58	maldi	1.475	0.002	6/12	36.0	0.001
	catechol-O-methyltransferase								
2089	phosphatidylethanolamine binding protein	5.5	20.78		1.255	0.01		#DIV/0!	0.024
	PEBP								
	HCNPpp								
	23 kDa morphine-binding protein								
	P23K								

Table A1 (continued). See previous description.

8/4 Expt	Protein Name	pI	MW (kDa)	ID method	Expression Change (%)	P-value of change	Peptide matches	% Coverage	P-Value of ID
555	pyruvate kinase, M2 isozyme	7.15	58.35	MS/MS	1.765	<0.001	7	18	
666	protein disulfide isomerase A3 precursor ERp60	5.9	56.61	MS/MS	1.71	<0.001	10	21	0.091
	Protein disulfide isomerase								
	Disulfide isomerase ER-60								
	ERp60								
	58 kDa microsomal protein								
	p58								
	ERp57								
	HIP-70								
	Q-2								
	pyruvate kinase, M2 isozyme			MS/MS			5		
861	keratin 18	4.6	16.94	maldi	1.34	0.00123	10/1	54.8	0.0655
944	heat shock protein 8	5.37	71.1	MS/MS	2.47	<0.001	2	6	
1011	hypothetical protein XP_346694	4.5	37	MS/MS	-1.245	0.025	1	4	
1015	CBP-50 protein	4.4	36.98	maldi	-1.325	0.003		26	0.117
	Calumenin [Precursor]								
	Crocalbin								
1085	actin	5.3	41.73	maldi	2.06	<0.001	7/13	25.45	0.063
1487	pyruvate dehydrogenase E1 component beta subunit	5.9	38.83	MS/MS	2.045	<0.001		2.9	0.021
	Pyruvate dehydrogenase (lipoamide)								
	PDHE1-B								
2211	phosphatidylethanolamine binding protein	5.5	20.78	MS/MS	1.93	<0.001	4	25	
228	Not ID'd				1.78	0.014			
299	Not ID'd				2.275	<0.001			
331	Not ID'd				1.4	<0.001			
427	Not ID'd				-1.405	0.007			
470	Not ID'd				1.72	0.002			
607	Not ID'd				-1.3	0.001			
721	Not ID'd				1.755	<0.001			
1113	Not ID'd				-1.245	0.022			
1036	Not ID'd				-1.395	0.001			
1105	Not ID'd				-1.445	0.007			
1102	Not ID'd				1.535	0.003			
1261	Not ID'd				-1.425	0.005			
1304	Not ID'd				1.335	<0.001			
1341	Not ID'd				1.61	0.002			
2050	Not ID'd				1.275	0.020			
2520	Not ID'd				1.4	<0.001			
1949	ubiquitin carboxyterminal hydrolase L1/ubiquitin thiolesterase	5.1	24.76	maldi	-1.105	0.31	8/14	45.3	0.000
1984	ubiquitin carboxyterminal hydrolase L1/ubiquitin thiolesterase	5.1	24.76	maldi	-1.095	0.67	8/16	48.4	0.005
2189	phosphatidylethanolamine binding protein	5.5	20.78	maldi	-1.05	0.86	10/1	66.3	0.0295

