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EPIGENETIC AND GENE EXPRESSION MECHANISMS OF DEVELOPMENTAL MANGANESE EXPOSURE NEUROTOXICITY AND EFFICACY OF CHRONIC METHYLPHENDIDATE ADMINISTRATION ON GENE EXPRESSION

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by

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Dissertation Abstract

EPIGENETIC AND GENE EXPRESSION MECHANISMS OF DEVELOPMENTAL MANGANESE EXPOSURE NEUROTOXICITY AND EFFICACY OF CHRONIC METHYLPHENDIDATE ADMINISTRATION ON GENE EXPRESSION

Nicholas A. Santiago

The etiology of neurodevelopmental disorders, such as Attention Deficit Hyperactivity Disorder (ADHD), are complex as it is known that both genetic and environmental factors influence the development of attentional dysfunction. One known environmental risk factor for developing attention deficits is having elevated exposure to the toxicant manganese (Mn) during childhood and adolescence, however, the underlying mechanisms that may contribute to the risk of attention deficits remain unresolved. In Chapter 1 of my dissertation I present a review of the current understanding of cellular Mn regulation in mammalian systems, the importance of studying early life development as a period of vulnerability to environmental insults, potential epigenetic and neuroinflammatory mechanisms for how developmental Mn exposure may cause attention deficits, and finally how our developmental Mn exposure rodent model may be used to investigate ADHD etiology to inform therapeutic interventions for treating attention disorders. In my Chapter 2, I propose a novel mechanism of our hypofunctioning catecholaminergic system and associated attention deficit Mn neurotoxicity phenotype, involving lasting epigenomic and gene transcriptomic dysregulation of mTOR and Wnt signaling. Lastly, my Chapter 3 addresses how chronic administration of a low dose of methylphenidate that is associated with ameliorating developmental Mn-induced attentional function in a rat

model alters gene expression pathways related to epigenetic regulation, inflammation, cell development (Wnt and mTOR), and hypofunctioning catecholaminergic systems in the prefrontal cortex. The following findings may aid future studies to target therapeutic interventions for children at risk of elevated Mn exposure.

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After undergrad I then worked as a laboratory manager for a couple years. This experience brought with it a lot of growth for me as I ended up navigating many difficult social and professional issues that almost prevented me from continuing my higher education journey. I would like to thank all those friends I made during that time that really helped me understand that even with the best intentions sometimes you have to just accept things as they are to move on in your own journey.

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Chapter 1

DEVELOPMENTAL MANGANESE EXPOSURE NEUROTOXICITY: HISTORY AND CURRENT VIEWS

1.0 Objective

Although manganese (Mn) is an essential nutrient for proper cellular function, human epidemiological studies have reported associations of elevated Mn exposure during late gestation and early childhood with inattention, impulsivity, hyperactivity, and fine-motor impairments (1-17). However, these epidemiological studies cannot establish causality due to their cross-sectional design and limited ability to reconstruct environmental exposure histories and control for confounding variables and genetic factors. Studies from controlled animal model experiments have established causality between Mn exposure and neurobehavioral deficits and they elucidate the behavioral and biochemical implications of developmental Mn exposure (18-25). The focus of this literature review is to integrate the evidence from human and animal studies on the impacts of developmental Mn exposure on behavioral deficits. This review covers the current understanding of cellular Mn regulation in mammalian systems, and the importance of studying early life development as a susceptibility period of lasting Mn neurotoxicity. Furthermore, this review proposes an epigenetic and neuroinflammatory hypothesis for how developmental Mn exposure may mechanistically alter brain regions and neurotransmitter circuits associated with attentional function. Lastly, gaps in understanding of how our developmental Mn exposure rodent model for ADHD may help elucidate the mechanisms underlying attention disorders, and potential therapeutic interventions for treating attention disorders, are addressed.

1.1 Properties of Mn and its essential biological role

1.1.1 General Mn background and environmental sources of exposure

Manganese is the 12th most abundant element and 5th most abundant transition metal in Earth's crust (26-28). Manganese naturally enters the environment through erosion, volcanic eruptions, and hydrothermal vent activity (27-28). These natural processes may allow Mn to accumulate in terrestrial environments (28). Additionally, there are several anthropogenic sources of Mn in the environment. Most notably, Mn is used as a metal strengthening agent in steel production; in the United States, Mn ore used for steel production accounts for approximately 80% of all industrial Mn emissions (29). Anthropogenic sources of Mn may also accumulate in the environment through mining, fossil fuel combustion, gasoline additive methylcyclopentadienyl Mn tricarbonyl, fungicides, and pesticides (28-30). Other anthropogenic sources of Mn to the environment include fireworks, dry-cell batteries, fertilizer, animal feed, paint, ceramics, leather, glass, textile, and cosmetics – all materials that in part are made of Mn (29-30). With global Mn reserves estimated to be approximately 570 million tons most widely distributed throughout South Africa, Ukraine, Australia, Brazil, India, China, Gabon, and Mexico, anthropogenic sources are a significant source of Mn contamination of the air (27-28).

1.1.2 Mn chemical properties and biological role

Mn exhibits complex reduction-oxidation chemistry and, depending on its oxidation state, Mn serves different biological functions (31-33). Thus, understanding of Mn metal speciation, oxidation state and ligand environment is important for

determining how Mn will function in biological systems (34-35). Mn is known to exist in 11 oxidation states as a transition metal, ranging from -3 to +7. However, in mammalian systems Mn has been shown to exist only as the Mn(II) and Mn(III) oxidation states (36-43). Mn(II) exhibits functional similarities to Ca(II) and Mg(II) due to its valence electron configuration and plays an essential role as a cofactor for several metalloenzymes in the metabolism of amino acids, lipids, proteins, and carbohydrates (44). Arginase, for example requires Mn for the elimination of ammonia by hydrolyzing arginine into ornithine and urea (45-46). Proper arginine homeostasis is critical for neuronal health as nitric oxide synthase (NOS) can convert arginine into nitric oxide and citrulline. Excess nitric oxide, as discussed later in this review, may propagate pro-inflammatory signals harmful for neuronal survival (47-48). Additionally, Mn is required for glutamine synthetase to catalyze the ATP-dependent condensation of glutamate with ammonia. This catalyzation is necessary to synthesize the amino acid glutamine in the glutamine/glutamate-γ-aminobutyric acid cycle within astrocytes (49-50).

Due to its valence electron configuration Mn(III) can function similarly to Fe(III), which is essential for hemoglobin distribution of oxygen and mitochondrial metabolism (44, 51-52). Mn and Fe homeostasis are tightly coregulated due to their chemical similarities (53-55). The transition from Mn(II) to Mn(III) at the active site of the key antioxidant enzyme Mn superoxide dismutase (MnSOD) allows the intracellular reactive oxygen species (ROS) super oxide anion produced during mitochondrial respiration to be metabolized (56-63).

1.2 Mn uptake, transportation, and elimination

1.2.1 Mn dietary absorption pathways

Humans primarily acquire Mn through their diet in foods, such as nuts, whole wheat bread, tofu, beans, fish, mussels, clams, spinach, whole grains, and black tea (64). For adults the daily intake of Mn ranges from 0.9 to 10 mg/day, while the recommended daily intake is 2.3 mg/Kg for men and 1.8 mg/Kg for women (65-66). These recommendations, however, have been questioned based on the scientific data used to set the Mn intake guidelines, as well as the increasing number of studies reporting associations between Mn exposure and the development of neurological impairments in infants and children (67). Taken together, about 1-5% of consumed Mn is absorbed by the gastrointestinal tract in healthy adults, whereas children are believed to absorb 15 – 20% of oral Mn intake (68). Mn enters the blood after absorption by the gastrointestinal tract Mn, where average blood Mn concentrations range from 4-15 μg Mn/L in adults (69). Prior to reaching the systemic circulation, ~80% of Mn that is absorbed into the portal blood is transported by liver hepatocytes into the biliary duct with bile back into the large intestine for fecal elimination, while <0.1% is excreted through the pancreas and kidneys (68, 70-72).

Manganese may enter the central nervous system through several possible routes. Mn that enters the systemic circulation may cross the blood-brain barrier through the cerebral capillary endothelium of the choroid plexus, the choroid plexus into cerebrospinal fluid (CSF), and then into the brain. Aerosol or particulate Mn that enters the nasal cavity may enter the brain (olfactory lobe) via the olfactory nerve (73-

75). Additionally, Mn from the air can directly be absorbed through the alveolar ducts in the lung, where 60-70% of respired particulate Mn may be expelled through mucociliary action or swallowing (76). Inhaled Mn particulates, typically <5 μm in size, can be absorbed across the lung epithelia into the systemic circulation and transported directly into the brain via the circulation (77-78). Under normal environmental conditions, these respiratory and nasal routes of Mn uptake are considered minor if not negligible compared to the oral route of intake. Normal brain intracellular Mn concentrations are believed to be 5.32–14.03 ng Mn/mg protein or 20.0 – 52.8 μM Mn, although several factors involving the homogeneity of cell size and Mn distribution in the brain make determining precise intracellular Mn levels complex (79).

1.2.2 Mn blood-brain barrier transport

Just as the biological role of Mn depends on its redox state, how Mn is taken up and transported across the blood-brain barrier also depends on its oxidation state. The uptake of Mn(II) into the brain is believed to rely most notably on the divalent metal cation transporter (DMT-1), which is a solute carrier protein that may also transport divalent Fe(II), zinc (Zn), cobalt (Co), copper (Cu), cadmium (Cd), nickel (Ni), and lead (Pb) (80-83). DMT1 is expressed in several brain regions that correspond with Mn accumulation, such as the basal ganglia, striatum, substantia nigra, globus pallidus, and subthalamic nucleus (84-86). Mn(III) uptake and transfer across the blood-brain barrier is believed to happen through Mn(III)–ligand complexes (51, 74, 87). Mn(III) is believed to be primarily bound to and transported by circulating transferrin, which

facilitates Mn(III) entry into the brain space via the transferrin receptor transport system (88). The liver synthesizes transferrin present in the systemic circulation, however, neurons, astrocytes, and microglia also express transferrin in the CNS (89). In maintaining cellular and systemic Mn homeostasis SLC30A10, ATPase 13A2 (ATP13A2), ferroportin, and secretory pathway Ca2+-ATPase 1 (SPCA1) are believed to be important, primarily by facilitating cellular Mn efflux (90-114).

1.3 Neurodevelopment and vulnerability to Mn toxicity

The prenatal and early postnatal periods may be unique windows of susceptibility for Mn overexposure as Mn absorption is up-regulated during pregnancy (12). As an essential nutrient necessary for the proper development of bone, cartilage, and tendons in the fetus, Mn is actively transported across the placenta from mother to fetus and across the developing blood-brain barrier (64, 115). Although Mn deficiency during fetal development is rare as adequate Mn is found in many foodstuffs, the limited cases of Mn deficiency suggest impaired bone growth, birth defects, lowered fertility, and altered metabolism of lipids and carbohydrates outcomes (116-117).

Instead, numerous cases of excess Mn exposure with impacts on health have been reported. For example, several epidemiological studies have reported associations of elevated infant mortality risk and decreased birthweights with high levels of Mn in groundwater and maternal and cord blood (118-123). Thus, determining the levels of Mn intake needed for optimal fetal development vs. the levels that may produce adverse neurological effects is important but poorly understood. As a biologically essential element that is also toxic at elevated exposures, Mn is known to produce a classic

inverted U-shaped dose-response profile. For instance, two studies report an inverted U-shaped association between neonate blood Mn levels at delivery and at 12 months of age with mental development index scores, consistent with the notion that Mn acts as both an essential element and a toxic metal (12). Maternal blood Mn levels of 24–28 μg/L were reported as a transition value from beneficial to adverse effects of Mn reflective in mental development scores of six-month old children (124). In a large prospective cohort study of neonates from China, a cord serum Mn threshold of 5 μg/L was identified, above which three-day-old neonates showed cognitive deficits as measured by the Neonatal Behavioral Neurological Assessment (125).

The underdeveloped homeostatic mechanisms of Mn absorption and excretion in infants may contribute to the complex threshold of optimal vs. dysfunctional levels of Mn for fetal development (29, 126-129). Although there is believed to be an increased nutritional need for Mn in early life, it is still unclear what level of Mn exceeds that beneficial need and becomes toxic for fetal development (67, 130-131). As noted above, recommended adequate intake levels for Mn in adults is 2.3 mg/day for men and 1.8 mg/day for women, but these amounts appear to differ with age depending on physiological need (64). The National Academy of Sciences extrapolated a reduced recommended intake range of 1.2-1.5 mg/day for children between 1-8 years of age and 3 ug/day for infants less than 6 months of age (64, 130). These recommendations, however, have been questioned based on the scientific data used to set the guidelines, as well as increasing number of studies reporting associations

between Mn exposure in drinking water and the development of neurological impairments in infant and children (3, 67, 132).

While adults have fully functioning hepatic (and possibly gastrointestinal) homeostatic mechanisms in place for regulating Mn uptake and elimination, these homeostatic controls are not fully developed in infants and young children. Specifically, infants absorb approximately 20% of ingested Mn, whereas adults are thought to absorb only ~5% (127). Studies in rodents support these findings in which neonate rats (<15 days of age) absorb and retain over 40% of ingested Mn compared to less than 5% retained by adult rats (76, 133-135). For further review on excretory processes and development please see Gurol et al., 2022 (136). In humans the blood-brain barrier remains semi-permeable to Mn and other potential toxins until its complete formation around 3-4 months of age or older (137-138). Interestingly essential neurochemical systems, such as dopaminergic synapses, experience rapid growth during the first 2-3 weeks of postnatal life (139). Taken together, these important neurochemical systems are developing while the biliary/gastrointestinal pathways necessary for effective excretion of Mn, and the mechanisms that regulate Mn transport across the blood-brain barrier are not well established. These factors make the early postnatal window a period of increased vulnerability to elevated Mn exposure.

1.4 Basic behavioral neurobiology, Mn neurotoxicology, and neurodevelopment

1.4.1 Attention, impulse control, and fine-motor function neurobiology

Attentional function may be thought of as the ability to self-monitor and respond to internal and external stimuli (140). This process is believed to occur in a

'Top-down' fashion regulated by the prefrontal cortex (PFC) and connecting brain circuitry defined as the fronto-striatal circuit (141-142). Moreover, the fronto-cortico-striatal circuit is additionally composed of the basal ganglia (BG), which is a group of related subcortical nuclei including the striatum, globus pallidus (GP), subthalamic nucleus (STN), and substantia nigra (SN) (142). The striatum is considered the input region of the BG as it receives substantial glutamatergic input from the cerebral cortex and intralaminar nuclei of the thalamus, dopaminergic inputs from the SN, norepinephrinergic inputs from the locus coeruleus (LC), and serotonergic afferents from the dorsal raphe nucleus (143). These signals provide feedback to the PFC and cingulate cortex as part of the fronto-cortico-striatal circuit that regulates attentional, arousal, and impulse control functions (140).

Dysfunction in sustained attention and arousal regulation found in individuals with ADHD have additional effects on the individual's working-memory, ability to organize, problem solve, and self-monitor (140-141, 144-147). These functional deficits have been largely attributed to PFC function as patients with PFC lesions are easily distracted, have impaired ability of shifting attentional set, impairment at gating sensory stimuli, have poor concentration and organization, and are more vulnerable to disruption from proactive interference (148-154). In accordance with these deficits, structural and functional brain imaging studies on individuals with ADHD demonstrate that they have smaller PFC volume, decreased PFC activity, weaker PFC white matter connections, and weaker resting connectivity compared to healthy controls (140, 146, 155-160). Furthermore, studies also suggest that striatal dysfunction is associated with

the etiology of ADHD, which is supported by experimental evidence in animal models where striatal lesions mimic symptoms of hyperactivity and poorer performance on tasks of response inhibition (161-162). These deficits are consistent with structural imaging studies in humans, where ADHD patients have notably smaller caudate volumes than healthy controls (163-164). Altogether, current evidence supports the notion that dysfunctional fronto-striatal circuitry is involved in, if not responsible for cognitive impairments in ADHD patients (140).

1.4.2 Mn associated behavioral deficits in children

Epidemiological studies have revealed that high Mn exposure during development increases the risk of inattention, impulsivity, hyperactivity, and fine-motor impairments similar to symptoms of ADHD (1-17). A recent population based registry study in Denmark followed a cohort of 643,401 children born 1992–2007 and aimed to associate clinical diagnoses of ADHD-Inattentive and ADHD-Combined subtype diagnoses with Mn measurements from drinking water samples to estimate longitudinal exposure during the first 5 year of life with high spatiotemporal resolution (165). After adjustment for age, birth year, socioeconomic status, and urbanicity the authors found that exposure to increasing levels of Mn in drinking water was associated with an increased risk of ADHD-Inattentive subtype, but not ADHD-Combined subtype (165). The importance of this finding becomes even more vital as the increased risk for ADHD the authors determined is associated with Mn levels below the WHO's health-based drinking water guideline value of 400 lg=L, which have implications for creating stricter drinking water guidelines for children (67).

Additional evidence of Mn exposure and attention deficit development comes from, a pilot study of 46 Canadian children using the Revised Conner's Rating Scale for Parents and for Teachers found that higher drinking water and hair Mn levels were associated with hyperactivity (2). This group subsequently performed larger scale studies using the California Verbal Learning Test-Children's Version (CVLT-C), Conner's' Continuous Performance Test (CPTII), Digit Span, Santa Anna Test, and manual Fingertapping, and found lower IQ scores, inattention, and motor-function impairments associated with higher hair and water Mn levels (3, 9). However, hyperactivity was no longer associated with hair and water Mn levels (9). More recently, this group used the Wechsler Intelligence Scale for Children, 4th edition and found that higher Mn concentrations in water, hair, and toenails were associated with Performance IQ deficits in girls, but surprisingly better Performance IQ scores were observed in boys (4). The authors reasoned their conflicting results may have occurred due to sample size and that the water Mn levels were lower compared to previous studies (4).

Other studies have similarly reported associations between elevated water Mn levels and poorer cognitive performance. Wasserman and colleagues reported an association between higher well water Mn levels and learning and memory impairments, including reduced Full-Scale, Performance, and Verbal raw IQ scores in 10-year old children. (132). Additionally, greater hair Mn levels have been associated with reduced verbal IQ and working memory scores in children living near a hazardous waste site, but only after adjusting for arsenic exposure (166). Haynes et al., 2018

assessed 106, 7-9-year-old children from an area of Ohio known for high historic industrial air Mn emissions; hair Mn was negatively associated with child IQ scores, while blood Mn was not associated with any functional outcomes (15). The authors believe this may be due to differences in Mn metabolism in which hair Mn may reflect chronic exposure and blood Mn may represent current exposure (2, 15, 167-168).

An ongoing study in Brazil has been following a cohort of 70 children between 7 and 12 years of age living near a ferro-manganese alloy plant (169-173). Results showed high levels of Mn in hair inversely associated with intellectual function (168-169). Furthermore, this group has shown an association of higher Mn in hair with increased aggressive behavior, impulsivity, inattention, and decreased working memory (173-174). More recently this group found an association between higher Mn in hair and hyperactivity using three subtests of the Developmental Neuropsychological Assessment Battery Second Edition – NEPSY II (Inhibition, Word Generation and List Memory), Grooved Peg Board Test, and the Brazilian Conner's Abbreviated Teacher Rating scale (172).