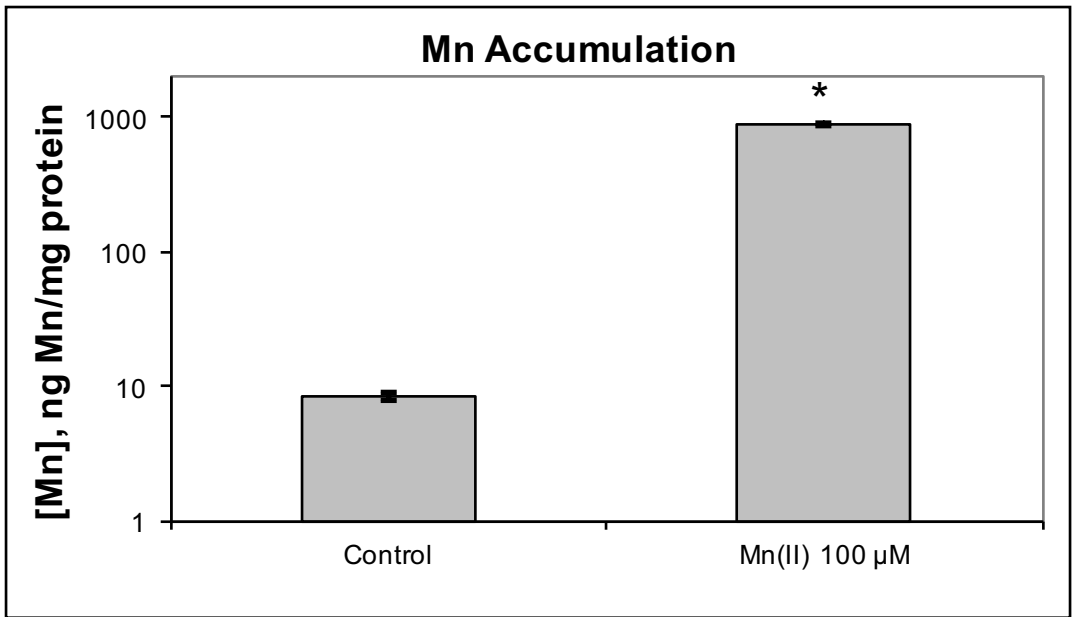
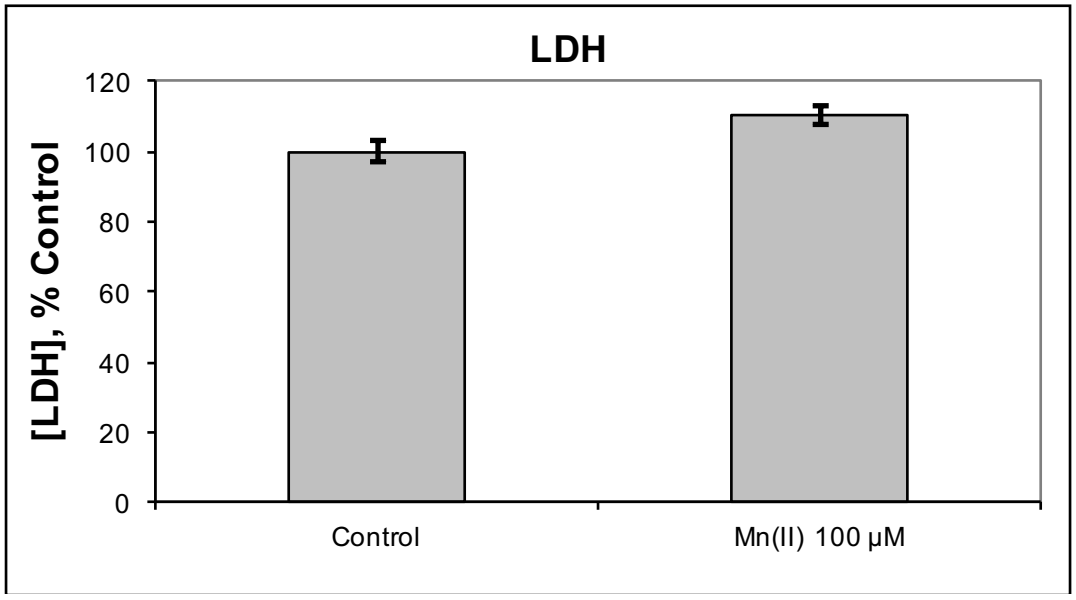
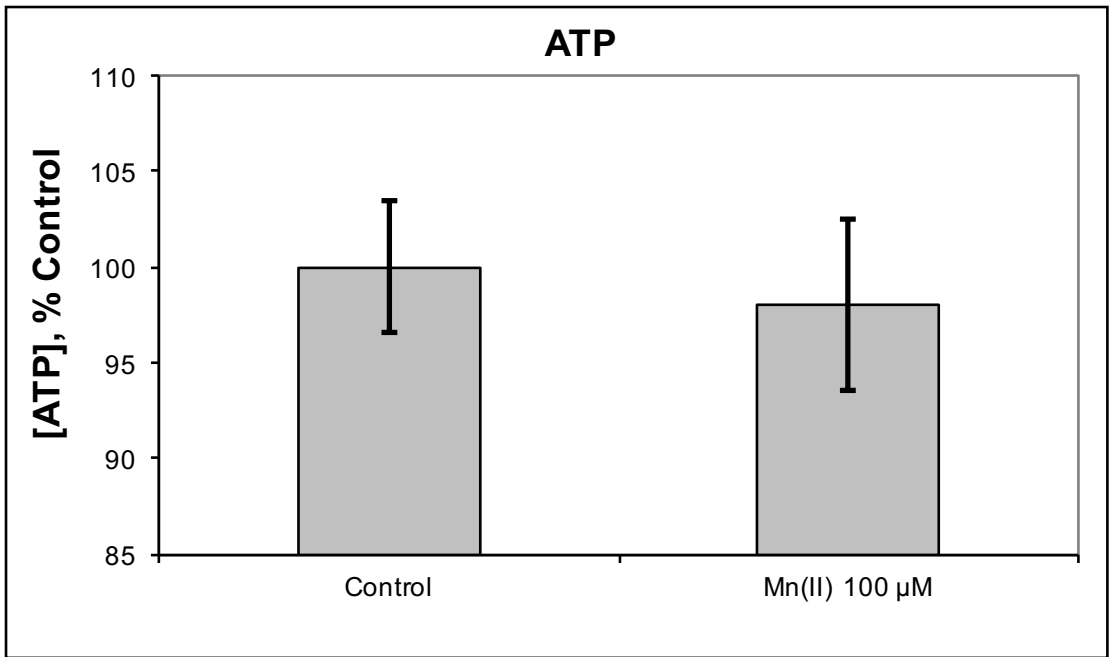


Figure A1. Cellular Mn levels increased ~100-fold following exposure to 100 µM $MnCl_2$ for 24 hours. Bar chart shows Mn concentrations in ng Mn/mg protein (n=4-6 independent replicates/treatment group); data are means ± SEM. * represents statistically different from controls ($p<0.0001$) based on Student's t-test.

a.



b.



c.

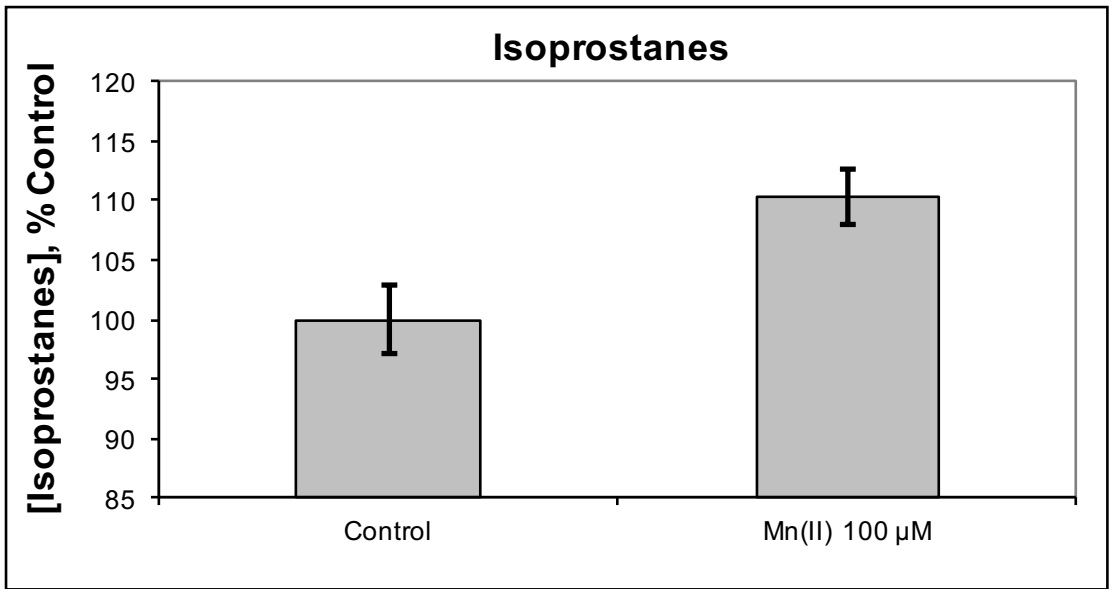
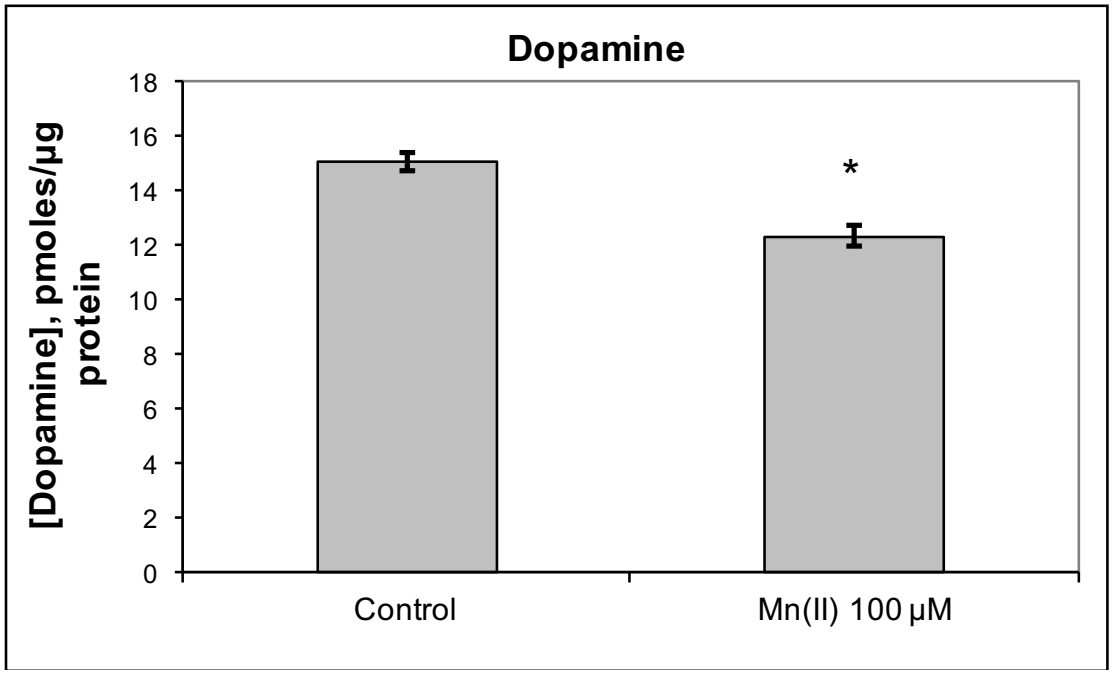


Figure A2. Exposure to 100 µM $MnCl_2$ had no effect on cell viability, based on assessment of LDH, ATP, and 8-isoprostanes. Bar charts show levels of (a) LDH, (b) ATP, and (c) 8-isoprostanes as percent of control (n=4-6 independent replicates/ treatment group); data are means ± SEM. There was no effect of treatment (p's>0.05) based on Student's t-test.

a.



b.

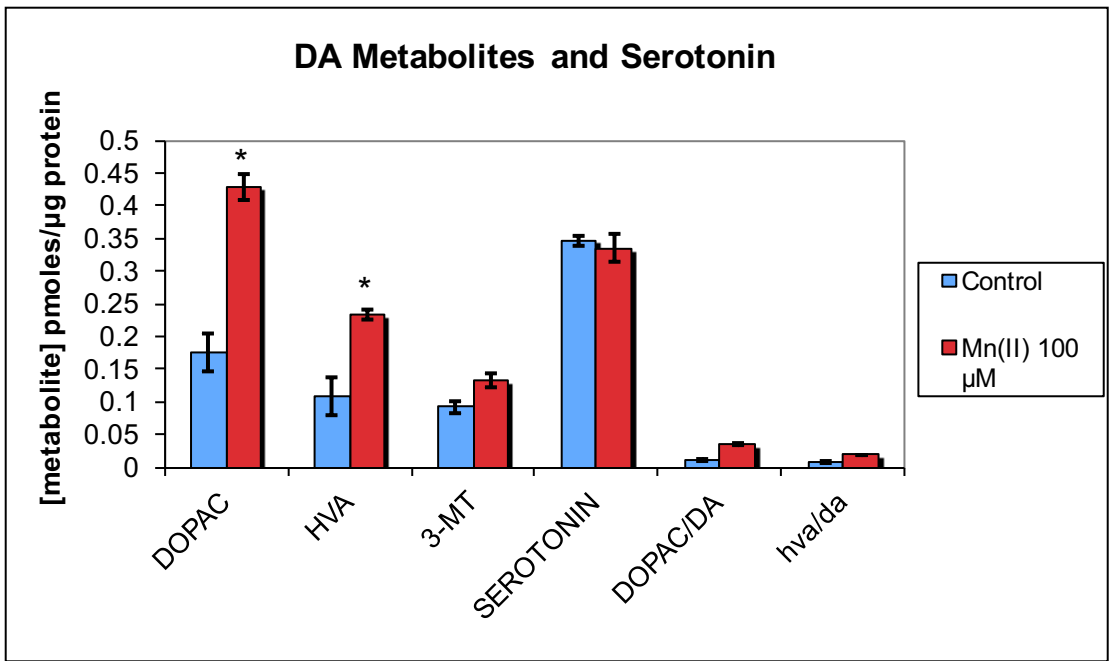
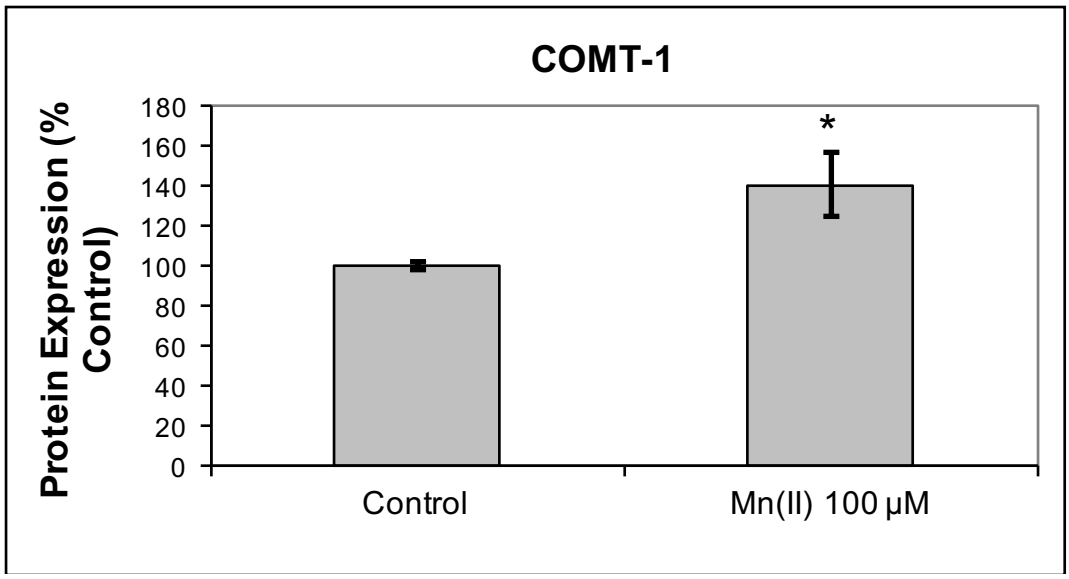