One study of 248 children from 7-10.5 years of age investigated the association of behavior, cognition, memory, and motor function in children living near agriculture fields that use Mn-containing fungicides (10). This study used prenatal and early postnatal dentine of shed teeth as a biomarker for Mn levels. The dentine in teeth represent an interesting biomarker as they can be used to distinguish between late gestation and early childhood Mn exposure, because it is believed that metal accumulation in developing teeth occurs in a daily pattern like tree growth rings that

may be processed for accurate exposure assessment from the second semester of pregnancy to 1 year (175-176). Higher prenatal and early postnatal Mn levels in teeth were associated with internalizing, externalizing, and hyperactivity in boys and girls. Unexpectedly, boys with higher Mn levels in prenatal and postnatal dentine had better memory function at ages 9 and 10.5, and better cognitive and motor function at ages 7 and 10.5 years of age (10).

Fine-motor impairments in children exposed to high levels of Mn have also been reported. Luchinni et al., 2012 found that adolescents 11-14 years of age from a region of historic ferro-manganese alloy plant in Italy had motor coordination and hand dexterity impairment associated with higher soil Mn levels, as well as tremor impairments associated with high blood and hair Mn levels (7). Hernández-Bonilla et al. (2011) used the Grooved Pegboard Test, Finger Tapping Test, and Santa Ana Test for assessing motor performance in children and similarly observed a negative relationship with blood Mn levels, speed, and motor coordination. These authors didn't find an association between hair Mn levels and motor function performance (177). Additionally, there was not an association between children living near a ferro-manganese alloy plant in Brazil with Mn levels in hair and motor function (172).

While some of these findings are mixed, it is nonetheless evident that higher environmental levels of Mn and higher Mn levels in exposure biomarkers are associated with impairment in behavioral and cognitive functions in children. However, it is difficult to establish causality of developmental Mn exposure and these cognitive and fine-motor impairments from the epidemiologic evidence because of poorly known

exposure history. This is in part due to the fact that there is no consensus in the field on what is the best exposure biomarker to assess developmental Mn exposure and impaired cognitive and motor function (16). Further, many of the discussed studies are also limited by their small sample size, cross-sectional design, control of confounding variables of multifaceted environmental exposure histories, and genetic factors. Moreover, the sex-specific findings complicate the interpretation of these results. Thus, these limitations underscore the importance of well-conducted animal model studies to characterize the effects and potential mechanisms of developmental Mn exposure on neurodevelopment.

1.4.3 Insight from animal models

Animal models using a developmental Mn exposure paradigm demonstrate similar deficits in learning and memory, attentional dysfunction, impulse control, and fine-motor movements reminiscent to that seen in humans (18-25, 178-180). For example, our group has shown 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 using the Montoya staircase test, and that rats exposed throughout life (25 mg Mn/kg/day dose) were more severely impaired than those exposed only in early life, though the effects of the higher 50 mg/kg/day dose restricted to early life were not worsened by continued lifelong exposure to that dose (19-20). Using the same developmental exposure paradigm, we have found lasting impairments in attentional preparedness, selective attention, arousal regulation, and altered impulse control in an adapted 5-choice serial reaction time attention task (21-22). These findings indicate that

developmental Mn exposure can cause attention deficits and reduced impulse control, somewhat consistent with a predominantly ADHD-inattentive phenotype (21-22).

The catecholamine dopamine (DA) is a neurotransmitter in the brain associated with learning, motivation, and reward and its dysfunction is implicated in the etiology of ADHD (144). DA activates five types of cell surface metabotropic receptors, DRD1-D5, to regulate the dopaminergic system. These five receptors are divided into two classes of functionality, the excitatory D1-like (DRD1 and DRD5), which activates adenylyl cyclase, and the inhibitory D2-like (DRD2, DRD3, and DRD4), which inhibits adenylyl cyclase (181). Research from our group has shown that postnatal Mn exposure from PND 1-21 reduced D1 and dopamine transporter (DAT) protein levels in the striatum and nucleus accumbens, and increased D2 protein levels in the striatum, nucleus accumbens, and PFC (23). Another study using an identical time-period of Mn exposure, but at a higher dose of 750 µg Mn/day reported similar reductions in DAT expression in the striatum and nucleus accumbens (180). Furthermore, we have reported that chronic postnatal Mn exposure reduced K+-stimulated dopamine release in the prefrontal cortex and striatum (20). Additionally, our lab has shown that developmental Mn exposure restricted to the pre-weaning period caused a lasting (into adulthood) reduction in K^+-evoked release of DA and norepinephrine from the PFC and striatum (measured in PND 500 – 600 animals), as well lasting changes in multiple proteins comprising the catecholaminergic synapse, including reductions in tyrosine hydroxylase, D1, DAT, and NET, and an increase in D2 and astrocytic GFAP in the PFC (18, 25). These rodent studies indicate that developmental Mn exposure causes

lasting changes to the catecholaminergic synapse in the PFC and striatum, and thus provide insight into causality of Mn-induced lasting cognitive and behavioral changes through regulating the dopaminergic system.

Additionally, adult Mn exposure has been shown to affect the uptake of another catecholamine, norepinephrine (NE), in a dose-dependent manner in rat forebrain synaptosomes, with selectivity for inhibition of NE uptake versus that of DA or GABA, and in the rat whole brain synaptosomes as well (182-183). Furthermore, weanlings exposed to Mn in their diet have shown Mn exposure decreased extracellular concentrations of norepinephrine (NE), uptake of NE, and expression NET in the striatum, as well as the uptake of NE (184). This decrease in extracellular NE concentrations may reduce the electrical activity of medium spiny neurons GABA projecting from the striatum to the GP, which may further increase GABAergic inhibitory firing from the GP to the STN, that ultimately decreases the firing activity of STN excitatory glutamatergic neurons projecting to the SNc. This signaling cascade can lead to decreased glutamatergic excitation in the SNc and decreased GABAergic inhibition from the striato-nigral projection neurons that may dysregulate dopaminergic input to the striatum (184). We have shown postnatal Mn exposure does reduce K+-stimulated NE release as well in the prefrontal cortex and striatum (18, 25). Interestingly, we have also found lasting changes in decreased NET in the PFC, making it important to further elucidate how NE contributes to Mn-exposure induced behavioral deficits (18).

The altered functionality of the cholinergic, adrenergic, and dopaminergic systems due to Mn may contribute to the attentional, impulsivity, and fine-motor impairments caused by developmental Mn exposure (185). Mn exposure decreases choline acetyltransferase (ChaT) and increases acetylcholinesterase (AChe) enzyme expression and activity in the hippocampus, frontal and parietal cortices, and the caudate and the putamen, and the cerebellum in animal models (185-191). Furthermore, the stimulatory effect of Mn on AChE activity promotes increased levels of neuronal oxidative stress and neuroinflammatory biomarkers as well as decreased antioxidant status (187-188). Since these enzymes are responsible for the synthesis and degradation of acetylcholine (ACh), and given ACh's important role in the regulation of neuronal proliferation, differentiation, migration, maturation, plasticity, and synapse formation, this may be an additional mechanism underlying how developmental Mn exposure impairs cognition (187-188, 193).

1.5 Mechanisms developmental Mn lasting effects on attentional function

1.5.1 Mn, oxidative stress, and the catecholaminergic system

Manganese is believed to exert cellular toxicity via a number of mechanisms, including disruption of cellular Ca homeostasis, mitochondrial function, cellular Fe homeostasis, and impaired endosomal trafficking (28, 193). There is substantial evidence that at elevated cellular levels Mn may act as a prooxidant and directly or indirectly increase formation of reactive oxygen species (ROS) (194-195). Studies have demonstrated in vitro that elevated Mn leads to inhibition of complexes within the mitochondrial electron transport chain and can induce ROS and nitrogen oxide synthase

(NOS) levels in neuronal cells (196-200). Superoxide radical, hydrogen peroxide, and hydroxyl radical are the most common ROS and they may all cause oxidative damage to nucleic acids, proteins, and phospholipids to cellular mitochondria (201-202). Mitochondrial DNA is sensitive to ROS damage because of the lack of protective histones and the lack of repair mechanism found in the nucleus (201-202). Intramitochondrial Ca^{2+} activates a series of proteins within the mitochondrion that increase ATP production by oxidative phosphorylation, such as by activating three dehydrogenases in the TCA cycle that catalyze production of NADH (203-205). It is also known that Mn(II) inhibits the rate of ATP production through inhibition sites in the electron transport chain (ETC), the TCA cycle, and at other loci (206-208). Inhibition of the ETC, TCA cycle, and other biochemical pathways by Mn induces buildup of O_2^- production in the mitochondria that increases ROS levels (209).

Oxidative stress can damage and alter the function of biomolecules like lipids, proteins and DNA, thus inducing signal transduction and gene expression events, inhibiting protein function, and promoting cell death (210). Guanine is the most susceptible to oxidation of all of the nucleobases (210). When deoxyguanosine is oxidized, a hydroxyl group is added to the 8^{th} position of guanine forming 8-hydroxy-2'-deoxyguanosine (8-OHdG) (210). This oxidation causes a change of a G-C base pair into an A-T base pair during DNA repair (210). Oxidative stress can alter DNA structure by degradation or modification of bases, single or double strand breaks, or cross-linkage of other proteins. If not repaired by DNA repair mechanisms, this damage may lead to mutations within DNA, leading to transcription alterations and genomic

instability that then modifies gene expression (210). Mn can also directly catalyze autoxidation of DA and serotonergic (SE) neurons, producing DA/SE-o-quinone, ROS, and hydrogen peroxide (H2O2) that decrease cellular DA and SE levels (211-218). These released ROS and prooxidant biomolecules may contribute to proinflammatory and epigenetic disruptions of the catecholaminergic system as well, as discussed below.

1.5.2 Mn-neuroinflammatory hypothesis and mechanistic contribution

Microglia and astrocytes are two important cell types that mediate the inflammation response in the CNS and influence the functionality of neurons. Microglia are the resident tissue macrophages of the CNS and comprise about 10% of all cells within the mammalian brain (219). As an integral part of the CNS, microglia play important roles in the development and maturation of the CNS as well as in tissue homeostasis (219-220). Astrocytes play a significant supportive and protective role in the CNS by releasing neurotrophic compounds that mediate proper neuronal development, normal synapse maintenance, formation of the blood-brain barrier, and nutrient and neurotransmitter concentrations in the brain (220-227). Animal model and cell culture studies have shown that Mn exposure results in activated microglia and astrocytes, as well as increased expression of pro-inflammatory cytokines, ROS, and NOS, suggesting neuroinflammation may play a role in the behavioral and biochemical phenotype of Mn exposure (24, 208, 220-221, 223, 228-243). Chronic or uncontrolled release of cytokines, ROS, and NOS can in turn activate additional immune cells, setting up a self-propagating cycle of inflammatory response (222, 244-250).

In light of the above, it is possible that elevated Mn exposure may induce a self-perpetuating pro-inflammatory environment, which may contribute to increased oxidation of DA, altered expression of DA proteins, and the lower DA levels associated with Mn exposure (18, 20, 24). For instance, in the DA biosynthesis pathway phenylalanine conversion to tyrosine and tyrosine conversion to levodopa by neurons requires the co-factors tetrahydrobiopterin BH4 and Fe – both of which may be sensitive to proinflammatory/pro-oxidizing environments (251-252). One possibility is that elevated ROS and NOS levels downregulate BH4 production and may cause a decrease in DA concentrations in the pre-synaptic neuron (253-257). Elevated ROS and NOS levels can also activate the janus kinase signal transducers and signal transducers and activators of transcripts (JAK-STAT) pathway. Janus kinase signal transducers (JAK) are cytoplasmic tyrosine kinases that activate when cytokines bind to JAK receptors. When cytokines bind to JAK receptors, phosphate groups are transferred to transcription factors, called signal transducers and activators of transcripts (STAT). Phosphate group transfer to STAT can alter gene expression. Thus, elevated ROS and NOS levels can stimulate JAK-STAT signaling and indirectly downregulate BH4 synthesis through genes regulated by STAT (245, 255, 257-259). Mn can activate JAK-STAT cytokine response, but it is unclear how this response is activated and whether Mn exposure downregulates BH4 and DA production (260).

1.5.3 Epigenetic hypothesis and mechanistic insight

How developmental Mn exposure creates life-long changes in the catecholaminergic system of the fronto-cortico-striatal loop, including the lasting

changes in catecholaminergic proteins noted above (i.e., TH, D1, D2, DAT, NET, etc.), and impairments in attentional and impulse control function remains unclear. One hypothesis is that developmental Mn may induce these lasting changes in protein expression levels via epigenetic mechanisms, such as DNA methylation and post-translational histone alterations of gene expression due to the pro-oxidized environment generated by Mn (261). It has been suggested that the DNA oxidation structure 8-OHdG can induce DNA hypomethylation by inhibiting DNA methylation at nearby cytosine bases, and the DNA oxidation structure 5-hydroxymethylcytosine may cause DNA hypomethylation by inhibiting active DNA demethylation processes (262). Reactive radical species can also function as catalysts of DNA methylation and can even induce site-specific hypermethylation by the up-regulation of expression of DNA methyltransferases (DNMTs) or the formation of a new DNMT containing complex with Sirtuin1, a histone deacetylase enrolled to DNA damage sites, or polycomb repressive complex 4 an enhancer of Zeste protein-2 that may facilitate DNA hypermethylation. (262).

Epigenetics is the study of heritable changes in gene expression that occur independent of modifications to nucleotide sequences (263). One epigenetic mechanism affecting gene expression involves DNA methylation of cytosine residues by DNA methyltransferases (DNMTs), which add a methyl group to the 5′ position of cytosines within CpG dinucleotides. About 70% of CpGs sites are methylated in the human genome and these largely, although not always, occur in promoter regions of genes (264). DNMT1 is mostly active as a maintenance DNMT, ensuring that a newly

synthesized DNA strand obtains the same methylation pattern as the parental strand during DNA synthesis (265). In contrast to maintenance methylation, DNMT3a and DNMT3b have been predominantly implicated in de novo DNA methylation (266). In general, hypermethylated DNA binds transcriptional repressors and leads to chromatin condensing and gene silencing, whereas hypomethylation stimulates DNA transcription and hence gene expression by preventing the binding of transcriptional repressors (267-268).

A second class of epigenetic mechanism is post-translational histone modifications including acetylation, methylation, phosphorylation, and ubiquitination (269-271). Histones are classified into H1, H2A, H2B, H3 and H4 histone families, each of which comprise several variants. Histone acetylation is achieved through the activity of histone acetyltransferases (HATs). Although hypoacetylation catalyzed by histone deacetylases (HDACs) is known for its repressive effect on gene expression, histone methylation can lead to transcriptional activation or repression, depending on the position of the histone lysine acceptor residue (272-273). Histone methylation is achieved through lysine methyl-transferases (KMTs) and demethylation is achieved by lysine demethylases (KDMs) (273). The third epigenetic regulation mechanism involves micro RNAs (miRNAs), which are small non-coding RNAs that participate in post-transcriptional regulation of gene expression by pairing with complementary sequences within messenger RNA (mRNA) molecules, and siRNAs, which play a role in the RNA interference (RNAi) pathway where they interfere with specific genes expression by the destruction of specific mRNA molecules (274).

Limited studies have been done to assess Mn exposure induced epigenetic changes on the catecholaminergic system. It is known, however, through neuronal cell culture, rodent brain, and human serum analysis that *Th*, *Dat*, *Drd1*, *Drd2*, *Net*, *Chat*, and *Ache* gene expression can be regulated by promotor region methylation and histone acetylation (261, 273, 275-293). One study found that after 30 days of 100 uM Mn exposure in SH-SY5Y neuroblastoma cells, methylation densities of CpG sites increased from 70.4 to 73.6% and non-CpG sites (CHG and CHH sites H=A/C/T) 3.4 to 19.7% and 3.7 to 25.4%, respectively (261). Furthermore, the *Th* promoter was hypermethylated and gene expression was downregulated (261). It was subsequently shown, using this same treatment paradigm, that the gene transcript *Hat1* was upregulated (293). Mn may also significantly suppress the acetylation of histone H3 and H4 as it was found Mn exposure significantly induced a time-dependent increase in *Hdac3* and *Hdac4* mRNA expression and protein levels at 6 h, 12 h and 24 h in PC12 cells and SH-SY5Y cells (294). The state of histone acetylation is dynamically regulated by *Hat* and *Hdac*, so it is believed Mn exposure may induce histone hypoacetylation through up-regulation of HDAC activity and protein expression levels, and down-regulation of HAT activity and protein levels in neuronal cells (294).

In the context of developmental Mn exposure, it has been shown in humans that Mn exposure during late gestation was associated with 713 CpG loci DNA methylation changes in the placenta and 4 of the 5 loci that reached Bonferroni-adjusted significance are crucial for neurodevelopment (295). Another study exposed pregnant mice to 800-ppm Mn(II) in their diet from gestational day 10 through day 21 after delivery on

weaning and searched epigenetically downregulated genes by global promoter methylation analysis in the hippocampal dentate gyrus of male offspring on postnatal day PND 21 and PND 77 (271). The authors found CGI hypermethylation of 24 gene promoter regions and transcript downregulation lasting into adulthood (271). Thus, these findings establish the potential of developmental Mn exposure to induce epigenetic changes that may mechanistically drive the lasting catecholaminergic changes observed in our model of developmental Mn exposure (18-25).

1.5.4 Epigenetic and neuroinflammation feedback loop

In theory, epigenetic methylation alterations and a highly oxidized, inflammatory environment may together be the driving mechanism of the lasting changes in catecholamine system proteins and resulting behavioral phenotype shown in our recent animal studies. Thus, it is important to understand how these two phenomena impact brain region neurocircuitry of neurotransmitter function. It has been demonstrated that expression of certain pro-inflammatory cytokines, including *Il-1b* and *Il-6*, are epigenetically regulated by differential DNA methylation of their promoters in conditions such as hypoxia, small cell lung cancer, and arthritis (296-299). Additionally, DNA methylation in astrocytes during development indicate that astrocytes express *DNMT*s and that astrocyte specific gene expression can be reduced by methylation as conditional knockout of *Dnmt1* in nestin-positive NPCs induces *Gfap* and *S100β* gene expression along with activation of the JAK-STAT pathway, including STAT1 and STAT3 (300). The *Gfap* gene promotor is methylated at a specific CpG site known to bind STAT3 during development. However, in late-stage

neural progenitor cells this particular cytosine is demethylated, allowing for leukemia inhibitory factor-induced activation of STAT3 to increase gene expression of *Gfap*, driving astrocyte differentiation (301). Additionally, astrocytes express enzymes that regulate histone methylation, including HMTs and histone demethylases (299, 302). This finding in particular suggests an important role of epigenetics in astrocyte function, and thus a potential role for epigenetics and inflammation to intersect.

1.6 Epigenetics, neuroinflammation, and ADHD

The prevalent ADHD symptoms include problems in maintaining attention, excessive motor activity, and impulsivity, which often lead to poorer academic performance and impaired social interactions (304). These symptoms were detected as early as age six in up to 5% of children (303-304). ADHD research has focused on the dopamine transporter (DAT), since modification of expression and/or function of its gene/gene product has been associated with ADHD symptoms (305-307). The dopamine transporter gene (*Dat1* or *Slc6a3*) is significantly associated with ADHD (308). The activity of this transporter is 14% higher in ADHD patients than in healthy controls (309). As a result, dopamine in ADHD patients takes less time to exert an effect in the synaptic cleft than dopamine does in controls (309). One study found a sample of ADHD children have altered methylation of CpG site 1 of the *Drd4* gene compared to healthy controls and might play a role in ADHD development (310).

Interestingly, the cell types responsible for the release of cytokines contributing to a proinflammatory environment were studied in ADHD children. One study found that the neutrophil/lymphocyte ratio, platelet/lymphocyte ratio, monocyte/lymphocyte

ratio, and mean platelet volume were significantly higher in ADHD children than healthy controls (311). In accordance with this, ADHD patients are reported to have a cerebrospinal fluid and blood serum cytokine profile of increased TNF-α, IL-2, IL-4 IL-6, IL-8, IL-10, IL-13, IL-16, while IL-1β showed a small decrease (311). Moreover, STAT6, involved in the expression of IL-4 signaling plays an important role in brain development, as STAT6-deficient mice have fewer dopamine transporters and increased locomotor activity (311). An increase in placental IL-6 leads to a reduction of growth hormone and insulin-like growth factor and activates the JAK-STAT3 pathway, resulting in the expression of several genes that may affect fetal neurodevelopment (312). An association of elevated cytokine levels and ADHD, specifically, increased IL-13 (anti-inflammatory) and inattention and increased IL-16 (proinflammatory) and hyperactivity have been suggested (313). A recent genome-wide scan of 958 ADHD child–parent trios reported that two single nucleotide polymorphisms (SNPs) in the IL-16 gene were associated with the inattentive phenotype, whereas another analysis reported a nominal association for IL-3 with earlier-onset ADHD (313). In ADHD, a meta-analysis including six studies with a total of 231 patients showed that this population have increased oxidative status, supposedly leading to increase oxidative damage; decreased antioxidant enzymes like CAT and increased oxidative stress markers like MDA (313). Taken together, these studies suggest inflammation may contribute to the development of ADHD.

Recent evidence suggests that dysregulation of the neurodevelopmental signaling pathways mammalian/mechanistic target of rapamycin (mTOR) may be one

underlying mechanism of our developmental Mn exposure altered catecholaminergic phenotype (18-25, 314). Persistently activated mTORC1 signaling can lead to blocked dopamine receptor D1 signaling and is associated with dopamine signaling pathway gene expression, cortico-striatal plasticity, and sociability behavioral deficits in mice (315). Additionally, mTORC1 activation is known to alter DNA methylation regulation of gene expression through altering gene and protein expression of the epigenetic modulators *Dnmt1* and *Dnmt3A* (316-318). The alteration of the Wingless-Int (Wnt) pathway may also lead to a hypofunctioning catecholaminergic system and an ADHD-like behavioral phenotype similar to that of our developmental Mn exposure rat model (314). Inhibition of Wnt signaling has been shown to decrease dopaminergic differentiation, myelination, dendrite morphogenesis, and is able to alter mTORC1 and mTORC2 activation outcomes (314). Furthermore, mTOR regulation is known to be activated and Wnt regulation is known to be inhibited due to glial cell induced proinflammatory environments(314, 319-325). However, whether developmental Mn exposure alters mTOR and Wnt pathway genes DNA methylation, and whether these changes are associated with catecholaminergic system gene and protein expression levels warrants future investigation as explored in Chapters 2 and 3.

1.7 Methylphenidate and epigenetics in ADHD treatment

Methylphenidate (MPH) is among the most commonly prescribed therapeutic treatments for ADHD. One pharmacological level mechanism of MPH is to act as a central nervous system stimulant that increases attention and reduces hyperactivity and impulsivity by inhibiting DAT and hence dopamine reuptake in the brain, without

triggering DA release (326). Interestingly in humans, MPH is believed to have a 65–70% overall efficacy rate in improving ADHD symptoms (327-330). One potential reason for the variations in individual treatment effectiveness may be due to epigenetic differences. For instance, polymorphisms in *Dat1* promotor region DNA methylation is correlated with the efficacy of MPH treatment of oppositional, hyperactivity, and impulsivity symptoms (331). Since polymorphisms in *Dat1* make it a strong candidate gene for both ADHD and its treatment and is the gene that codes for DAT their finding of less methylation being associated with greater response to MPH treatment would be consistent with this, given that greater promoter sequence methylation is generally associated with lower gene expression (309, 332-334).

Our group is the first to investigate the ability of MPH to alleviate the behavioral deficits associated with developmental Mn exposure (20-22). Male Long-Evans rats exposed to an environmentally relevant oral dose of 0 or 50 mg Mn/kg/day from PND 1-145 and treated with oral MPH administration (2.5 mg/kg/day, ~1 hr prior to testing, 16 days) were alleviated of Mn exposure induced sensori-motor deficits.(20). Additionally, by using a 5-choice serial reaction time attention task we found that MPH treatment 20 minutes prior to testing alleviated the impulse control deficits, but not the attentional deficits caused by developmental Mn exposure (22). Here in Chapter 3, I investigate how MPH treatment affects prefrontal cortex gene expression.

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Chapter 2

DEVELOPMENTAL MANGANESE EXPOSURE CAUSES LASTING ATTENTION DEFICITS ACCOMPANIED BY DYSREGULATION OF MTOR SIGNALING AND CATECHOLAMINERGIC GENE EXPRESSION IN PREFRONTAL CORTEX

2.1 Abstract

Elevated manganese (Mn) exposure is associated with attentional deficits in children, and is an environmental risk factor for attention deficit hyperactivity disorder (ADHD). We have shown that developmental Mn exposure causes lasting attention and sensorimotor deficits in a rat model of early childhood Mn exposure, and that these deficits are associated with a hypofunctioning catecholaminergic system in the prefrontal cortex (PFC), though the mechanistic basis for these deficits is not well understood. To address this, Male Long-Evans rats were exposed orally to Mn (50 mg/kg/d) over PND 1-21 and attentional function was assessed in adulthood using the 5-Choice Serial Reaction Time Task. Targeted catecholaminergic system and epigenetic gene expression, followed by unbiased differential DNA methylation and gene regulation expression transcriptomics in the PFC were performed in young adult littermates. Results show that developmental Mn exposure causes lasting focused attention deficits that are associated with reduced gene expression of tyrosine hydroxylase, dopamine transporter, and DNA methyltransferase 3a. Further, developmental Mn exposure causes broader lasting methylation and gene expression dysregulation associated with epigenetic regulation, inflammation, cell development, and hypofunctioning catecholaminergic neuronal systems. Pathway enrichment analyses uncovered mTOR and Wnt signaling pathway genes as significant

transcriptomic regulators of the Mn altered transcriptome, and Western blot of total, C1 and C2 phospho-mTOR confirmed mTOR pathway dysregulation. Our findings deepen our understanding of the mechanistic basis of how developmental Mn exposure leads to lasting catecholaminergic dysfunction and attention deficits, which may aid future therapeutic interventions of environmental exposure associated disorders.

2.2 Significance Statement

Attention deficit hyperactivity disorder (ADHD) is associated with environmental risk factors, including exposure to neurotoxic agents. Here we used a rodent model of developmental manganese (Mn) exposure producing lasting attention deficits to show broad epigenetic and gene expression changes in the prefrontal cortex, and to identify disrupted mTOR and Wnt signaling pathways as a novel mechanism for how developmental Mn exposure may induce lasting attention and catecholaminergic system impairments. Importantly, our findings establish early development as a critical period of susceptibility to lasting deficits in attentional function caused by elevated environmental toxicant exposure. Given that environmental health threats disproportionately impact communities of color and low socioeconomic status, our findings can aid future studies to assess therapeutic interventions for vulnerable populations.

2.3 Introduction

Attention and impulse control disorders, such as Attention Deficit Hyperactivity Disorder (ADHD), are the most prevalent neurodevelopmental disorders in children, affecting ~6-11% of all U.S. children aged 6-17 years (1). Elevated

environmental exposure to manganese (Mn) from contaminated well-water, ferroalloy industry emissions, and fungicide/pesticide use is associated with ADHD-like symptoms in children (2-18). The primary brain regions known to regulate attentional function are the prefrontal cortex (PFC) and striatum of the fronto-cortico-striatal brain circuit 19-21). The fronto-cortico-striatal brain circuit is believed to regulate attention through the catecholaminergic system, which is composed of neurotransmitters and receptors, such as dopamine and norepinephrine (19-21). Animal model studies have been important in establishing a link between developmental Mn exposure, lasting impairments in attention, impulse control, and sensorimotor function, and lasting hypofunction of the PFC and dorsal striatum catecholaminergic system (22-26). These Mn-associated effects include altered tyrosine hydroxylase (TH), dopamine transporter (DAT), dopamine D1 and D2 receptors (D1R, D2R), and norepinephrine transporter (NET) protein levels, and decreased K^+-evoked release of dopamine and norepinephrine (22, 27-29) in fronto-cortico-striatal brain regions. However, the molecular mechanisms leading to these developmental Mn-induced catecholaminergic system changes are not well-understood.

Mn-induced alterations of the epigenome and transcriptome may occur due to the role of Mn as a pro-oxidant that can increase oxidative stress levels (30-32). Increased oxidative stress can increase DNA oxidation (e.g., as 8-OHdG and 5-hmc) to induce DNA hypomethylation, or produce elevated prooxidants that can alter DNA methyltransferase (DNMT) protein expression and form DNMT-containing complexes that facilitate DNA hypermethylation (30-32). It is known that DNA methylation of

cytosine residues by DNMTs and histone deacetylation by hypoacetylhistone deacetylases (HDACs) regulate expression of key catecholaminergic genes TH, D1R, D2R, DAT, and NET (32-49). However, there is limited data on whether developmental Mn exposure may alter the epigenetic profile of catecholaminergic system genes (49-53). One example is that male mice exposed to perinatal Mn had hypermethylation of 24 gene promoter regions and transcript downregulation lasting into adulthood (51).

Recent evidence suggests that dysregulation of the neurodevelopmental signaling pathways mammalian/mechanistic target of rapamycin (mTOR) and Wingless-Int (Wnt) may lead to a hypofunctioning catecholaminergic system and an ADHD-like behavioral phenotype similar to that of our developmental Mn exposure rat model (54-61). Inhibition of Wnt signaling can cause hypofunctioning catecholaminergic system dysfunction similar to our Mn phenotype as Wnt inhibition, for example, has been shown to decrease dopaminergic cell differentiation, myelination, dendrite morphogenesis, and activate mTORC1 signaling – and persistently elevated mTORC1 signaling blocks canonical D1R signaling that is dependent on DARPP-32 (dopamine- and cAMP-regulated neuronal phosphoprotein) (54-58). Further, mTORC1 activation can alter DNA methylation gene expression regulation through regulating DNMT1 and DNMT3A gene and protein expression (59-61). However, it remains unclear whether developmental Mn exposure may alter DNA methylation of mTOR and Wnt pathway genes, and whether these changes are associated with altered catecholaminergic system gene and protein expression levels.

Here, we sought to determine whether lasting attention deficits caused by developmental Mn exposure are associated with DNA methylation and gene expression changes in PFC genes in categories of epigenetic regulation, inflammation, cell development, and catecholaminergic system functions, including the Wnt and mTOR signaling pathways. Given that our observed Mn exposure associated deficits persist into adulthood long after developmental Mn exposure has ended, we postulate that developmental Mn exposure creates lasting epigenome and gene expression changes of key PFC catecholaminergic and broader neuronal system functions. Male Long-Evans rats were exposed orally to Mn over PND 1-21 and attentional function was tested in adulthood using the 5-Choice Serial Reaction Time Task. Targeted gene expression analyses of the catecholaminergic system and epigenetic regulation genes, followed by unbiased differential DNA methylation, transcriptomics, and Western blot analyses were performed in adult littermates. Our findings show that developmental Mn exposure causes lasting attention deficits that are associated with differential methylation and gene expression of inflammation, epigenetic regulation, cell development, and hypofunctioning neuronal systems. Furthermore, mTOR and Wnt pathways are identified as causal and upstream regulators of our Mn altered transcriptome, and altered mTOR pathway function was evidenced by increased mTORC1 protein levels. Our findings deepen our understanding of mechanisms that underly our lasting developmental Mn exposure hypofunctioning catecholaminergic system and attention deficits. These findings suggest altered mTOR signaling as a

potential target of therapeutic interventions for environmental exposure associated neurological disorders.

2.4 Methods

2.4.1 Subjects

Subjects were 128 neonate male Long-Evans rats born in-house from nulliparous timed-pregnant females (from Charles River gestational day 18). 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. Rats (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 the rats designated for behavioral testing were pair-housed with an animal of the same 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 rats' diurnal cycle. Littermates that were designated for molecular analysis were grouped by treatment and fed *ad libitum* until PND 66 when sacrifice and brain tissue collection was performed for molecular analysis.

Males were exclusively used because attentional dysfunction is two to three times more prevalent in boys than girls (62-63), and because our prior studies have established that early postnatal Mn exposure causes lasting impairments in learning, attention, and fine motor function in male rats (22-29). All animal procedures were

approved by the institutional IACUC (protocols Smithd1803) 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 rats from the study were based on overt signs of poor rat health, including loss of body weight, absence of grooming, impaired function, and death; no rats were excluded from the study based on these criteria. This study was not pre-registered.

2.4.2 Manganese dosing regimen

Neonatal rats were orally exposed to a Mn dose of 50 mg Mn/kg/day starting on PND 1 through weaning on PND 21 (early postnatal Mn exposure) (Supplemental Methods 2.2). This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water and/or diet; pre-weaning exposure to 50 mg Mn/kg/day produces a relative increase in Mn intake about the level of increase reported in infants and young children exposed to Mn-contaminated water or soy-based infant formulas (22-29).

2.4.3 Focused attention behavioral testing

The 5-Choice Serial Reaction Time Task (5-CSRTT) was used to assess attentional function as previously described (24-26). Briefly, rats began testing at about PND 45 (60-62 rats/treatment), with food magazine and nose-poke training for 1 week, followed by two 5-choice visual discrimination tasks using fixed cue durations of 15 and 1 second, respectively. Once the animals attained 80% correct on two of three successive sessions in the visual discrimination tasks, they moved on to the focused

attention tasks. Two successive focused attention tasks were used to assess the ability of the rats to detect and respond to a brief visual cue presented unpredictably in time and location (i.e., one of the five response ports). The first focused attention task was administered over PND 73–86 and used pre-cue delays of 0, 1, 2, and 3 seconds. The second and more challenging focused attention task was conducted over PND 88–102, and used pre-cue delays of 0, 3, 4, and 5 seconds (Supplemental Methods 2.3). All rats were weighed and tested 6 days/week throughout training and testing. Behavioral assessment occurred during the active (dark) period of the diurnal cycle at the same time each day and in the same operant chamber for each individual rat. All behavioral testing was conducted by individuals blind to the treatment condition of the subjects. All rats were maintained on a food restriction schedule with water available *ad libitum* throughout behavioral assessment, as described previously (23-26).

2.4.4 Targeted catecholamine and epigenetic system gene expression

Gene expression of key catecholaminergic (*Th*, *Dat*, *Drd2*) and epigenetic modulator genes (*Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Hdac3*, and *Hdac4*) was determined through RT-qPCR on PFC tissue from PND 66 control and developmental Mn-exposed littermates of the behaviorally tested rats (6-7 rats/treatment). This age was selected because it coincides with the age at the start of behavioral testing in the focused attention tasks (22-29). Briefly, partially thawed fresh-frozen PFC tissue punches (1.39 mm thickness, 1.5 mm diameter, 25-30 mg) were taken from the anterior cingulate cortex/prefrontal cortex area (Paxinos and Watson Rat Brain Atlas: 2.16-0.77 mm anterior to bregma) over dry-ice and underwent Dounce Homogenization and total

RNA extraction, DNAse treatment, and Reverse Transcription. RT-qPCR cycle quantity thresholds were measured using the ThermoFisher Scientific TaqMan Advanced Master Mix (Applied Biosystems: Cat. #4444556) and TaqMan primers on the Bio-Rad CFX95 instrument (Supplemental Table 1) following the manufacturer's protocol. *Gapdh*, *ActB*, and *Ubc* expression were assessed as reference genes and the tool Normfinder confirmed the most stable reference gene combination for gene expression analysis (64-65). RT-qPCR was performed with two RT reaction replicates per animal, and three replicates per RT for each animal (66) (Supplemental Methods 2.4).

2.4.5 Differential DNA methylation and functional pathway analysis

We conducted reduced representation bisulfite sequencing (RRBS) to determine whether developmental Mn exposure led to lasting alterations of genome methylation status that may associated with the Mn-induced hypofunctioning catecholaminergic system identified in our prior studies (22-29). DNA was extracted from the same PND 66 fresh-frozen PFC samples detailed in Section 2.4, bisulfite converted, and sequenced at the University of California Davis Genome Center (n=3 rats/treatment). Galaxy pipeline analysis was conducted to obtain differentially methylated regions (DMRs) between control and Mn groups. Significant DMRs (unadjusted and adjusted $p<0.05$) were annotated by chromosome to the rat (rn6) genome, and then visualized and manually confirmed using the Integrative Genomics Viewer (IGV) sliding window (67). DMR fold-change values were calculated for each differentially methylated gene as the difference of the mean methylation value of the

control versus Mn PFC samples. All significant ($p<0.05$) DMR genes were then ranked and analyzed using Fast Preranked Gene Set Enrichment Analysis (FGSEA) to determine the associated functions of the differentially methylated gene products through Gene Ontology biological processes (68). Gene Ontology biological processes groups were further reduced, filtered, and visualized using REVIGO ontology's algorithm for semantic similarity of parent and child terms (69). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were conducted on all significant DMR associated genes ($p < 0.05$ unadjusted) using EnrichR (70) (Supplemental Methods 2.5).

2.4.6 Differentially expressed genes and functional pathway analysis

The ability of developmental Mn exposure to alter global gene expression was determined by 3' Tag RNA-sequencing. RNA aliquots from the same PND 66 PFC RNA extraction in Method 2.4 (n=5-6 rats/treatment) were analyzed for Bioanalyzer quality assessment, library preparation, and 3' Tag RNA-sequencing by the University of California Davis Genome Center. FastQ files were processed using established Geneious Prime RNA-sequencing tools (Geneious Prime 2022.0). DESeq2 was used to calculate differentially expressed gene (DEG) values between control and developmental Mn-exposed PFC brain samples (71). Next, all DEGs were ranked and analyzed using Fast Preranked Gene Set Enrichment Analysis (FGSEA) to determine the DEG's Gene Ontology-associated biological processes, molecular functions, and cellular components subtypes. Gene Ontology biological processes groups were further reduced, filtered, and visualized using REVIGO's algorithm for semantic similarity of

parent and child terms (69). The reduced Gene Ontology terms were then categorized into the four functional categories of inflammation, epigenetics, cell development, and neuronal function, based on our *a priori* hypotheses and known mechanisms of Mn neurotoxicity (22, 27-30, 49-52, 72). KEGG analysis was conducted on all significant DMR associated genes ($p < 0.05$ unadjusted) using EnrichR (70)(Supplemental Methods 2.6).

2.4.7 Differential methylation and differential expression integration

Integrated analysis of DMR and DEG data was conducted to determine the Gene Ontology biological processes associated with both differential methylation and differential gene expression of our Mn neurotoxicity attention deficit phenotype. We used the Genomics Tools Venn-Diagram web program and REVIGO to compare and visualize the upregulated and downregulated Gene Ontology biological processes group lists shared by both DMR associated genes and DEGs, and the four *a priori* hypothesized categories of biological dysfunction (inflammation, epigenetics, cell development, and neuronal function) described in Methods 2.5. Genomics Tools Venn-Diagram web program comparison was performed on the differentially methylated and expressed genes to further determine genes that were both differentially methylated and differentially expressed. Clustergrammer was then used to visually display DMR and DEG expression levels, and the gene region locations of the DMRs (e.g., promoter, exon, intron, etc.) were determined using the UCSC Genome Integrator and IGV slide sorting noted in Methods 2.5.