Figure A3. Levels of cellular DA, DOPAC, and HVA were altered by Mn exposure, but there was no effect on 3-MT or serotonin. Bar charts show concentrations of neurotransmitters and metabolites as pmoles/µg protein. a. DA was significantly decreased by 20% relative to controls (n=4-6

independent replicates, p=0.001). b. DA-metabolites, DOPAC and HVA increased by 45% (p=0.0004) and 145%, (p=0.006) respectively, while no other metabolites or serotonin were changed (p's>0.05). Data are means ± SEM. * represents statistically different from controls (p<0.05) based on Student's t-test.

a.



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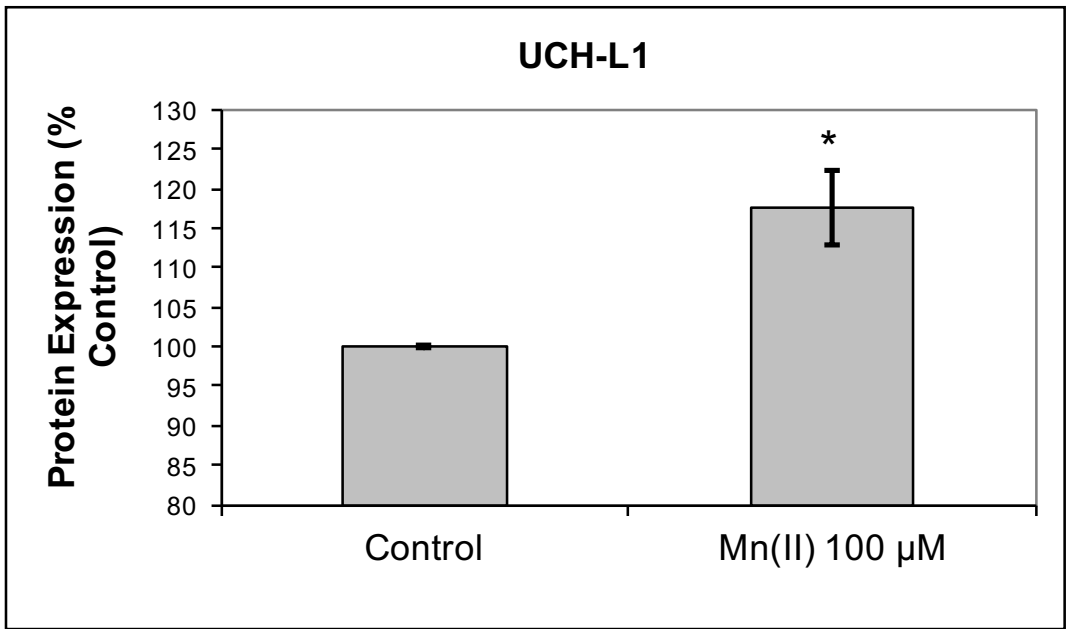


c.