Ingenuity Pathway Analysis (IPA) was performed at the UCLA Technology Center for Genomics & Bioinformatics using all p-unadjusted DEGs from DESeq2 to identify significant causal and upstream regulator genes. Genes that were identified as upstream and causally-related to the DEGs were then integrated with the differentially methylated and expressed genes using the Genomics Tools Venn-Diagram web program comparison to identify genes that may be mechanistically associated with the Mn-neurotoxicity DEG phenotype. Finally, in order to further strengthen our assessment of the significance of the identified causal and upstream regulators, we compared the DMR and DEG lists to the most significant activation value regulator Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) gene interaction pathway lists (73)(Supplemental Methods 2.7).

2.4.8 Total and phosphorylated mTOR protein levels

Western blots were conducted for total mTOR, mTORC1, mTORC2, and β-Catenin protein levels to determine whether developmental Mn exposure led to lasting dysfunction of the mTOR and Wnt signaling pathway. Briefly, protein was extracted from PFC brain punches of PND 66 control and Mn rats, homogenized, and protein lysates were prepared for Western blot analysis (Supplemental Table 2 for antibodies). Western blot band densitometry was quantified using Bio-Rad Image Lab volume quantification software (5-6 rats/treatment) (Supplemental Methods 2.8).

2.4.9 Mn Blood levels

Blood Mn concentrations were measured in PND 24 littermates and the same PND 66 rats used in the reported molecular outcomes using methods previously

reported (n=7 rats/treatment/age) (22-29). Mn levels were determined using a Thermo Element XR inductively coupled plasma–mass spectrometer. The analytical detection limit for Mn in blood was 0.04 ng/mL, respectively. (Supplemental Methods 2.9).

2.4.10 Statistical analysis

The behavioral data were modeled by way of structured covariance mixed models. Fixed effects included in the model were Mn exposure (two levels corresponding to the control and Mn-treated groups), pre-cue delay, cue duration, and session block depending on the outcome analyzed. The 12 day duration of testing for each focused attention task were divided into four 3-day test session blocks each. In all models, rat was the random effect to account for correlations within observations from the same animal. Statistical tests used a Kenward-Roger correction. Plots of residuals by experimental condition were used to examine the assumption of homogeneity. Additional random effects with high variance in the residuals across levels of the factor (e.g., session block) were added to achieve homogeneity. The distribution of each random effect was inspected for approximate normality and presence of outliers. The significance level was set at $p \leq 0.05$. Significant main effects or interaction effects were followed by single-degree of freedom contrasts to clarify the nature of the interactions, using the Student's t-test for pairwise comparisons of least squared means. All behavioral data analyses were conducted using SAS (version 9.4) for Windows. RT-qPCR gene expression data statistical analyses were performed on ΔCt values by non-parametric Wilcoxon rank-sum/Kruskall Wallis test analysis and $p \leq 0.05$ deemed significance (74). Western blot protein expression data were analyzed by non-

parametric Wilcoxon rank-sum/Kruskall Wallis test to compare two paired groups, with significance set at $p \leq 0.05$. Mn blood levels were analyzed by ANOVA and Tukey-Post hoc analysis to establish $p \leq 0.05$ statistical significance. These latter analyses were performed using JMP Pro (version 16.1.0; SAS Institute, Inc.).

2.5 Results

2.5.1 Developmental Mn exposure impairs focused attention

We performed two successive focused attention tasks using the 5-CSRTT to demonstrate that developmental Mn exposure causes lasting deficits in attentional function in these animals, consistent with our prior studies (25-26). Focused attention can be defined as the ability to maintain attentional focus on an impending visual cue, in the face of unpredictable delays between trial onset and presentation of the visual cue (25). The first focused attention task 1 (FA1) used variable pre-cue delays of 0, 1, 2, or 3 and a fixed visual cue duration of 0.700 sec (See Methods 2.3.). The analysis of percent accurate response revealed a significant interaction between Mn exposure and testing session block ($F(3, 269.5)=4.33$, $p=0.005$). Specifically, the Mn group performed significantly worse than controls across all four test session blocks (p's = 0.035, 0.002, <0.001, and < 0.001 for session blocks 1-4, respectively) (Figure 1A). Also, both control and Mn animals showed improvement in attentional accuracy over the four test session blocks (3 days/session block); however, control animals improved more over testing than the Mn animals, based on within treatment group contrasts across session blocks (Figure 1A). In the second focused attention task, which is more challenging with variable longer pre-cue delays of 0, 3, 4, or 5 sec and visual cue

durations of 0.400 or 0.700 sec, we found that the Mn-exposed animals continued to exhibit lower attentional accuracy compared to controls, based on a significant main effect of Mn exposure ($F(1, 228.9)=9.7$, $p=0.002$) (Figure 1B). However, there was no higher order interaction between Mn and block ($F(3, 579.4)=1.73$, $p=0.1597$), or between Mn and pre-cue delay ($F(3, 508)= 0.50$, $p=0.685$). Altogether these results establish that developmental Mn exposure causes lasting deficits in attention, consistent with our previous published studies (22, 25-26).

2.5.2 Developmental Mn exposure alters expression of key catecholaminergic system and epigenome associated genes

To determine whether developmental Mn exposure leads to lasting changes in the expression of key catecholaminergic system genes that are known to be epigenetically regulated, and that code for proteins known or suspected to be altered by Mn exposure and involved in attentional function (22, 27-29, 32-49), we conducted RT-qPCR gene expression analysis on *Th*, *Dat*, and *Drd2* and their known epigenetic regulators *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Hdac3*, and *Hdac4* in the prefrontal cortex (PFC) of PND 66 littermates to the behaviorally-tested animals. Results show that developmental Mn exposure causes lasting ~50% and ~83% reductions in *Th* and *Dat* gene expression, respectively, relative to controls (p's =0.05 and 0.03), while *Drd2* gene expression was unaffected (p=0.25) (Figure 2). Developmental Mn exposure also decreases *Dnmt3a* gene expression by 31% (p=0.05), but does not significantly change *Dnmt1*, *Dnmt3b*, *Hdac3*, or *Hdac4* gene expression levels (p's = 0.10, 0.20, 0.32, and 0.31, respectively) (Figure 2). These findings establish developmental Mn exposure

causes lasting alteration in gene expression of key catecholaminergic system genes, consistent with our prior studies showing changes in catecholaminergic system proteins and a potential role for epigenetic mechanism underlying our Mn exposure associated attentional deficits (22, 27-29).

2.5.3 Developmental Mn exposure leads to global hypermethylation of genes associated with inflammatory, epigenetic, cell development, and neuronal system changes in the PFC

We conducted reduced representation bisulfite sequencing (RRBS) to determine whether developmental Mn exposure leads to lasting alterations of PFC genome methylation status that may contribute to the Mn-induced hypofunctioning catecholaminergic system. Overall, Mn exposure induces differentially methylated regions (DMRs) associated with 4250 genes (p-unadjusted < 0.05), of which ~83% (i.e., 3541/4250) are hypermethylated and 17% (709/4250) hypomethylated. After adjusting for multiple comparisons, 1027 genes remain hypermethylated and 192 genes remain hypomethylated (p-adjusted < 0.05) (Figure 1A; Supplemental Sheet 1). In regard to our hypothesis that developmental Mn exposure causes lasting disruption of epigenetic and catecholaminergic system gene methylation state, specifically, we found that the epigenetic modulators DNA methyltransferase (1, 3a, and 3b) and histone deacetylase (4 and 7) are hypermethylated, while histone deacetylase 3 is hypomethylated (Supplemental Sheet 1). Moreover, several catecholaminergic system and Mn transport-related genes are differentially methylated, including hypermethylation of dopamine receptor D4 (*Drd4*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta (*Ywhaz*),

catechol-O-methyltransferase (*Comt*), solute carrier family 30 member 10 (*Slc30a10*), and protein phosphatase 1 regulatory inhibitor subunit 1B (*Ppp1r1b*/DARPP-32), and hypomethylation of 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*Ywhag*) and GABA transporter 1 (*Slc6a1*) (p-adjusted $p<0.05$) (Supplemental Sheet 1).

Gene Ontology biological processes analyses were conducted on all significant 4250 DMR genes ($p < 0.05$ unadjusted) to elucidate the overall biological processes associated with our Mn-induced differentially methylated genes. The results identify 814 hypermethylated and 239 hypomethylated Gene Ontology terms ($p < 0.05$), many of which align with our *a priori* hypothesized mechanistic categories of Mn neurotoxicity (i.e., alteration of inflammation, epigenetic regulation, cell development, and neuronal function-related processes) (Figure 1B; Supplemental Sheet 2). Of particular relevance to pathways known to regulate catecholaminergic system function is the identification of the hypermethylated *(GO:0060070) canonical Wnt signaling pathway* and the hypomethylated *(GO:0032008) positive regulation of TOR signaling* (Figure 1B). Subsequent KEGG pathway analysis revealed that the differentially methylated genes are associated with neurodegenerative disease and that the *mTOR signaling pathway* is a top hypermethylated pathway caused by developmental Mn exposure (Supplemental Figure 1).

2.5.4 Developmental Mn exposure induces lasting gene expression changes associated with altered inflammatory, epigenetic, cell development, and neuronal system changes in the PFC

To assess whether broader gene expression changes could be associated with the differential methylation changes noted above and inform the mechanistic basis for the lasting deficits in attentional function, we conducted RNA-seq analysis on PFC brain tissue. Findings show developmental Mn exposure leads to lasting differential expression of 501 (p-unadjusted < 0.05) or 27 (p-adjusted < 0.05) genes (Figure 4A; Supplemental Sheet 3). Of the 27 p-adjusted differentially expressed genes, 13 are upregulated and 14 are downregulated (Figure 4A). All 27 genes can be readily categorized into the four *a prior* hypothesized biological function categories identified in the differential methylation analyses above using the Rat Genome Database, as follows: inflammation (eight genes), epigenetics (five genes), cell development (four genes) and neuronal function (10 genes) (Figure 4A). Notably, several of the p-adjusted differentially expressed genes are associated with catecholaminergic function and Wnt and mTOR signaling pathways. Specifically, the encoded proteins of *Crebbp*, *Prkacb*, *Gabarapl1*, and *Ywhaz* are known regulators and intermediates of the Wnt and mTOR pathway regulating neuronal cell proliferation and differentiation, and specifically, their gene and protein expression may alter dopamine synthesis, release, and transport pathways (Supplemental Sheet 4).

Gene Ontology biological processes enrichment analysis was conducted to gain further insight into broader gene expression pathway changes caused by developmental Mn exposure. Results identify 192 down- and 211 up-regulated (p-unadjusted < 0.05) Gene Ontology biological processes terms (Supplemental Sheet 5). These Gene Ontology terms were further refined, filtered, and visualized using REVIGO, leading

to identification of 69 down- and 63 up-regulated terms that were then categorized into the four *a priori* hypothesized mechanistic categories noted above (i.e., inflammation, epigenetic regulation, cell development, and neuronal function), and their respective leading edge contributing genes as defined by Gene Ontology enrichment analysis and fold-changes were determined. Consistent with our hypothesis, one notable neuronal function term of relevance that has genes with reduced expression is *(GO:0033605) positive regulation of catecholamine secretion*, while cell development terms with genes with increased expression are *(GO:0031929) TOR signaling* and *(GO:0030178) negative regulation of Wnt signaling* (Figure 4B, C; Supplemental Figure 4). Additional Gene Ontology molecular function and cellular component and KEGG pathway analyses illustrates that developmental Mn exposure is associated with lasting proinflammatory and hypofunctioning neuronal systems (Supplemental Figure 2, 3). Overall, the Gene Ontology differential gene expression analysis further supports a significant pattern of mTOR, Wnt, and hypofunctioning neuronal pathways caused by developmental Mn exposure.

2.5.5 Integration of Mn induced differential methylation and gene expression further supports mTOR and Wnt pathway dysregulation is associated with hypofunctioning neuronal systems

We conducted an integrated analysis of the Gene Ontology terms shared by both differentially methylated and differentially expressed genes to evaluate whether there were altered neuronal functions that may inform our Mn attentional deficit phenotype. We found that 27% (41/148) of our downregulated gene expression-associated Gene Ontology terms are hypermethylated, while about 7% (14/204) of our upregulated Gene

Ontology terms are hypomethylated (Figure 5A). In a venn diagram comparison of all significant Gene Ontology terms we see that the *(GO:0032006)* regulation of *mTOR signaling* and *(GO:0035567) non-canonical Wnt signaling* pathways are present in both hypermethylated and hypomethylated Gene Ontology term lists and the Gene Ontology term *(GO:0010467) gene expression* is present in the hypermethylated, gene expression upregulated, and gene expression downregulated Gene Ontology term lists (Figure 5B). Overall, DMR and DEG integrated Gene Ontology analysis shows that developmental Mn exposure is associated with mTOR and Wnt signaling dysregulation that is also associated with genes and pathways that regulate gene expression, as determined by the shared Gene Ontology terms.

Subsequently, we assessed which genes of our DMR and DEG analysis were both differentially methylated and expressed and we found that developmental Mn exposure leads to the lasting differential methylation and expression of 155 genes in the PFC ($p < 0.05$-adjusted) (Figure 5C, D; Supplemental Sheet 6). Notably, of these 155 genes, 85% are differentially methylated within their promoter region, while the remaining 15% are differentially methylated across various exons or their terminator region (Supplemental Sheet 6). Gene Ontology biological function, molecular function, and cellular components analysis of the 155 differentially methylated and differentially expressed genes were easily categorized into the same inflammatory, epigenetic, cell developmental, and hypofunctioning neuronal pathways functional categories described above (Figures 3, 4; Supplemental Figure 5). Also of note is that subsequent KEGG pathway analysis using the 155 differentially methylated and expressed genes

identified the *mTOR signaling pathway* as the most significant upregulated term by p-value (p = 0.003), along with several downregulated neuronal pathways, such as the *GABAergic synapse* (Supplemental Figure 6). Altogether, the functions of the 155 differentially methylated and expressed genes support our hypothesis that developmental Mn exposure leads to lasting mTOR signaling dysregulation and hypofunctioning neuronal systems.

2.5.6 IPA causal and upstream regulator integration with DMRs and DEGs corroborate Wnt and mTOR pathways are novel mechanistic targets of developmental Mn exposure

We performed Ingenuity Pathway Analysis (IPA) on the 501 (p-unadjusted < 0.05) genes that emerged from the DESeq2 analysis reported above, in order to determine whether causal and upstream regulator gene(s) may help explain the lasting gene expression phenotype caused by developmental Mn exposure. IPA causal analysis reveals 447 (p-unadjusted < 0.05) significant genes, while IPA upstream analysis reveals 179 (p-unadjusted < 0.05) genes that are associated with (i.e., regulate) our transcriptomic findings (Supplemental Sheet 7, 8). Of these, 129 genes are identified as both upstream and causal regulator genes, and eight of these genes (*Dnajc5*, *Qki*, *Lrrc4*, *Immt*, *Chchd6*, *Gabbr2*, *Prkacb*, *Uchl3*) are themselves differentially expressed (p-unadjusted) (Figure 6B; Supplemental Figure 7). The top 15 significant causal and upstream regulator genes, based on their IPA-predicted activation value, are shown in Figure 6A, along with a brief description of their function. Notably, these regulator genes are also readily categorized within the four *a priori* hypothesized mechanistic categories of Mn neurotoxicity noted above (inflammation, epigenetic regulation, cell

development, and neuronal function), and they share several of the same Gene Ontology biological process and KEGG terms from the DMR and DEG analysis (Figures 3-5; Supplemental Figures 1-6).

It is highly noteworthy that the mTOR signaling pathway is consistently identified as being altered by, as well as mediating many of the lasting molecular changes caused by developmental Mn exposure, as it has the largest IPA activation value and a high frequency of interactions (8/15other top master regulators) defined in the gene interaction pathways (Figure 6A). The Ctnnb1/Wnt signaling pathway is also identified as a top regulator, with regulation interactions with several of the other top 15 regulators identified in the IPA analyses (Figure 6A). Together, the mTOR and Wnt master regulator gene interaction networks can account for (i.e., regulate) 15 and 32 of the 501 differentially expressed genes, respectively (Supplemental Figure 8).

Finally, to further strengthen our assessment of the significance of Wnt and mTOR regulatory pathways altered by developmental Mn exposure, we compared our lists of differentially methylated and expressed genes to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) gene interaction pathway lists for both mTOR and Wnt signaling pathways (Figure 6C, D). This analyses shows that, of the 4250 p-unadjusted differentially methylated genes, 50 genes were determined through STRING to be associated with mTOR signaling pathways and 69 genes were associated with Wnt signaling pathways (Figure 6C, D; Supplemental Sheet 9). In particular, five differentially expressed genes were associated with Wnt STRING genes and nine differentially expressed genes were associated with mTOR STRING genes

(Figure 6C, D; Supplemental Table 9). These results further highlight that developmental Mn exposure is associated with important lasting gene expression changes mediating neuronal function, particularly the Wnt and mTOR signaling pathways.

2.5.7 Developmental Mn leads to decreased total mTOR and increased mTORC1 protein levels in rat PFC, which establishes impaired mTOR signaling as a novel mechanistic target of Mn neurotoxicity

We conducted Western blot analysis for total mTOR, phosphorylated-mTORC1, phosphorylated-mTORC2, and β-Catenin (proxy for Wnt signaling) protein levels in brain PFC to determine whether the lasting differential methylation and gene expression changes caused by developmental Mn exposure reported above translated to functional dysregulation of the mTOR and Wnt signaling pathways. We found that developmental Mn exposure leads to a 28% decrease in total mTOR ($p = 0.008$) and a 51% increase in mTORC1 ($p = 0.036$), compared to controls; protein levels of mTORC2 ($p = 0.927$) and β-Catenin ($p = 0.936$) were unchanged (Figure 7). By assessing the protein levels of the main mTOR signaling pathway, we confirm that developmental Mn exposure causes lasting alterations to the mTOR signaling pathway in the PFC, which provides important insight into the molecular mechanisms of catecholaminergic hypofunctioning and attentional dysfunction in our animal model of childhood developmental Mn exposure and inattention.

2.5.8 Developmental Mn exposure increased blood Mn levels and these levels normalize by PND 66

Blood Mn analyses in control and developmentally Mn-exposed shows that, following weaning (PND 24) the oral Mn exposure regimen used here increased blood Mn to levels comparable to environmentally-exposed infants, and that by young adulthood (PND 66), blood Mn levels had returned to control levels. Specifically, our developmental Mn exposure regimen over PND 1 – 21 significantly increased blood Mn levels of PND 24 rats to ~496 ng/mL, compared to 24 ng/mL in controls ($p < 0.001$) (Supplemental Figure 9). Following cessation of Mn exposure on PND 21, blood Mn levels in the Mn group (15 ng/mL) declined to levels comparable to controls (11 ng/mL) in PND 66 animals. The data support that the attention deficits and associated molecular changes associated with Mn exposure described are due to lasting neurochemical changes caused by developmental Mn exposure, and cannot be attributed to elevated Mn levels concurrent with behavioral testing and molecular measurements.

2.6 Discussion

Elevated Mn exposure is associated with inattention, impulsivity, and psychomotor deficits in children, and is recognized as an environmental risk factor for ADHD (2-18, 75). Animal model studies have demonstrated that developmental Mn exposure can cause these ADHD-like attention deficit symptoms, which are associated with lasting hypofunctioning of the catecholaminergic system in the PFC and striatum (22-29). However, the molecular mechanism(s) that underlie these lasting Mn deficits remain poorly understood. To address this, we used a rat model of childhood environmental Mn exposure and lasting attentional dysfunction to investigate whether

Mn exposure leads to lasting alterations of PFC epigenomic and transcriptomic domains that may help explain the lasting catecholaminergic and attention deficits reported previously.

Here, we found that developmental Mn exposure causes lasting attentional deficits that are associated with reduced expression of the catecholaminergic system genes tyrosine hydroxylase and dopamine transporter, as well as the epigenetic regulator DNA methyltransferase 3a, in the PFC, the brain region mediating attentional function. These findings are consistent with our previous findings showing reduced tyrosine hydroxylase and dopamine transporter protein expression in Mn-exposed animals, while also opening up the possibility of epigenetic regulation mechanisms as an underlying feature of how developmental Mn may cause attention deficits that last into adulthood long after elevated exposure has ended (22). In gaining a broader understanding of how developmental Mn exposure alters the epigenome and transcriptome, our Gene Ontology results showed changes in the broader categories of inflammation, epigenetic regulation, cell development, and hypofunctioning neuronal systems. These findings support our *a priori* understanding of potential developmental Mn exposure mechanisms.

Upon investigating potential causal and upstream regulators of our transcriptome further our findings showed specific dysregulation of mTOR and Wnt signaling pathways. Although β-Catenin protein levels did not significantly change as expected from our IPA findings, the altered mTOR pathway protein levels we found confirm our hypothesis of altered mTOR signaling as an underlying mechanism of

developmental Mn associated catecholaminergic system hypofunction. Our findings corroborate the limited data on Mn and mTOR pathway activation; specifically, one prior rodent study found that low dose developmental Mn exposure increased mTORC1 protein expression (76).

The identification of altered mTOR signaling as a key upstream regulator that can account for, to a notable extent, our developmental Mn exposure phenotype of catecholaminergic gene and protein expression changes that are associated with attentional deficits is a significant finding of our study. mTOR functions as a core component of two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (78-79). These two complexes are known to regulate different neurodevelopmental processes, such as neuron size, dendritic growth and maturation, and axon elongation (80-81).

Our differential methylation, differential gene expression, IPA integration, and protein level findings provide evidence that mTORC1 is an activated IPA causal regulator of our Mn-exposure transcriptomic phenotype that also has increased protein expression. Prior studies have demonstrated that persistently elevated mTORC1 signaling blocks canonical D1R signaling, which has been shown to alter dopamine signaling pathway gene expression, cortico-striatal plasticity, and cognitive functions in mice (54-58). Furthermore, DA-Tsc1-KO mice that have a loss of tuberous sclerosis complex 1 (*Tsc1*) function, an upstream regulator of mTOR activity, leads to constitutive activation of mTORC1, striatal dopamine neuron somatodendritic hypertrophy, reduced intrinsic excitability, altered axon terminal structure, and reduced

dopamine release (82). These mice, interestingly, have increased tyrosine hydroxylase synthesis, but reduced dopamine release due to decreased vesicle function that is associated with reduced cognitive flexibility outcomes and in turn is rescued by genetic reduction in activated mTORC1 signaling (82). These published catecholaminergic system deficit findings contribute to our current understanding of activated mTORC1 signaling dysregulation as a mechanism of Mn-neurotoxicity, but additionally need the context of our additional mechanistic findings further discussed below to establish a mechanistic pathway on how developmental Mn can alter the catecholaminergic system to produce our observed catecholaminergic and attention deficit Mn exposure phenotype (22-29).

Although we were unable to detect a significant reduction in mTORC2 protein levels, our differential methylation, gene expression, and Ingenuity Pathway Analysis indicates inhibited mTORC2 signaling as a consequence of developmental Mn exposure. This predicted mTORC2 dysregulation is evident through Rictor, an essential component of the mTORC2 complex, measured here as hypermethylated and IPA predicted inhibited. Our findings of *Wnt*, *Gsk3b*, *Akt,* and *Tsc1-Tsc2* complex differential methylation and IPA dysregulation as well as *Tsc2* differential gene expression give evidence of dysregulated mTORC2 based on their respective pathway interactions (Figure 8) (89-92). Mice without functioning *Rictor* (nRictor KO) have demonstrated novelty-induced hyperactivity, decreased dopamine bioavailability, and elevated dopamine receptor D2 protein expression (83). These findings specifically mirror our previous findings that developmental Mn exposure significantly decreases

dopamine and norepinephrine evoked release, and that developmental Mn exposure increases dopamine receptor D2 protein expression levels in the prefrontal cortex by about 200% (22, 29). Interestingly, based on previous research (93-94), elevated dopamine receptor D2 protein expression and/or the inhibition of *Akt* caused by inhibited mTORC2 can contribute to our ~80% Mn-induced decrease in dopamine transporter gene expression (present study) and a similarly decreased protein expression findings shown in our prior studies (22).

We propose a potential mechanism of Mn neurotoxicity that can lead to decreased attentional function in our animal model that is based on our integration of our identified Mn exposure altered differentially methylated, expressed, causal, and upstream regulator genes with peer reviewed scientific literature (Figure 8). Briefly, Mn exposure may induce oxidative stress and DNA damage leading to altered epigenetic regulation of inflammatory response as well as direct change to Akt activity (Figure 8, Step 1-3) (30-32, 84-90). This altered regulation leads to reduced Wnt signaling and as a result increased mTORC1 and decreased mTORC2 regulation (Figure 8, Step 4-6) (56, 91-94). Altered mTORC1/mTORC2 impair the catecholaminergic system and perpetuate a continued inflammatory environment of activated glial cells reflecting our Mn-induced catecholaminergic system hypofunction and attention deficits (Figure 8, Step 4-6) (22-29, 54-58, 95-98). This activation of glial cells is consistent with our prior findings that developmental Mn exposure leads to lasting rat PFC GFAP and pro-inflammatory astrocyte activation (22).

In summary, our findings demonstrate that developmental Mn exposure causes lasting differential methylation and gene expression changes in inflammatory, epigenetic regulation, cell development, and hypofunctioning neuronal systems with reduced expression of the catecholaminergic system genes tyrosine hydroxylase and dopamine transporter, as well as DNA methyltransferase 3a, in the prefrontal cortex. Additionally, developmental Mn exposure causes lasting methylation, gene expression, and protein level dysregulation in mTOR and Wnt signaling pathways and these resulting dysregulations can induce lasting prefrontal cortex catecholaminergic system hypofunctioning, and may underlie or contribute to the lasting deficits in attention and impulse control reported here and in our prior studies (22, 27-29). Importantly, we establish early development as a critical period of susceptibility to lasting deficits in attentional function caused by elevated environmental toxicant exposure. Our findings deepen our understanding of the lasting epigenetic and gene transcriptomic changes caused by developmental Mn exposure, and how these changes may lead to lasting catecholaminergic system dysfunction. Specifically, our findings of altered mTOR pathway regulation may aid potential therapeutic studies on the environmental exposure contribution to neurological disease.

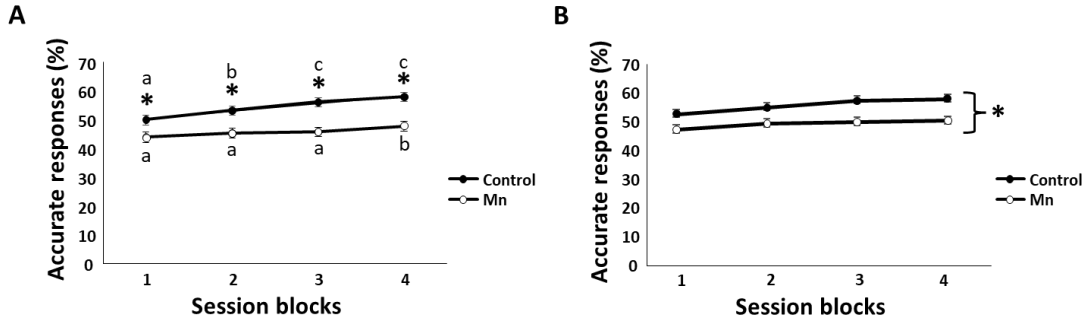
2.7 Acknowledgements

The broader experimental design of developmental Mn treatment, behavioral testing, MPH dosing, and use of antagonists were led by Donald Smith, Barbara Strupp, and Stephane Beaudin. In making this dissertation chapter the molecular approaches (transcriptomic, methylomic, and proteomic) experimental design was made primarily

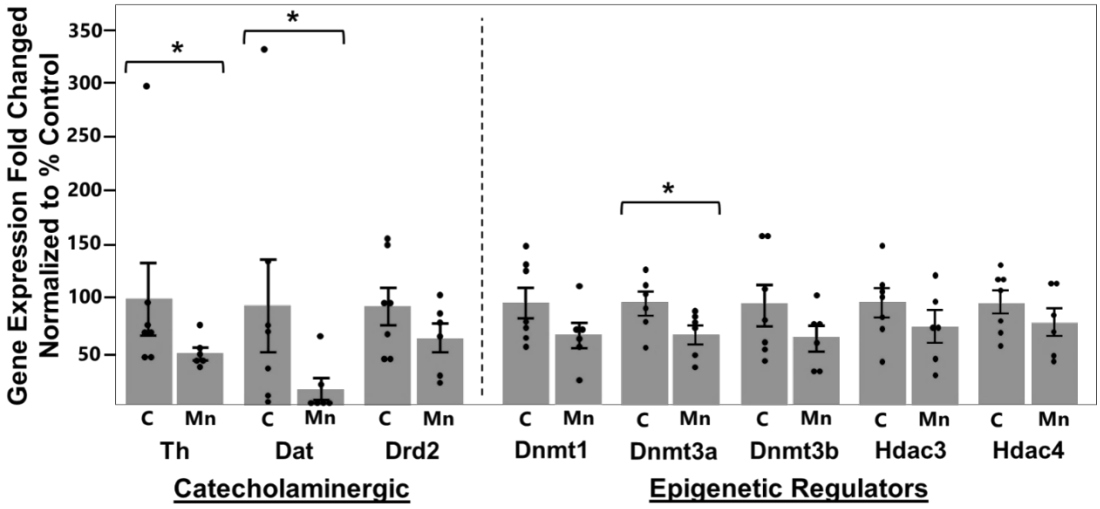
by Nicholas Santiago and Donald Smith. The writing was led by Nicholas Santiago and Donald Smith with feedback from Stephane Beaudin, Shanna Howard, and Barbara Strupp. The behavioral testing was led primarily by Stephane Beaudin, Donald Smith, Nicholas Santiago, and Shanna Howard with many helpful undergraduate student volunteers. The behavioral analysis and interpretation was led by Stephane Beaudin with feedback from Donald Smith, Barbara Strupp, and Nicholas Santiago. The methylation and gene expression tissue collection and extraction was led by Nicholas Santiago and Shanna Howard. The methylation and gene expression sequencing was done by UC Davis Genome Core. Ingenuity Pathway Analysis was conducted by UCLA Bioinformatic Core. The methylation and gene expression bioinformatics and interpretation was performed and interpreted by Nicholas Santiago with input from Donald Smith. The RT-qPCR was performed by Nicholas Santiago. The Western Blot analysis was performed by Nicholas Santiago and Alex He. Mn levels in blood were performed by Thomas Jursa. Funding was from the National Institutes of Environmental Health Sciences (#R01ES028369).

2.8 Figures

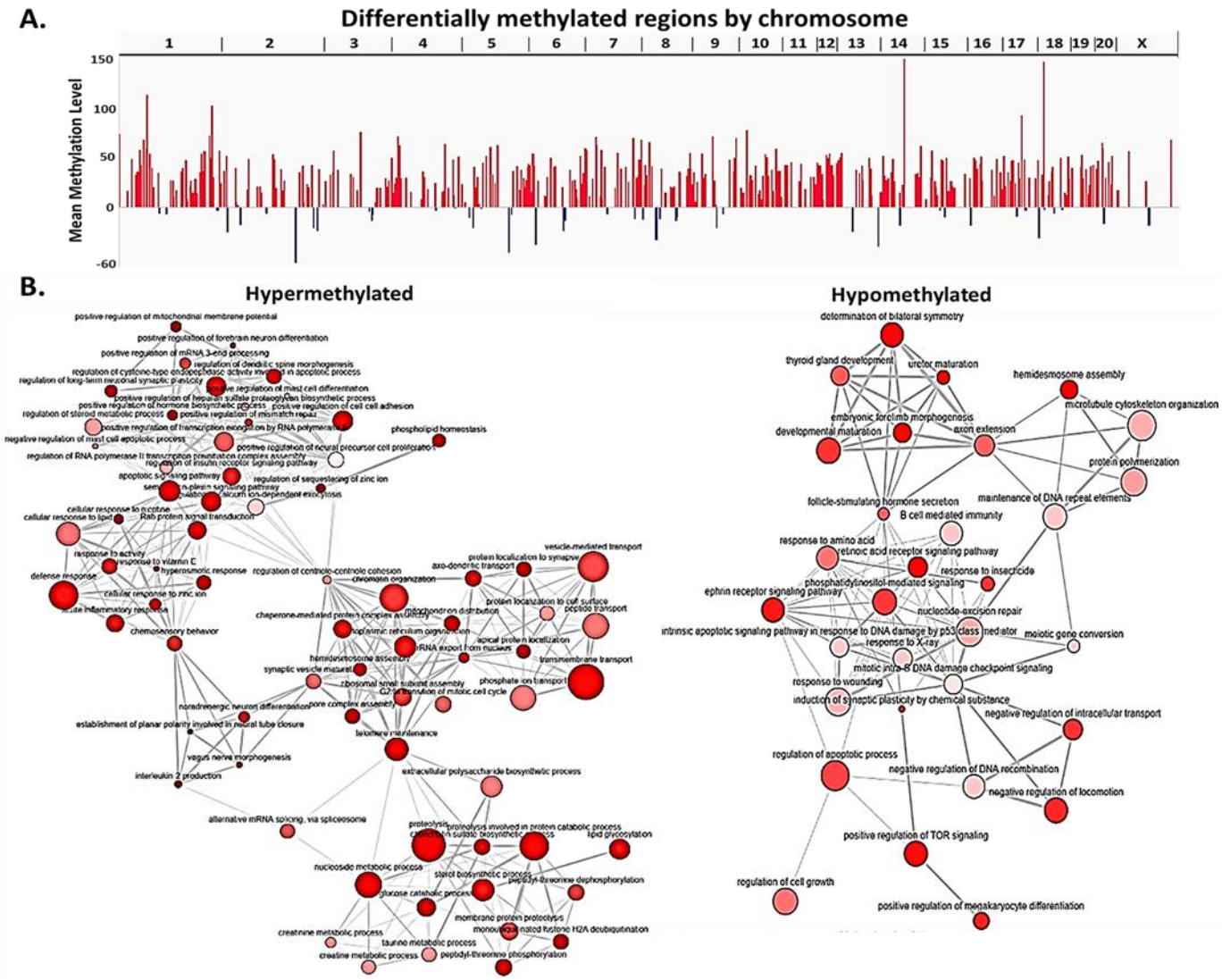
2.8.1 Figure 1: Developmental Mn exposure impairs focused attention. Percent accurate responses of the control and Mn groups in the first (A) and second (B) focused attention tasks as a function of testing session block (3 days of testing/block). * $p < 0.05$ versus controls in 'a' and 'b'. Letter superscripts in 'a' indicate within treatment group differences ($p < 0.05$) between session blocks; within treatment group, session blocks with different superscript letters are significantly different ($p < 0.05$). Data are lsmeans ± SEM (n=60-62/treatment).



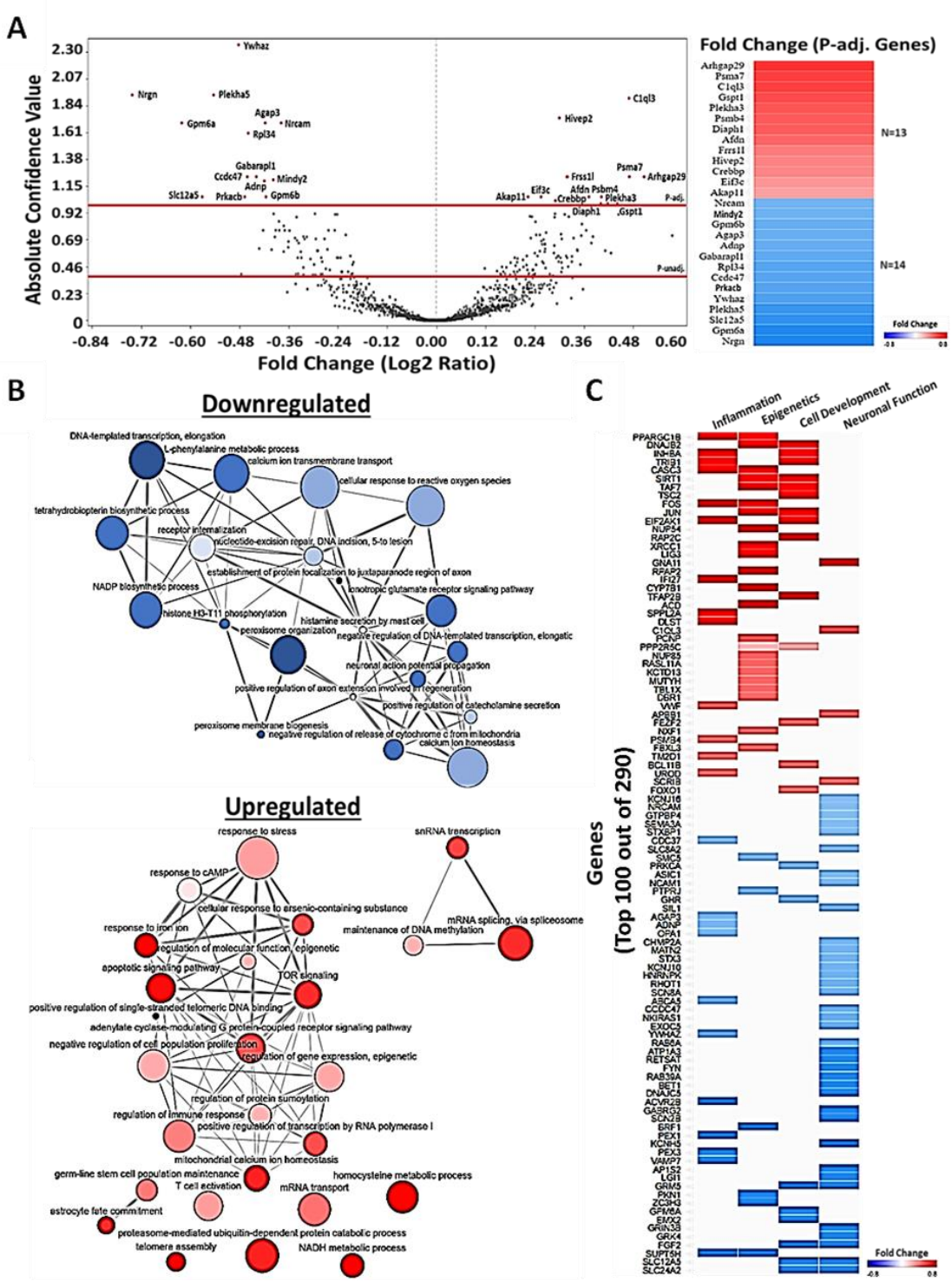
2.8.2 Figure 2: Developmental Mn exposure alters key catecholaminergic and epigenetic gene expression. PND 66 prefrontal cortex RT-qPCR gene expression results conducted on catecholaminergic and epigenetic modulator genes. Gene expression fold-change values, as % of control, were calculated using ΔΔCt values. Pair-wise statistical comparisons were performed on ΔCt values. * indicates significant $p<0.05$ versus control, based on non-parametric Wilcoxon analysis (n=6-7 rats/treatment).