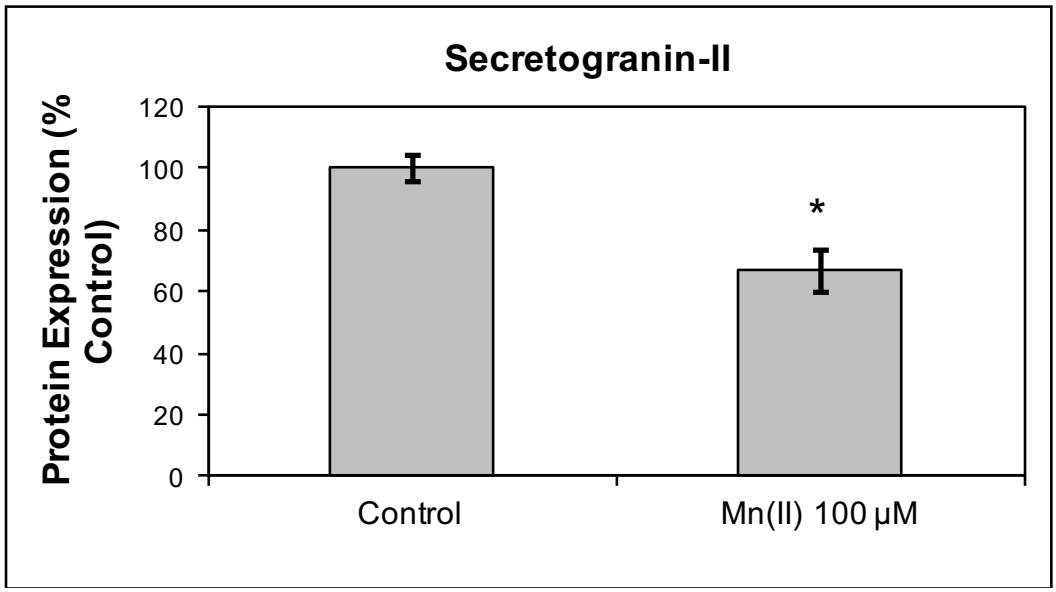


Figure A4. Mn exposure increased levels of cellular membrane bound COMT-1 and UCH-L1, but decreased levels of secretogranin-II. Bar charts show protein levels of (a) COMT-1, (b) UCH-L1, and (c) secretogranin-II as percent of control (n=4-6 independent replicates/treatment group). a. COMT-1 was significantly increased by ~40% relative to controls ($p=0.03$); b. UCH-L1 was increased by ~20% ($p=0.009$); c. secretogranin-II was decreased by ~35% ($p=0.001$). Data are means ± SEM. * represents statistically different from controls ($p<0.05$) based on Student's t-test.

References

Freeman K., Lin P., Lin L., Blank L. C. (1993) Monoamines and metabolites in the brain, in *High Perform. Liq. Chromatogr. Neurosci. Res.*, pp. 25–55. Wiley, England.

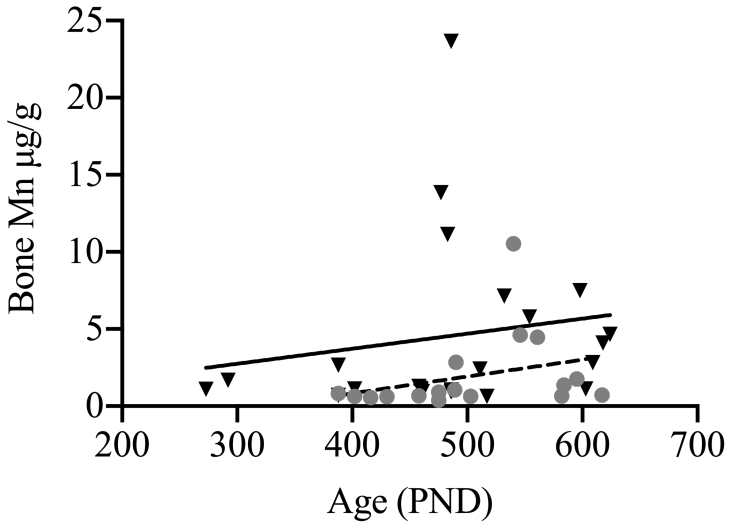
Gwiazda R. H., Lee D., Sheridan J., Smith D. R. (2002) Low Cumulative Manganese Exposure Affects Striatal GABA but not Dopamine. *Neurotoxicology* **23**, 69–76.

Witholt R., Gwiazda R. H., Smith D. R. (2000) The neurobehavioral effects of sub-chronic manganese exposure in the presence and absence of pre-Parkinsonism. *Neurotoxicol. Teratol.* **22**, 851–861.

Supplementary Information for Chapter 2

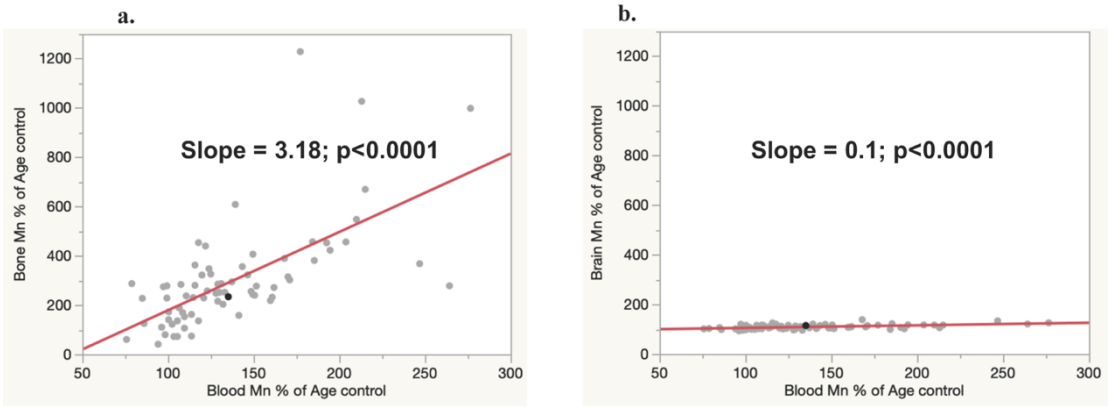
S1 Table 1. Statistical analysis outcomes of elevated Mn exposure across blood, brain, and bone tissue by age. Data are outputs from one-way ANOVA comparing the main effect of Mn exposure (three to five treatment groups) within an age group and tissue (n = 10-16 animals/treatment group). See Results, section 3.2.

Tissue	Age (PND)	ANOVA Output	P-value
Blood	24	$F(2, 40) = 98.48$	< 0.0001
	66	$F(4, 69) = 22.53$	< 0.0001
	500	$F(4, 83) = 37.61$	< 0.0001
Brain	24	$F(2, 39) = 22.52$	< 0.0001
	66	$F(4, 70) = 16.59$	< 0.0001
	500	$F(4, 68) = 11.17$	< 0.0001
Bone	24	$F(2, 39) = 67.35$	< 0.0001
	66	$F(4, 70) = 35.18$	< 0.0001
	500	$F(4, 103) = 17.09$	< 0.0001

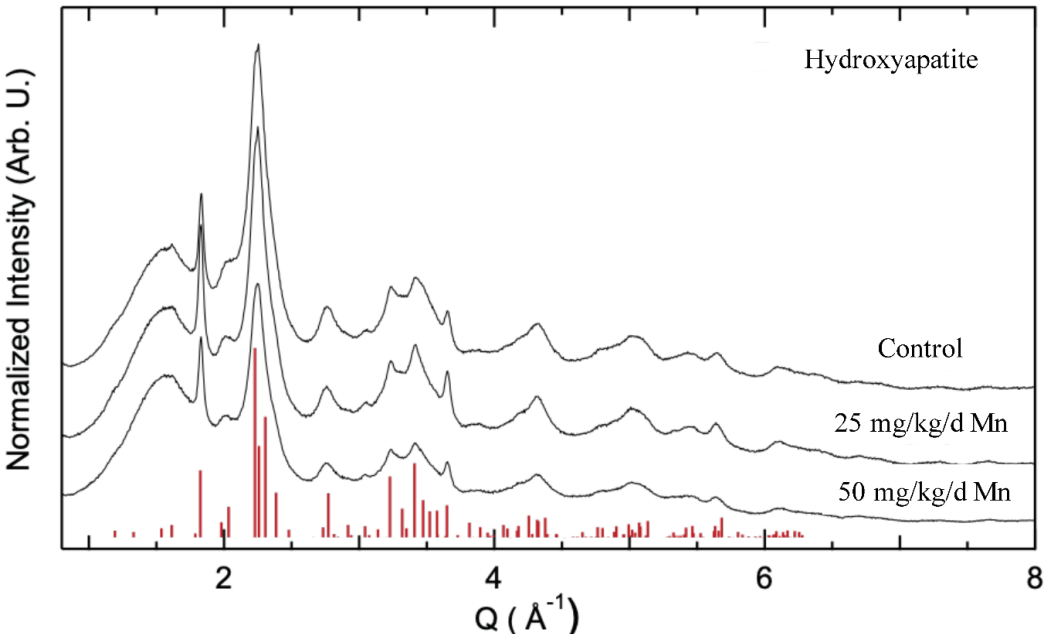


S1 Figure 1. There is no significant relationship between bone Mn levels and PND sacrifice age vs. bone Mn levels in aged adult animals. Data are bone Mn concentrations (ug/g dry weight) by PND age at sacrifice of aged adult continuous lifelong 25 and lifelong 50 Mn exposure group animals (n = 21 – 23/group). Regression plots are bivariate fit for each exposure group. There is no significant relationship between sacrifice age and bone Mn levels for lifelong 25 ($R^2 = 0.091$, $p = 0.24$) or lifelong 50 exposure groups ($R^2 =$

0.029, $p = 0.46$) (section 3.2). Gray circles with broken line represents the lifelong 25 group data, while black triangles with solid line represents the lifelong 50 group data.

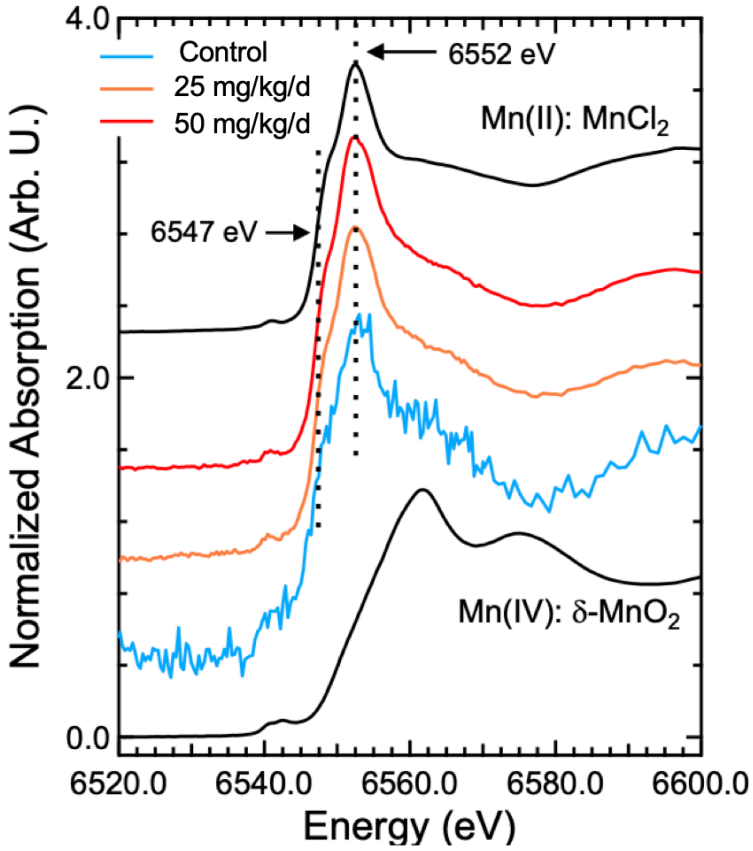


S1 Figure 2. Sample regression plots of bone vs. blood Mn and brain vs. blood Mn. Data are a subset of sample regression plots from Figure 3 using Pearson's R values of tissue Mn concentrations converted to percentage of age-matched controls for **(a)** bone Mn vs. blood Mn, and **(b)** brain Mn vs. blood Mn, respectively, across all treatment groups and ages (PND 24, 66, ~500). See description of analysis in text (section 3.3.1.)



S1 Figure 3. Elevated bone Mn does not significantly alter the particle size or gross crystalline structure of hydroxyapatite mineral compared to

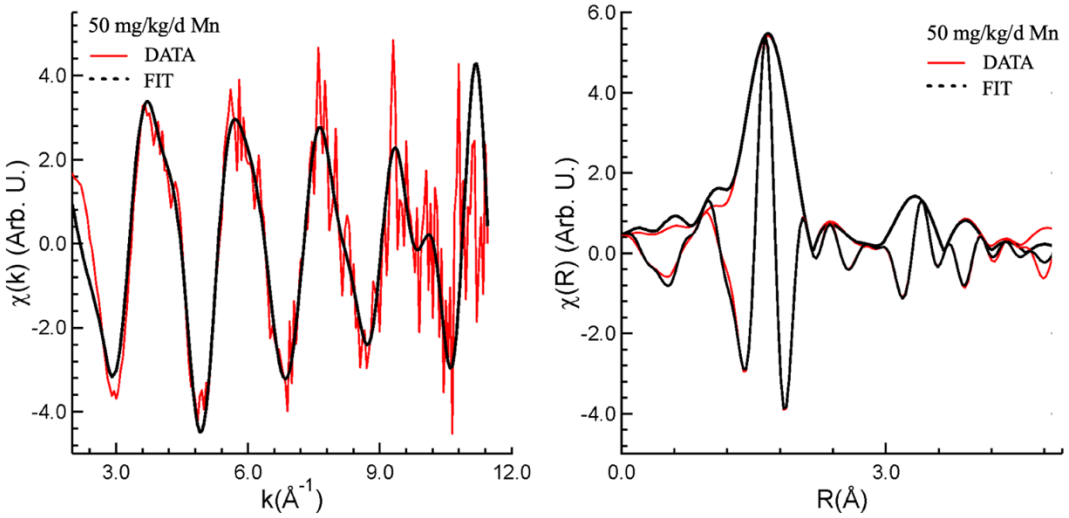
the peak positions of hydroxy apatite. Data are XRD absorption spectra for three PND 24 rat femurs from control, 25 and 50 mg Mn/kg/day treatment groups. X-axis shows wavelength-dependent x-ray diffraction, Q, per angstrom; y-axis is the x-ray spectra normalized intensity in arbitrary units (arb. u.). Red bars along the x-axis are where hydroxyapatite spectral peaks should appear for the mineral.