2.8.3 Figure 3: Developmental Mn exposure leads to differential methylation in inflammatory, epigenetic regulation, cell development, and neuronal systems. (A). RRBS of PND 66 PFC tissue, all significant (p-adj.<0.05) differentially methylated regions. Red=hyper-/Blue=hypo-methylated, mean methylation levels. (B). All $p < 0.05$ unadjusted Gene Ontology term similarity connections maps. All circles are $p < 0.05$ significant GO terms. Smaller circle = smaller p-val/Darker = more child terms included in parent term. Thicker connecting line between terms indicates more child terms within that share a functional connection. Figure is shown on separate page below.

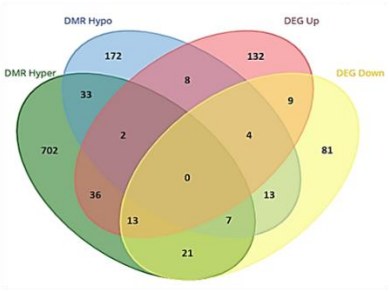

A.
Differentially methylated regions by chromosome
Mean Methylation Level
150
100
50
0
-60
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
X
B.
Hypermethylated
Hypomethylated
positive regulation of mitochondrial membrane potential
positive regulation of forebrain neuron differentiation
positive regulation of mRNA 3-end processing
regulation of dendritic spine morphogenesis
positive regulation of mast cell differentiation
positive regulation of heparan sulfate proteoglycan biosynthetic process
positive regulation of cell cell adhesion
positive regulation of mismatch repair
phospholipid homeostasis
negative regulation of mast cell apoptotic process
positive regulation of neural precursor cell proliferation
regulation of insulin receptor signaling pathway
regulation of sequestering of zinc ion
cellular response to lipid
Rab protein signal transduction
response to activity
response to vitamin E
hyperosmotic response
defense response
cellular response to zinc ion
chemosensory behavior
vesicle-mediated transport
protein localization to synapse
axo-dendritic transport
regulation of centriole-centriole cohesion
chromatin organization
protein localization to cell surface
peptide transport
chaperone-mediated protein complex assembly
mitochondrion distribution
endoplasmic reticulum organization
apical protein localization
mRNA export from nucleus
hemidesmosome assembly
transmembrane transport
synaptic vesicle maturation
phosphate ion transport
ribosomal small subunit assembly
cell cycle translation of mitotic cell cycle
nonadrenergic neuron differentiation
pore complex assembly
establishment of planar polarity involved in neural tube closure
telomere maintenance
vagus nerve morphogenesis
interleukin 2 production
extracellular polysaccharide biosynthetic process
alternative mRNA splicing, via spliceosome
proteolysis
proteolysis involved in protein catabolic process
lipid glycosylation
nucleoside metabolic process
sterol biosynthetic process
peptidyl-threonine dephosphorylation
glucose catabolic process
membrane protein proteolysis
creatinine metabolic process
monoubiquitinated histone H2A deubiquitination
taurine metabolic process
creatine metabolic process
peptidyl-threonine phosphorylation
determination of bilateral symmetry
thyroid gland development
ureter maturation
hemidesmosome assembly
microtubule cytoskeleton organization
embryonic forelimb morphogenesis
developmental maturation
axon extension
protein polymerization
follicle-stimulating hormone secretion
maintenance of DNA repeat elements
B cell mediated immunity
response to amino acid
retinoic acid receptor signaling pathway
response to insecticide
phosphatidylinositol-mediated signaling
ephrin receptor signaling pathway
nucleotide-excision repair
intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator
meiotic gene conversion
response to X-ray
mitotic intra-S DNA damage checkpoint signaling
response to wounding
induction of synaptic plasticity by chemical substance
negative regulation of intracellular transport
regulation of apoptotic process
negative regulation of DNA recombination
negative regulation of locomotion
positive regulation of TOR signaling
regulation of cell growth
positive regulation of megakaryocyte differentiation

2.8.4 Figure 4: Developmental Mn exposure induces lasting gene expression changes associated with altered inflammatory, epigenetic regulation, cell development, and neuronal system biological function in the PFC (A). Fold change expression levels of developmental Mn exposure induced differentially expressed genes (DEGs) are displayed as a volcano plot. Each red line is marked as the genes that are significant before and after multiple comparison adjustments. (B). The list of p-adjusted DEGs are displayed on the heatmap on the right by their respective fold changes. (B). Gene Ontology similarity connections maps are displayed by downregulated or upregulated genes that contributed to the Gene Ontology term results. All circles represent $p < 0.05$ GO terms. Smaller circle size=smaller p-value/Darker=more child terms that have been reduced to the parent term represented. Thicker connecting line between two Gene Ontology terms indicates more child terms within both terms. (C). A heatmap displays the leading edge DEGs and their log2 Fold Change levels of the categorized (inflammation, epigenetic, cell development, and neuronal function) Gene Ontology terms. Figure is shown on separate page below.


A
Absolute Confidence Value
Fold Change (Log2 Ratio)
Ywhaz
Nrgn
Plekha5
Agap3
Gpm6a
Nrcam
Rpl34
C1ql3
Hivep2
Gabarapl1
Ccdc47
Mindy2
Adnp
Slc12a5
Prkacb
Gpm6b
Frss1l
Psma7
Arhgap29
Afdn
Psbm4
Akap11
Eif3c
Crebbp
Plekha3
Diaph1
Gspt1
P-adj.
P-unadj.
Fold Change (P-adj. Genes)
N=13
N=14
Fold Change
B
Downregulated
DNA-templated transcription, elongation
L-phenylalanine metabolic process
calcium ion transmembrane transport
cellular response to reactive oxygen species
tetrahydrobiopterin biosynthetic process
receptor internalization
nucleotide-excision repair, DNA incision, 5-to lesion
establishment of protein localization to juxtaparanode region of axon
NADP biosynthetic process
ionotropic glutamate receptor signaling pathway
histone H3-T11 phosphorylation
histamine secretion by mast cell
peroxisome organization
negative regulation of DNA-templated transcription, elongatic
neuronal action potential propagation
positive regulation of axon extension involved in regeneration
positive regulation of catecholamine secretion
peroxisome membrane biogenesis
negative regulation of release of cytochrome c from mitochondria
calcium ion homeostasis
Upregulated
response to stress
snRNA transcription
response to cAMP
cellular response to arsenic-containing substance
response to iron ion
mRNA splicing, via spliceosome
maintenance of DNA methylation
regulation of molecular function, epigenetic
apoptotic signaling pathway
TOR signaling
positive regulation of single-stranded telomeric DNA binding
adenylate cyclase-modulating G protein-coupled receptor signaling pathway
negative regulation of cell population proliferation
regulation of gene expression, epigenetic
regulation of protein sumoylation
regulation of immune response
positive regulation of transcription by RNA polymerase I
mitochondrial calcium ion homeostasis
germ-line stem cell population maintenance
homocysteine metabolic process
T cell activation
mRNA transport
astrocyte fate commitment
proteasome-mediated ubiquitin-dependent protein catabolic process
telomere assembly
NADH metabolic process
C
Inflammation
Epigenetics
Cell Development
Neuronal Function
Genes
(Top 100 out of 290)
Fold Change

2.8.5 Figure 5: Mn exposure led to 155 PFC genes being differentially methylated and expressed in functions related to hypofunctioning neuronal systems. (A). Venn diagram of shared Gene Ontology groups between DMR hyper-and hypomethylated associated genes and DEG up-and downregulated genes. (B). Table of top relevant Gene Ontology groupings and their biological category significance. (C). DMR and DEG genes are compared to identify which genes are both differentially methylated and differentially expressed. (D). The 155 genes that are both DMR and DEG are displayed in a heatmap with their corresponding fold change values (See Supplemental Sheet 6 for clearer readability of genes in 'D').

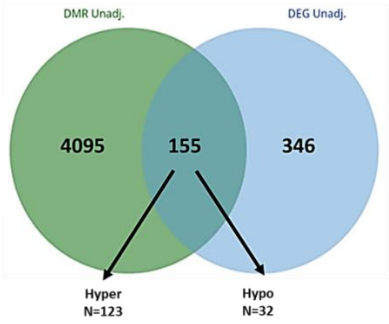
A.



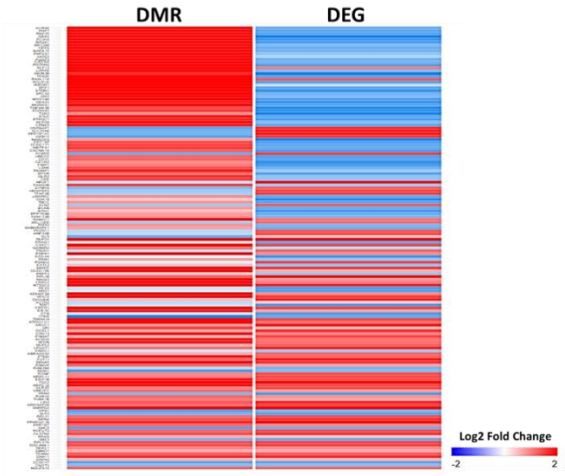
B.

Gene Ontology Name	Gene Grouping	Biological Function Category
Nervous system development	DMR Hyper/DEG Downregulated	Neuronal Function
Generation of neurons	DMR Hyper/DEG Downregulated	Neuronal Function
Axonogenesis	DMR Hyper/DEG Downregulated	Neuronal Function
Neurotransmitter transport	DMR Hyper/DEG Downregulated	Neuronal Function
DNA metabolic process	DMR Hypo/DEG Upregulated	DNA Damage and Epigenetics
Protein import into peroxisome membrane	DMR Hypo/DEG Upregulated	Inflammation and Oxidative Stress
Non-canonical Wnt signaling pathway	DMR Hyper/DMR Hypo	Cell Development
Regulation of TOR signaling	DMR Hyper/DMR Hypo	Cell Development
Cellular response to DNA damage stimulus	DMR Hyper/DMR Hypo	DNA Damage and Epigenetics
Gene expression	DMR Hyper/DEG Up/DEG Down	DNA Damage and Epigenetics

C.



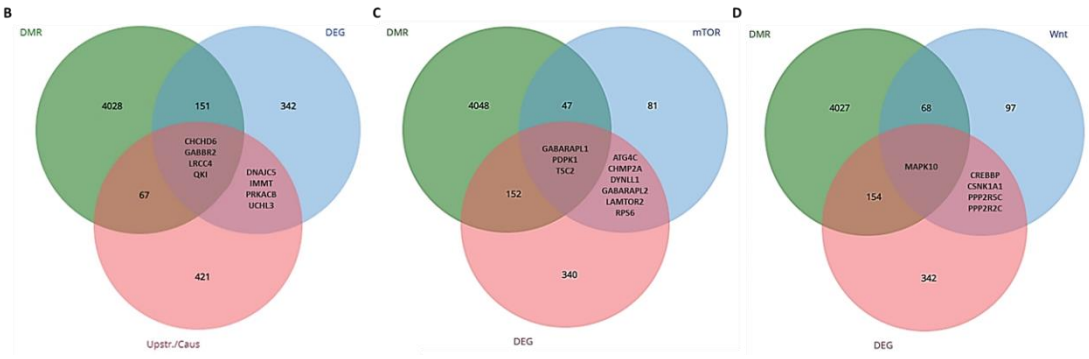
D.



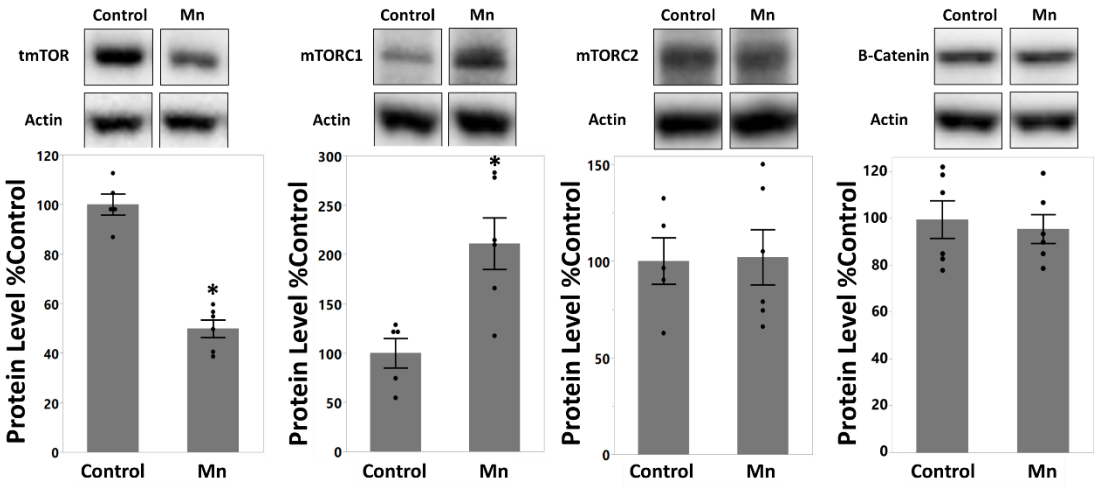
2.8.6 Figure 6: Integration of IPA causal and upstream regulators with significant DMRs and DEGs elucidates (A). The top 15 significant upstream and causal gene regulators determined by Ingenuity Pathway Analysis (IPA) are displayed with a brief definition of biological relevance to our Mn neurotoxicity phenotype, the biological function category that matches the Gene Ontology Group categories, and the IPA activation value. IPA activation values above 2 and below -2 are predicted statistically to be activated in their corresponding upregulated or downregulated direction. (B). A venn diagram of the resulting integration of IPA Causal and Upstream Regulators with DMR and DEGs. (C). Known genes regulated by mTOR compared to the significant Mn altered DMR and DEGs. (D). Known genes regulated by Wnt compared to the significant Mn altered DMR and DEGs. mTOR and Wnt pathway dysregulation as key Mn mechanistic targets.

A

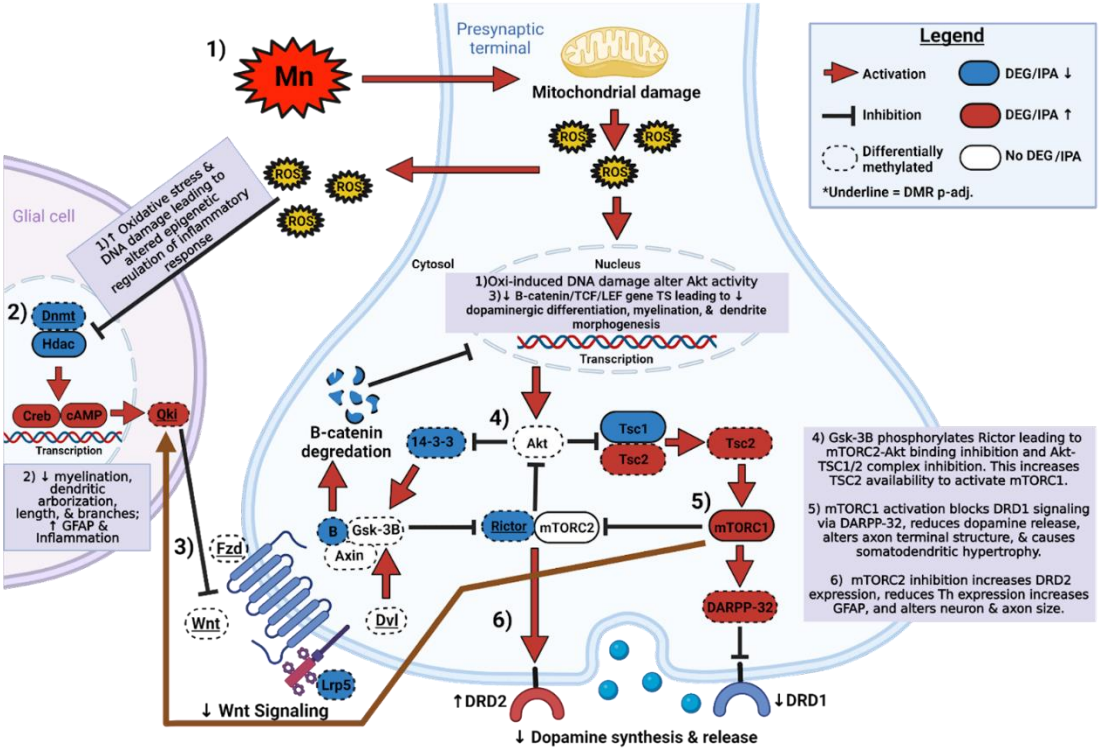
Gene	Function	Biological Function Category	IPA Activation Value
Mammalian Target of Rapamycin (mTOR)	Integrates growth factor stimulation and nutrient sensing to modulate cell growth, proliferation, protein translation, differentiation, and autophagy.	Cellular Development	2.89
Nuclear Factor Erythroid 2-related Factor 2 (NFE2L2)	Transcription factor that binds to antioxidant response (ARE) elements present in the promoter region of many cytoprotective genes.	Inflammation	2.58
Glutamate Ionotropic Receptor NMDA Type Subunit 3A (GRIN3A)	NMDA receptor subtype of glutamate-gated ion channels that plays a role in the synaptic refinement period, restricting spine maturation and growth.	Neuronal Function	2.11
DEAD-Box Helicase 5 (DDX5)	Involved in RNA structure alteration, plays a role as a coregulator of transcription, a regulator of splicing, and in the processing of small noncoding RNAs.	Epigenetics	1.63
Protein Tyrosine Phosphatase Receptor Type R (PTPRR)	Regulates cell growth, differentiation, mitotic cycle, and oncogenic transformation.	Cellular Development	1.63
CAMP Responsive Element Binding Protein 1 (CREB1)	Protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway.	Epigenetics	1.11
Transcription Factor AP-2 Gamma (TFAP2C)	Sequence-specific DNA-binding transcription factor involved in the activation of several developmental genes, such as those with neural tube development.	Epigenetics	1.10
Catenin Beta 1 (CTNNB1)	Encodes B-Catenin a key downstream component of the canonical Wnt signaling pathway that promotes cell proliferation and differentiation.	Cell Development	-0.97
Hepatocyte Nuclear Factor 4 Alpha (HNF4A)	A nuclear transcription factor which binds DNA as a homodimer. Plays a role in development of the liver, kidney, and intestines.	Cell Development	-1.51
BTB Domain And CNC Homolog 1 (BACH1)	With MAF, coordinates transcription activation and repression to control the NFE2L2 oxidative stress.	Inflammation	-1.63
RPTOR Independent Companion Of MTOR Complex 2 (RICTOR)	Subunit of mTORC2, which regulates cell growth and survival in response to hormonal signals. Deficiency can lead to decreased neuronal and axon size.	Cell Development	-1.65
Adenosine A2a Receptor (ADORA2A)	Plays an important role in cardiac rhythm and circulation, cerebral and renal blood flow, immune function, pain regulation, and sleep. Acts by increasing CAMP levels.	Neuronal Function	-1.89
Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (FOS)	Encodes a leucine zipper protein that can dimerize with proteins of the JUN family to form the transcription factor complex AP-1 to regulate cell proliferation, differentiation, and transformation.	Cell Development	-1.91
Quaking (QKI)	Regulates pre-mRNA splicing, export of mRNAs from the nucleus, protein translation, and mRNA stability. Is involved in myelinization and oligodendrocyte differentiation	Cell Development	-1.98
Carnitine Palmitoyltransferase 1B (CPT1B)	The rate-controlling enzyme of the long-chain fatty acid beta-oxidation pathway mitochondria.	Neuronal Function	-2.35



2.8.7 Figure 7: mTOR signaling is established as a novel mechanistic target of Mn neurotoxicity. Protein levels of total mTOR, mTORC1, mTORC2, and β-Catenin are measured in PND 66 prefrontal cortex samples. Mn reduced total mTOR, increased mTORC1, and did not detectably change mTORC2 or β-Catenin protein levels. Wilcoxon: Total mTOR ($p = 0.008$), mTORC1 ($p = 0.036$), mTORC2 ($p = 0.927$), β-Catenin ($p = 0.936$); n = 5 control/6 Mn per blot.



2.8.8 Figure 8: Proposed mechanism of Mn Neurotoxicity based on integration of p-unadjusted DMRs, DEGs, IPA upstream and casual regulators, and peer reviewed scientific literature. Briefly, Mn exposure may induce oxidative stress and DNA damage leading to altered epigenetic regulation of inflammatory response as well as direct change to Akt activity. This altered regulation leads to reduced Wnt signaling and as a result increased mTORC1 and decreased mTORC2 regulation. Altered mTORC1/mTORC2 impair the catecholaminergic system and perpetuate a continued inflammatory environment reflecting our Mn phenotype of lasting catecholaminergic system hypofunction and attention deficits. Legend: Red arrow is activation; Black Line with bar is inhibition; Dashed circle is differentially methylated; Blue Fill is DEG/IPA downregulated prediction; Red Fill is DEG/IPA upregulated prediction; No fill is no DEG/IPA evidence; Underlined name is DMR p-unadjusted and p-adjusted significance. Figure was made in Biorender Premium.



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Chapter 3

CHRONIC METHYLPHENIDATE NORMALIZES EXPRESSION OF KEY PREFRONTAL CORTEX CATECHOLAMINERGIC AND INSULIN-MTOR-PATHWAY GENES ALTERED BY DEVELOPMETAL MANGANESE EXPOSURE

3.1 Abstract

Elevated environmental exposure to manganese (Mn) is associated with attentional dysfunction in children. Previously, we have developed a rodent model establishing that developmental Mn exposure causes lasting impairments in attention, impulse control, and sensorimotor function, that these deficits are associated with lasting dysfunction in the catecholaminergic system in the prefrontal cortex and dorsal striatum, and that a low dose (0.5 mg/kg/day) of prolonged (but not pharmacological short term) oral methylphenidate (MPH) ameliorates the Mn associated attention deficits (1). However, the mechanism(s) underlying the efficacy of chronic oral MPH to improve the developmental Mn exposure attention deficits remains unclear. Our recent discovery in Chapter 2 that developmental Mn exposure induces lasting dysregulation of mammalian/mechanistic target of rapamycin (mTOR) and Wingless-Int (Wnt) signaling, as well as epigenomic, transcriptomic, and protein expression changes that may contribute to the Mn-induced hypofunctioning catecholaminergic system and attention deficits in our rat model suggests the mTOR signaling pathway as a potential therapeutic target. Thus, I hypothesize that chronic MPH administration normalizes the lasting gene expression changes of mTOR and Wnt pathways as a mechanism to improve Mn attentional function. To test this hypothesis, I conducted RNA-sequencing on prefrontal cortex brain tissue from PND 180 rats developmentally

exposed to oral Mn (50 mg/kg/day) over Postnatal Day 1-21, followed by chronic low dose MPH treatment (0.5 mg/kg/day, oral) and behavioral testing over PND 109-143 (1), and analyzed the differential gene expression data using Gene Ontology and Ingenuity Pathway Analysis to identify upstream and casual regulators. Results show that developmental Mn causes differential gene expression changes to epigenetic regulation, inflammation, cellular development, and hypofunctioning neuronal systems, generally consistent with findings in PND 66 animals exposed to the same Mn dose (Chapter 2). Additionally, pathway enrichment and Ingenuity Pathway Analysis shows that mTOR and Wnt signaling pathways remain altered, consistent with our prior findings in young adult (PND 66) rats developmentally exposed to Mn. Moreover, in these PND 180 adults, chronic 0.5 mg/kg MPH treatment enhances and normalizes several of these mTOR signaling pathway dysregulation at the gene, Gene Ontology, and upstream and causal regulator level. Interestingly I found that MPH's ability to normalize gene expression at the gene expression, pathway enrichment, and IPA causal and upstream regulator level affects the insulin receptor signaling pathway more upstream of mTOR signaling. Our present study suggests that MPH efficacy to ameliorate Mn-induced attention deficits may occur beyond the acute pharmacological response of inhibiting dopamine transporter (DAT) and norepinephrine transporter (NET) reuptake, and in addition normalizes lasting changes to the insulin-mTOR signaling pathway to improve catecholaminergic function and attention deficits. These findings deepen our understanding of developmental Mn exposure lasting effects on

the transcriptome and provide potential mechanisms of prolonged MPH treatment to ameliorate this gene expression dysfunction.

3.2 Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common neurological disorders in children and if left untreated presents a high societal burden of financial, mental health, and criminal implications (2). Methylphenidate (MPH) is among the most commonly prescribed therapeutic treatments for ADHD and related symptoms in children, and is believed to have a 65–70% overall efficacy rate in improving symptoms of inattention and impulsivity (2-5). MPH is believed to function as a central nervous system stimulant that increases attention and reduces hyperactivity and impulsivity in ADHD subjects by acting on the catecholaminergic system of the fronto-cortico-striatal brain circuit (6-9). The fronto-cortico-striatal catecholaminergic system is composed of a network of receptors and transporters that modulate the levels of the neurotransmitters dopamine and norepinephrine (6-9). MPH for instance is known on the pharmacological level to block dopamine transporter (DAT) and norepinephrine transporter (NET) from taking dopamine and norepinephrine back up into the nerve cells after being released. This results in higher levels of available extracellular synaptic noradrenaline and dopamine (10).

Elevated environmental exposure to the toxicant manganese (Mn) is believed to be a risk factor for developing attentional dysfunction as it is associated with symptoms of ADHD, such as inattention, impulsivity, hyperactivity, and fine-motor impairments in children (11-28). Our group has shown that developmental Mn

exposure restricted to the pre-weaning period caused a lasting (into adulthood) impairments in attention, impulse control, and sensorimotor function in a rat model (29-33). These behavioral deficits are associated with lasting dysfunction in the catecholaminergic system of the fronto-cortico-striatal brain areas, such as reduced dopamine receptor D1 (D1), dopamine transporter (DAT), and tyrosine hydroxylase (TH) protein levels and increased dopamine receptor D2 (D2) protein levels (29, 34-35). Furthermore, we have reported that developmental Mn exposure reduced K+-stimulated dopamine and norepinephrine release in the prefrontal cortex and striatum (36). These rodent studies indicate that developmental Mn exposure causes lasting changes to the catecholaminergic synapse in the PFC and striatum, and thus provide insight into causality of Mn-induced lasting cognitive and behavioral changes through regulating the dopaminergic system.

Our group is also the first to investigate the ability of MPH to alleviate the behavioral deficits associated with developmental Mn exposure in an animal model, and found that oral MPH administration (2.5 mg/kg/day) alleviated sensorimotor deficits caused by Mn exposure (31). We additionally found that this MPH treatment alleviated Mn-induced impulse control deficits, however, but not the Mn-induced attentional deficits (32). One study by others aimed at comparing the effects of what the authors deemed acute (2 mg/kg, i.p.) and chronic (2 mg/kg twice daily for 2 weeks) MPH treatment in rats did to key catecholamine protein targets (DAT, NET, TH, VMAT2, and D1) in the prefrontal cortex and striatum (67). These authors found that chronic but not acute administration of MPH increases levels of DAT, NET, TH,

VMAT2 and D1 (67). This demonstrates that prolonged MPH treatment may have benefits on catecholaminergic and behavioral outcomes due to additional mechanisms than just MPH's known immediate pharmacological effects on DAT and NET.

Clinical evidence of short term MPH efficacy , which may be due to MPH's pharmacological action as a dopamine and norepinephrine transporter antagonist, suggests varied results on improving attentional function in children with ADHD (37-43). One study of 57 children diagnosed with ADHD aged 6–13 years found that MPH (0.5–1.0 mg/kg) significantly improved subjects' Test of Variables of Attention (TOVA) performance in commission errors (measure of impulsivity; how many times the non-target is selected), response time and ADHD scores (comparison of the person's TOVA performance to an age/gender-specific group with ADHD), but not in omission errors (measure of inattention; how many times the target is not selected), response time variability, or response sensitivity (37). This study is consistent with two additional children studies using doses of 0.5 mg/kg that reported significant improvements in commission errors only but not omission errors (38-39). A follow up study to compared children TOVA performance while on MPH (0.5mg/kg) for 12 months, put them on placebo for 1 week and then measured TOVA performance again and reported significant improvement for both omission and commission errors (40), while another research group reported no beneficial effects at 0.3 mg/kg (41). A last study in children at 0.3 mg/kg mg reported omission errors and response time improved when MPH were given to individuals with ADHD (42). In terms of effects on ADHD information process, previous studies have demonstrated that shorter term

pharmacological effects of MPH treatment (0.3 mg/Kg) does not affect short-term memory, visual retention, baseline motor speed or encoding, but does increase timed tasked decision making (43).

Although shorter term MPH treatment studies on direct pharmacological effects show varied findings, MPH studies that look at prolonged MPH treatment have shown more consistent results that the ability of MPH to improve ADHD symptoms in children may be maintained for at least two years (44-49). For example, a study of ninety-four children and adolescents (ages 8-18 years) who had been treated with MPH for more than 2 years were randomly assigned to double-blind continuation of treatment for 7 weeks (36 or 54 mg/day of extended-release methylphenidate) or gradual withdrawal over 3 weeks, to 4 weeks of placebo confirmed that on average, MPH treatment after two or more years significantly improved children attention deficits compared to children that discontinued MPH treatment after two years and were administered a placebo (50). This benefit, however, had smaller effect sizes than those in short-term trials (50). This finding means that the children administered MPH for up to two years did not reach a point of treatment tolerance where MPH was no longer beneficial, which is important as sensitization or tolerance can to a prolonged treatment can be a problem. Interestingly, the attention ability of 60% of children did not deteriorate after withdrawing to placebo, suggesting that chronic MPH may be mechanistically altering additional systems than its immediate pharmacological action to maintain lasting attention benefits (50). In regard to our developmental Mn model, since we only began to see improved attention after at least 9 days of administration, I

believe this MPH efficacy to normalize attentional function mechanistically in developmental Mn exposed animals may be influenced by additional factors on the gene expression level than just acute exposure (dopamine transporter) DAT and (norepinephrine transporter) NET inhibition (1).

Recently we have discovered that developmental Mn exposure induces mammalian/mechanistic target of rapamycin (mTOR) and Wingless-Int (Wnt) signaling epigenomic, transcriptomic, and protein expression dysregulation and that these impairments are associated with the Mn-induced hypofunctioning catecholaminergic system and attention deficits in our rat model (Chapter 2). Interestingly, new evidence suggests that MPH may also alter mTOR and Wnt signaling neurodevelopmental pathways as a mechanism to improve attentional function, in addition to its classical method of inhibiting dopamine transporter function. (51, 60-65). For example, in vitro research has revealed that SH-SY5Y neuronal like cells when exposed to MPH and a dopamine transporter inhibitor have activated Wnt signaling (60) and in additional cell study MPH affected the mTOR pathway by decreasing the activity of Akt and mTOR substrates after short term treatment and increasing them after long-term treatment (65). Thus, the varied evidence I see of acute MPH effects, but consistent evidence of chronic MPH even after discontinued use suggests perhaps chronic MPH treatment leads to lasting changes in mTOR and Wnt signaling to create lasting attention benefits in developmental Mn exposed rats.

Here I aim to establish an association between attentional benefits of chronic low dose MPH treatment and prefrontal cortex gene expression in developmental Mn

exposed rats (1). I hypothesize that the attentional benefits of our chronic low MPH dose are associated with normalized prefrontal cortex gene expression related to mTOR, Wnt, and catecholaminergic system pathways. To test this prefrontal cortex brain tissue from behaviorally tested developmental Mn exposed and control rats were assessed for differential gene expression analysis and I will first establish the developmental Mn exposure phenotype of how developmental Mn exposure alters gene expression outcomes (e.g., differentially expressed genes, pathway enrichment Gene Ontology terms, and IPA upstream and causal regulators). I will compare this to our previous reported findings in younger adult animals (PND 66) from Chapter 2. In order to achieve the goal of identifying which Mn-induced gene expression outcomes are normalized and/or enhanced by MPH treatment I will use a Venn Diagram comparison approach of comparing each individual analysis (C v Mn, C v MnMPH, and Mn v MnMPH) as further detailed below. Here I report that developmental Mn causes similar differential gene expression changes to epigenetic, inflammation, cell development, and hypofunctioning neuronal systems as we have found previously in PND 66 rats (Chapter 2). Related to catecholaminergic function *solute carrier family 29 member 4 (Slc29a4*) and *cadherin 2* (*Cdh2*) decreased by developmental Mn exposure (C v Mn), but then increased in developmental Mn exposed rats with MPH compared to just developmental Mn exposed rats (Mn v MnMPH). Additionally, Wnt and mTOR signaling pathway related genes *LDL receptor related protein 5 (Lrp5)* and *tyrosine-protein kinase lyn (Lyn)* are revealed as also decreased by developmental Mn, but increased in MnMPH animals while the Wnt pathway gene *ring finger protein 43*

(Rnf43) shows the reverse effect. In particular, mTOR and Wnt signaling pathways remain altered in these PND 180 rats and interestingly chronic low dose MPH treatment normalizes this mTOR signaling pathway dysregulation more upstream at the insulin-PI3K-AKT-axis signaling pathway. These findings establish the importance of prolonged MPH treatment mode of action to target these key catecholaminergic and mTOR signaling pathway genes that elucidates our understanding of novel mechanisms outside of MPH's pharmacological mechanism, which may aid future therapeutic interventions for children at risk of elevated Mn exposure and the development of attention deficits.

3.3 Methods

The present study presents transcriptomics analyses of prefrontal cortex brain tissue from control and Mn-exposed rats treated with vehicle or MPH (0.5 mg/kg). The adult animals yielding the samples underwent developmental Mn (or vehicle) exposure over PND 1 – 21, followed by a series of attention, impulse control, and sensorimotor function tests over PND 47-109. Over the final stages of behavioral testing, animals were treated with MPH prior to testing (30 days total) in a 2 x 2 (-/+ Mn, -/+ MPH) study design, followed by a final catecholaminergic receptor antagonist phase of the study (PND 144-179) where all animals in the 2 x 2 design received three cycles of daily vehicle, D1R, D2R, or α2AR receptor antagonist following a Latin square design. Animals were sacrificed 3 days following the final MPH and receptor antagonist dose for brain tissue collection.

3.3.1 Subjects

All subjects (n = 128) were born in-house from nulliparous timed-pregnant Long Evans rats (from Charles River gestational day 18) as previously described (Chapter 2, Methods). Briefly, 12 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. Rats (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 the rats designated for behavioral testing were pair-housed with an animal of the same 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 rats' diurnal cycle.

Males were exclusively used because attentional dysfunction is two to three times more prevalent in boys than girls (52-53), and because our prior studies have established that early postnatal Mn exposure causes lasting impairments in learning, attention, and fine motor function in male rats (29-36). All animal procedures were approved by the institutional IACUC (protocols Smithd1803) 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 rats from the study were based on overt signs of poor rat health, including loss of body weight, absence of grooming, impaired function, and

death; no rats were excluded from the study based on these criteria. This study was not pre-registered.

3.3.2 Manganese dosing

Neonatal rats were orally exposed to a Mn dose of 50 mg Mn/kg/day starting on PND 1 through weaning on PND 21 (early postnatal Mn exposure) (29-36). 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 infant formulas (29-36).

3.3.3 Chronic methylphenidate and catecholaminergic receptor antagonist administration

Briefly, developmental Mn exposed and control rats were behaviorally tested using the 5-Choice Serial Reaction Time task from PND 45-108 to determine baseline Mn attention deficits. Next, methylphenidate hydrochloride (MPH) (Sigma-Aldrich Inc, St Louis, Missouri) was administered at oral doses of 0, 0.5, 1.5, and 3.0 mg/kg/d once daily for 30 days from PND 109-143 and rat attention testing continued to determine the efficacy of MPH treatment. Lastly, help elucidate the roles of key catecholaminergic receptors (individually) in the Mn deficits, and in the MPH efficacy to alleviate the deficits rats entered an antagonist administration phase for about 36 days from PND 144-179, getting a specific antagonist (dopamine receptor D1, dopamine receptor D2, alpha-2A adrenergic receptor) by subcutaneously at doses of 0.005, 0.015, and 1.0 mg/kg, respectively 10 minutes prior to oral MPH, and getting

MPH every 3rd day with 2 days of washout for antagonists between antagonist and MPH doses (e.g., MPH and antag on day 1, day 4, day 7, day 10 for cycle 1, day 13, 16, 19, 22 for cycle 2, and day 25, 28, 31, ,34 for cycle 3). While the Mn and MPH groups received all the same antagonists, it is important to recognize this treatment, since the tissue that is presently analyzed came from rats on MPH 6 day/week for 30 dosing days, then every 3 days for an additional 36 days or so. Animals were sacrificed on about PND 180.

Only the 0 and 0.5 mg/kg/d animals are being analyzed for gene expression analysis in the present study. Control- and Mn-exposed animals were randomized into the MPH or vehicle treatment groups prior to starting the MPH treatment phase of the study. MPH treatment was delivered using a food wafer delivery method. This, or similar, MPH dosing regimens have been shown to be safe to adolescent rats and juvenile monkeys, to produce a blood MPH half-life of ~2 h in adult rats, and to improve attention, working memory, inhibitory control, and forelimb skill deficits in animal models , thereby mimicking the human pharmacokinetic profile and clinical use of the drug (31, 33).

3.3.4 Differentially expressed genes and functional pathway analysis

The ability of developmental Mn exposure and chronic MPH administration to alter global gene expression was determined by 3' Tag RNA-sequencing as previously described (Chapter 2, Methods). Briefly, partially thawed fresh-frozen prefrontal cortex tissue punches (1.39 mm thickness, 1.5 mm diameter, 25-30 mg) were taken from the anterior cingulate cortex/prefrontal cortex area (Paxinos and Watson Rat Brain Atlas:

2.16-0.77 mm anterior to bregma) over dry-ice and underwent Dounce Homogenization and total RNA extraction, DNAse treatment, and Reverse Transcription. RNA aliquots from the RNA extraction (n=6 rats/treatment) were analyzed for Bioanalyzer quality assessment, library preparation, and 3' Tag RNA-sequencing by the University of California Davis Genome Center. FastQ files were processed using established Geneious Prime RNA-sequencing tools (Geneious Prime 2022.0.). DESeq2 was used to calculate differentially expressed gene (DEG) values between control and developmental Mn-exposed prefrontal cortex brain samples (54). Next, all DEGs were ranked and analyzed using Fast Preranked Gene Set Enrichment Analysis (FGSEA) to determine the DEG's Gene Ontology-associated biological processes, molecular functions, and cellular components subtypes. Gene Ontology biological processes were further reduced, filtered, and visualized using REVIGO's algorithm for semantic similarity of parent and child terms (55) (Chapter 2, Methods).