S1 Figure 4. Elevated bone Mn does not alter the valence state of Mn in bone. The energy position of the first inflection point (arrow at 6547 eV and left vertical dotted line), and the overlapping peaks at 6552 eV (arrow at right vertical dashed line), coincides with that of $MnCl_2$. This confirms the charge state of the Mn as +2. Data are XANES absorption spectra for three bone samples of PND 24 rat femurs from the control (blue line), 25 mg Mn/kg/day (orange line), and 50 mg Mn/kg/day (red line) Mn exposure groups. Spectra for black lines are standardized values for Mn^{2+} in $MnCl_2$ and Mn^{4+} in MnO_2, respectively. X-axis shows kinetic energy of photo electrons in electron volts (eV); y-axis is normalized absorption in arbitrary units (arb. u.).

S1 Table 2. EXAFS results and comparison with the interatomic distances between hydroxyapatite and a femur bone sample from a PND 24 rat exposed to Mn 50 mg/kg/d over PND 1 - 21. N is the number of atoms of oxygen (O), phosphorus (P), or calcium (Ca) in the central atom Mn coordination. D is the interatomic distance, and σ^2 is the Debye Waller Factor, which reflects the degree of x-ray scattering due to thermal motion; both of these values were unremarkable in this dataset.

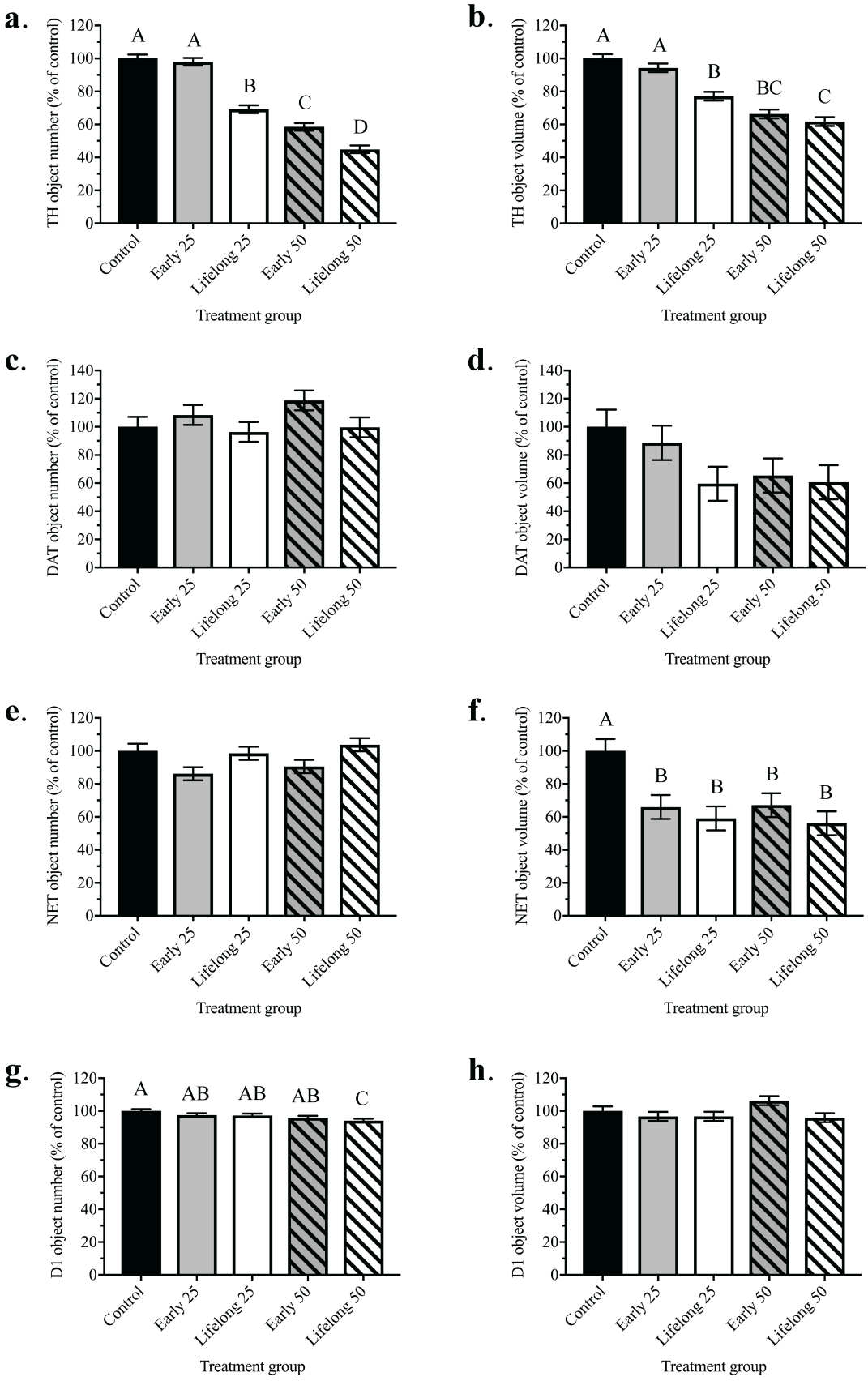
		Mn 50 mg/kg/d	**Hydroxy-apatite**
Mn-O1 (N=6)	*D(Å)*	2.15 (0.01)	2.40
	$\sigma^2(Å^2)$	0.0075 (0.0001)	
Mn-O2 (N=3)	*D(Å)*	2.41 (0.01)	2.80
	$\sigma^2(Å^2)$	0.0075 (0.0001)	
Mn-P1 (N=3)	*D(Å)*	2.91 (0.02)	3.21
	$\sigma^2(Å^2)$	0.0097 (0.0002)	
Mn-P2 (N=3)	*D(Å)*	3.13 (0.02)	3.60
	$\sigma^2(Å^2)$	0.0054 (0.0020)	
Mn-P3 (N=2)	*D(Å)*	3.42 (0.02)	3.55
	$\sigma^2(Å^2)$	0.0034 (0.0001)	
Mn-O3 (N=9)	*D(Å)*	3.93 (0.03)	3.72
	$\sigma^2(Å^2)$	0.0095 (0.0003)	
Mn-Ca1 (N=6)	*D(Å)*	3.94 (0.05)	4.19
	$\sigma^2(Å^2)$	0.0286 (0.0006)	



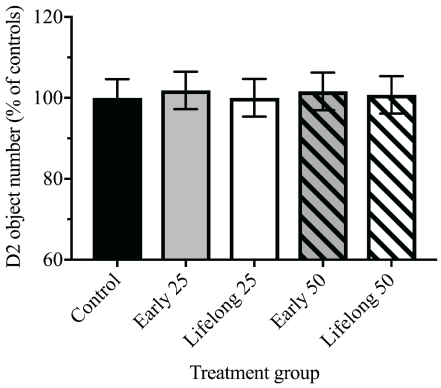
S1 Figure 5. Shell by shell fits for Mn K EXAFS and Fourier transform. EXAFS spectra for a femur bone sample from a rat exposed to 50 mg/kg/d over PND 1-21 (left) and Fourier Transform of that EXAFS spectra (right).

Supplementary Information for Chapter 3

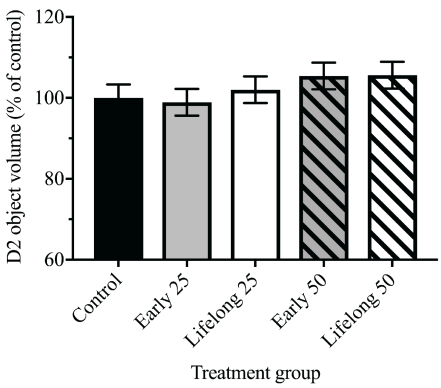
S2 Figure 1a – 1m. Imaris-rendered object number and object volume for proteins quantified by immunohistochemistry. Bar charts show total object number/image (left panels) and total object volume/image (right panels) of Imaris-quantified objects. Data (least squares means ± SEM) reflect 12-18 images/animal and n = 6 animals/treatment group, shown as percent of control values generated from the statistical model that included all five treatment groups. (**a, b**) tyrosine hydroxylase (TH), (**c, d**) dopamine transporter (DAT), (**e, f**) norepinephrine transporter (NET), (**g, h**) dopamine D1 receptor (D1), (**i, j**) dopamine D2 receptor (D2), (**k**) α_{2A} adrenergic receptor (α_{2A}), (**l, m**) glial fibrillary acidic protein (GFAP). Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test.


a.
TH object number (% of control)
A
A
B
C
D
b.
TH object volume (% of control)
A
A
B
BC
C
c.
DAT object number (% of control)
d.
DAT object volume (% of control)
e.
NET object number (% of control)
f.
NET object volume (% of control)
A
B
B
B
B
g.
D1 object number (% of control)
A
AB
AB
AB
C
h.
D1 object volume (% of control)
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

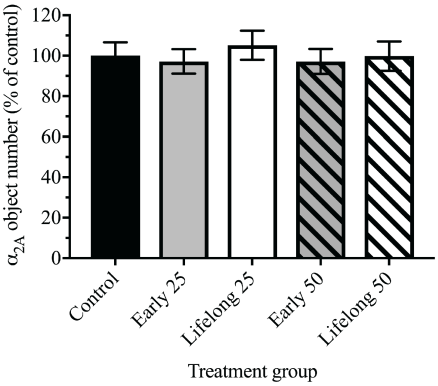
i.


D2 object number (% of controls)
120
100
80
60
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

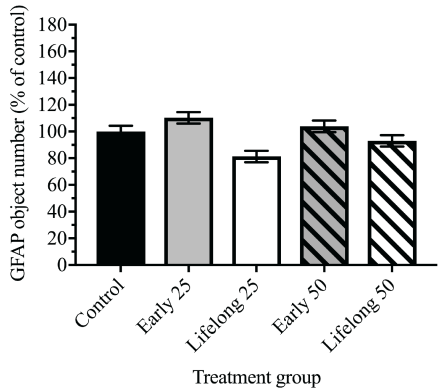
j.


D2 object volume (% of control)
120
100
80
60
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

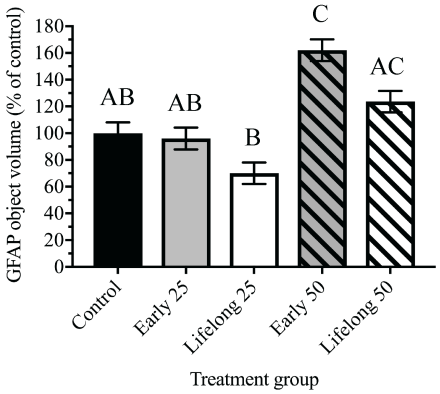
k.


α2A object number (% of control)
120
100
80
60
40
20
0
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

l.


GFAP object number (% of control)
180
160
140
120
100
80
60
40
20
0
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

m.


GFAP object volume (% of control)
180
160
140
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100
80
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20
0
AB
AB
B
C
AC
Control
Early 25
Lifelong 25
Early 50
Lifelong 50
Treatment group

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