3.3.6 Treatment Group Analysis Integration

Integrated analysis of the DEG data were conducted to determine which genes and Gene Ontology biological processes were altered by developmental Mn exposure and/or chronic methylphenidate exposure that associate with our Mn-attention deficit and subsequent chronic methylphenidate rescue. I used the Genomics Tools Venn-Diagram web program and REVIGO to compare and visualize the upregulated and downregulated Gene Ontology biological processes group lists shared by C v. Mn, C v. MnMPH, and Mn v. MnMPH associated genes. Genomics Tools Venn-Diagram web program comparison was performed on differentially expressed genes to further

determine genes that were changed due to chronic methylphenidate administration. Clustergrammer was then used to visually display DEG expression levels.

3.3.6 Upstream and causal gene identification by Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) was performed at the UCLA Technology Center for Genomics & Bioinformatics using all p-unadjusted DEGs from DESeq2 to identify significant causal and upstream regulator genes. Brief gene function descriptions were obtained from GeneCards: The Human Gene Database (Chapter 2, Methods).

3.4 Results

3.4.0 Results Overview

My objectives for the following result sections are to determine the lasting changes in prefrontal cortex gene expression caused by developmental Mn in behaviorally tested rats. I aim to identify differentially expressed genes and enriched pathways altered by Mn that may be associated with the lasting deficits in attention, impulse control, and sensorimotor function, including catecholaminergic system function, and higher regulatory pathways. I also aim to determine if prolonged oral MPH treatment, at a dose (0.5 mg/kg) shown to fully alleviate the Mn behavioral deficits, i) restores differentially expressed genes/pathways altered by developmental Mn exposure, and/or ii) induces differentially expressed genes/pathways that may compensate for the altered differentially expressed genes caused by Mn and lead to amelioration of the Mn functional deficits (1).

To achieve these aims, I will first establish the developmental Mn exposure phenotype of how developmental Mn exposure alters gene expression outcomes (e.g., differentially expressed genes, pathway enrichment Gene Ontology terms, and IPA upstream and causal regulators) and I will compare this to our previous reported findings in younger adult animals (PND 66) from Chapter 2. I will primarily be highlighting gene expression outcomes related to a hypofunctioning catecholaminergic system phenotype caused by developmental Mn exposure, as well as epigenetic gene regulation, inflammation, and cellular development pathways, such as Wnt and mTOR signaling shown in our Chapter 2 findings. This analysis will be done through reporting the individual control v manganese (C v Mn) two-way RNA-seq analysis. In order to achieve the goal of identifying which Mn-induced gene expression outcomes are normalized and/or enhanced by MPH treatment I next report the findings from two other individual RNA-seq two-way comparisons: control v manganese with MPH (C v MnMPH) and manganese v manganese with MPH (Mn v MnMPH). Individual C v MnMPH analysis informs us of what gene expression outcomes remain different in MnMPH rats compared to control rats despite MnMPH rats no longer having attention deficits compared to controls while the individual Mn v MnMPH comparison informs us how MnMPH rats that no longer have attention deficits compared to control differ from Mn rats that do have attention deficits.

Although the C v MnMPH and Mn v MnMPH comparison individual analysis will be detailed further in the supplemental material, both comparisons are needed to create a Venn Diagram of gene expression outcomes from all three two-way

comparisons (C v Mn, C v MnMPH, Mn v MnMPH) to identity which gene expression outcomes are solely due to developmental Mn exposure, Mn altered outcomes normalized by MPH, and Mn altered outcomes enhanced by MPH. Supplemental Figure 1 details what we may learn from each section of the Venn Diagram comparison. Altogether through understanding these comparisons it will strengthen our understanding of lasting developmental Mn exposure induced transcriptomic changes and elucidate potential mechanisms of MPH treatment to rescue Mn-induced gene expression outcomes in developmental Mn exposed behaviorally tested rats.

3.4.1 Developmental Mn exposure induces lasting catecholaminergic, epigenetic regulation, inflammation, and cellular development related gene expression changes in the prefrontal cortex of Mn rats compared to control rats and prolonged MPH treatment alters similar gene expression outcomes

Here we report the individual 3' tag transcriptomic analysis gene expression two by two comparisons outcomes of C v Mn, C v MnMPH, and Mn v MnMPH analyses to establish developmental Mn and MPH transcriptomic phenotypes prior to an integrated comparison analysis to establish mechanism. Our first comparison of C v Mn rats determines what outcomes may relate to our Mn-induced hypofunctioning catecholaminergic system and attention deficit behavioral phenotype. In this comparison we found that Mn specifically upregulates 900 genes and downregulates 816 genes (Figure 1A, Supplemental Sheet 1). After adjusting for multiple comparisons (p-adjusted < 0.05) 58 upregulated and 31 downregulated DEGs remain (Figure 1A). Of note to our catecholaminergic phenotype we see downregulated *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*Ywhaz*) and

increased *catechol-O-methyltransferase* (*Comt*) similar to our PND 66 findings (Chapter 2).

Previously, we have uncovered that a chronic low dose of MPH ameliorated the attention deficits in developmental Mn exposed rats (1). We used RNA-sequencing analysis to determine what genes were differentially expressed in the prefrontal cortex of these same Mn exposed with MPH treatment (MnMPH) rats compared to controls. We found 1,664 significantly (p-unadjusted < 0.05) differentially expressed genes, 942 genes upregulated and 722 genes downregulated (Supplemental Sheet 3). After adjusting for multiple comparisons (p-adjusted < 0.05) 227 upregulated genes and 121 downregulated genes remain significant (Supplemental Figure 1A). Of importance to our Mn-induced catecholaminergic phenotype and mTOR signaling pathway established in Chapter 2, *tuberous sclerosis complex subunit 2* (*Tsc2*) and *glial fibrillary acidic protein* (*Gfap*) are downregulated while *insulin-like growth factor binding protein* (*Igfbp1*) is upregulated (Supplemental Sheet 3). These genes regulate mTOR signaling and can lead to altered expression of catecholaminergic genes (*Th*, *Drd1*, and *Drd2*) as well as altered dopamine levels (Chapter 2, Figure 8).

Individual analysis of the differential gene expression outcomes between developmental Mn exposed rats and developmental Mn exposed rats with chronic MPH treatment (Mn v MnMPH) were next analyzed to determine how MPH effects rat with Mn altered neurobiology. We determined developmental Mn exposure upregulates 592 and downregulates 496 prefrontal cortex genes (p-unadjusted < 0.05) with the addition of MPH administration (Supplemental Figure 2A, Supplemental Sheet 5). Only nine

upregulated and six downregulated genes remain significant (p-adjusted < 0.05) when adjusting for multiple comparisons (Supplemental Figure 2A). Downregulated genes relevant to our Mn-induced Wnt and mTOR dysregulation phenotype include *wingless INT 3a (Wnt3a), tuberous sclerosis complex subunit 1* (*Tsc1*), *tuberous sclerosis complex subunit 2* (*Tsc2*), and upregulated genes include *insulin growth factor binding protein 1* (*Igfbp1*), *and LDL receptor related protein 5* (*Lrp5*) (Supplemental Sheet 5). Taken together many of these relevant genes map well to our group's developmental Mn exposure proposed mechanism in Chapter 2 of developmental Mn increasing oxidative stress and inflammatory pathways to inhibit Wnt signaling and activate mTOR signaling thus creating a hypofunctioning catecholaminergic system. This further corroborates our PND 66 findings (Chapter 2). Additionally, we see that in the prolonged MPH treated groups these similar catecholaminergic, Wnt, and mTOR related genes are altered.

3.4.2 Developmental Mn exposure alters similar pathway enrichment Gene Ontology terms in the PFC of PND 180 rats compared to PND 66 rats and prolonged MPH treatment alters similar pathways

Here we establish the individual two by two (C v Mn, C v MnMPH, and Mn v MnMPH) RNA-seq comparison pathway enrichment transcriptomic phenotypes, which are necessary to understand prior to conducting our integrated two way comparison analysis of potential MPH gene expression rescue and enhancement mechanisms. Pathway enrichment Gene Ontology analysis of C v Mn p-unadjusted genes identified that developmental Mn exposure upregulates of 59 and downregulates 58 biological process terms (Figure 1B/C, Supplemental Sheet 2). Of the upregulated

terms *(GO:0032008) positive regulation of TOR signaling cascade*, *(GO:0010629) negative regulation of gene expression*, and *(GO:0019221) cytokine-mediated signaling pathway* stood out as relevant terms to our *a priori* Mn-hypothesized biological categories (e.g., epigenetic regulation, inflammation, cellular development, and hypofunctioning neuronal systems) similar to our PND 66 findings (Figure 1D and Chapter 2). The downregulated GO terms *(GO:0070989) oxidative demethylation*, *(GO:0001672) regulation of chromatin assembly*, and *(GO:0021954) central nervous system neuron development* (Figure 1D) additionally corroborate our understanding of our previously reported in Chapter 2.

Pathway enrichment analysis of C v MnMPH, which are developmental Mn exposed rats that are associated with rescued attention function, reveal 96 upregulated and 33 downregulated biological process terms associated related to similar categories of epigenetic regulation, inflammation, cellular development, and neuronal systems as previously documented (Supplemental Figure 1B/C, Supplemental Sheet 4) (Chapter 2) (1). Specifically, upregulated *(GO:0033603) positive regulation of dopamine secretion* and *(GO:00320080) positive regulation of TOR signaling cascade* as well as downregulated *(GO:0006974) DNA damage response* and *(GO:2001244) positive regulation of intrinsic apoptotic signaling pathway* stood out due to our previous Mn impaired catecholaminergic, mTOR, and inflammation findings (Supplemental Figure 1D, Supplemental Sheet 4) (Chapter 2).

Lastly, the individual comparison of Mn v MnMPH analysis reveals upregulation of 42 and downregulation of 59 biological process terms (Supplemental

Figure 2C/D, Supplemental Sheet 6). Relevant upregulated GO terms include *(GO:0006469) negative regulation of protein kinase activity* and *(GO:0006836) neurotransmitter transport* and relevant downregulated GO terms include *(GO:0030182) neuron differentiation*, and *(GO:0007268) synaptic transmission* (Supplemental Figure 2D). Overall, these individual two by two comparisons suggest developmental Mn exposure creates lasting changes to epigenetic gene regulation, inflammation, cellular development, and hypofunctioning neuronal pathways similar to our previous PND 66 findings. Additionally, MPH treated groups (C v MnMPH and Mn v MnMPH) have altered pathways related to similar functions, specifically in benefits of dopaminergic function, however, the integrated analysis shows that pathways upstream of mTOR signaling, such as with the insulin regulation may be more important to MPH's gene expression effects as mTOR signaling is seen in both C v Mn and C v MnMPH and is not different in Mn v MnMPH.

3.4.3 Integration of developmental Mn exposure and MPH differential gene expression analyses suggests that chronic administration of low dose MPH normalizes gene expression as mechanisms of ameliorating attention dysfunction in the prefrontal cortex of developmentally Mn exposed adult rats

Now with each individual comparison phenotype complete I am able to integrate all three analyses comparisons (C v Mn, C v MnMPH, and Mn v MnMPH) to determine which genes and Gene Ontology terms can be attributed to developmental Mn outcomes and also which may be normalized by MPH administration (Supplemental Figure 1). To start, my analysis reveals 124 genes (p-unadjusted) that are not only altered by developmental Mn in the C v Mn comparison, but that are also

enhanced by MPH in Mn exposed animals in the Mn v MnMPH comparisons. For instance, their fold change is not the same as control as they are up or downregulated by Mn, but then the directionality of the fold change is reversed by MPH in Mn exposed animals (Supplemental Figure 1A, Figure 2A, Supplemental Sheet 7). These genes are considered enhanced (up or downregulated foldchange from Control). Of particular note to our catecholaminergic phenotype we see both *solute carrier family 29 member 4* (*Slc29a4)* and *cadherin 2 (Cdh2)* decreased by developmental Mn exposure (C v Mn), but then increased in developmental Mn exposed rats with MPH compared to just developmental Mn exposed rats (Mn v MnMPH). These genes can regulate the release and transport of dopamine. Additionally, Wnt and mTOR signaling pathway related genes *LDL receptor related protein 5* (*Lrp5*) and *tyrosine-protein kinase Lyn* (*Lyn*) are revealed as also decreased by developmental Mn, but increased in MnMPH animals while the Wnt pathway gene *ring finger protein 43* (*Rnf43*) is increased by developmental Mn exposure compared to control, but decreased in MnMPH animals compared to Mn animals (Figure 2B,Supplemental Figure 1A, Supplemental Sheet 7).

Secondarily, in assessing which genes were altered by developmental Mn exposure, but now rescued by prolonged MPH treatment we see 1005 genes (p-unadjusted) through the Venn Diagram genes only in the C v Mn group that were no longer DEG in the C v MnMPH or Mn v MnMPH groups (Figure 2C, Supplemental Figure 1B) Examples of these normalized genes related to our Mn altered phenotype are *wingless INT 6/7b* (*Wnt6/7b*), *tyrosine 3-monooxygenase/tryptophan 5-*

monooxygenase activation protein zeta (*Ywhaz*), and *janus kinase 3* (*Jak3*) (Figure 2, Supplemental Figure 7).

Lastly, 591 genes (p-unadjusted) are identified solely in differentially expressed in the Mn v MnMPH comparison (Figure 2D, Supplemental Figure 1C, Supplemental Figure 7). Of these genes *axin 1* (*Axin1)*, *tuberous sclerosis complex 1* (*Tsc1*), and *wingless INT 3a* (*Wnt3a)* are related to Wnt and mTOR signaling function and *calcium voltage-gated channel subunit alpha1 C* (*Cacna1c*) is related to catecholaminergic system function (Figure 2D). For additional viewing all p-unadjusted genes in all three comparisons (C v Mn, C v MnMPH, and Mn v MnMPH) are charted by their associated gene expression fold change in each individual two-way comparison (Supplemental Sheet 8). Overall, the pattern continues of genes related to catecholaminergic, Wnt, and mTOR pathway function being changed by developmental Mn, but then enhanced or normalized by MPH treatment.

3.4.4 Gene Ontology term integration reveals significant epigenetic, inflammation, cell development and hypofunctioning neuronal pathways normalized by MPH administration

To further define the functional processes normalized by MPH administration, we integrated the prior comparisons (C v Mn, C v MnMPH, and Mn v MnMPH) Gene Ontology terms. The analysis first reveals that 10 Gene Ontology terms (p-unadjusted) are both altered by developmental Mn exposure and also enhanced by prolonged MPH treatment (Figure 3A). These Gene Ontology terms include several related to hemoglobin function, such *as (GO:0010999) regulation of eif2 alpha phosphorylation by heme, (GO:0046501) protoporphyrinogen ix metabolic process, (GO:0046986)*

negative regulation of hemoglobin biosynthetic process, and several terms related to neuronal processes, such as *(GO:0006874) cellular calcium ion homeostasis* and *(GO:0045947) negative regulation of translational initiation*. The overarching connector of both the hemoglobin and neuronal processes here is in *Eif2a,* which functions in coordination with mTORC1 under cellular stress to initiate translation and the downregulation of *Eif2a* (as seen here) is viewed as a neuroprotectant and antioxidative for the brain (Figure 3B) (66).

We see 97 Mn altered biological process terms normalized by MPH administration (Figure 3A, Supplemental Sheet 9). Some relevant terms of interest that were associated with downregulated genes by Mn exposure, but are now normalized are *(GO:0021954) central nervous system neuron development*, *(GO:0042417) dopamine metabolic process* and *(GO:0070989) oxidative demethylation*, while *(GO:0051898) negative regulation of protein kinase B signaling cascade (Akt)* and *(GO:0019221) cytokine-mediated signaling pathway* which were originally upregulated by Mn are now normalized. (Figure 3C, Supplemental Sheet 9). It is also interesting to note that *(GO:0007268) synaptic transmission*, *(GO:0014013) regulation of gliogenesis*, and *(GO:0032024) positive regulation of insulin secretion* are several pathways that show up as not normalized, but in fact being further altered by MPH in developmental Mn rats (Figure 3D, Supplemental Sheet 9).

3.4.5 Ingenuity Pathway Analysis integration indicates the top regulators of MPH normalized gene expression

Finally, we conducted Ingenuity Pathway Analysis (IPA) to uncover the upstream and causal regulators of these developmental Mn and chronic MPH transcriptomic changes so that we may have a better mechanistic understanding of developmental Mn and MPH effects on neurobiology (See Methods). Using our integrated comparison approach 69 IPA causal and upstream regulators are identified (p-unadjusted < 0.05) to be altered by developmental Mn exposure, but enhanced by prolonged MPH treatment (Figure 4A). These 69 regulators include the catecholamine and mTOR related gene *solute carrier family 7 member 5* (*Slc7a5*), the Wnt related genes *lymphoid enhancer binding factor 1* (*Lef1)* and *vascular endothelial growth factor A* (*Vegfa*), and the epigenetic modulator *histone deacetylase 2* (*Hdac2)*. Of these 69 IPA regulators only *insulin like growth factor 1* (*Igf1)* reached statistical power to have an inhibited activation value by IPA (Figure 4B, Supplemental Sheets 10-12).

IPA reveals 331 significant (p-unadjusted < 0.05) upstream and causal regulators found only in our C v Mn analysis, suggesting these genes are regulators of our MPH gene expression rescue(Figure 4A, Supplemental Sheets 10-12). Of these upstream and causal regulators, 73 had an activation value (+/-) 2, indicating they are predicted to be activated or inhibited (Figure 4B, Supplemental Sheet 10-12). The *catechol-o-methyltransferase* (*Comt*), *histone deacetylase 7* (*Hdac7*), *DNA methyltransferase 3a* (*Dnmt3a*), *insulin like growth factor 2* (*Igf2*), *quaking* (*Qki*), and *lymphoid enhancer binding factor* (*Lef1*) stand out as significant gene regulators of our normalization phenotype due to their role in epigenetic regulation of catecholaminergic function and/or upstream mTOR and Wnt pathway regulation (Supplemental Figure

4A, Supplemental Sheet 10-12). Additionally, several of these regulators such as *Comt* and *Igf2* were previously identified as being differentially expressed themselves in our present findings. Lastly, IPA upstream and causal regulators of our altered MnMPH rat altered phenotype from Mn rat (Figure 1C) show 438 causal and upstream regulators with 31 defined as activated or inhibited (Figure 4A/4B). These significant regulators include *AKT serine/Threonine kinase 1* (*Akt*), *NFE2 like BZIP transcription factor 2* (*Nfe2l2*), *tyrosine 3-monoxygenease/tryptophan 5-monoxygenase activation protein zeta* (*Ywhaz*), and *catenin beta interacting protein 1* (*Ctnnbip1*) as regulators, which are also connected to mTOR and Wnt signaling, related to our PND 66 gene expression findings, and many of which were previously shown differentially expressed themselves in our present analysis (Supplemental Figure 4B, Supplemental Sheet 10-12) (Chapter 2). As a whole we see from the IPA causal and upstream analysis that several regulators not only are present in our previously proposed Mn neurotoxicity mechanism and differential expression data (*Qki*, *Dnmt3a*, *Hdac7*) in Chapter 2, but are also differentially expressed genes (*Comt* and *Igf2*) that are found normalized by prolonged MPH treatment elucidating potential MPH treatment mechanisms.

3.5. Discussion

Attention Deficit Hyperactivity Disorder (ADHD) affects upwards of 6-11% of all U.S. children aged 6-17 years (56). Although the exact causes of neurological disorders such as ADHD are unknown, elevated exposure to environmental toxicants are believed to be a risk factor (28). Unfortunately, children from lower income and/or marginalized ethnic backgrounds have been shown to be disproportionately at risk of

elevated toxicant exposure compared to other identity groups and consequently, these children have a higher risk of developing neurological disorders (28). Thus, determining how toxicants, such as Mn alter the brain is vital to implementing therapeutic approaches to serve these vulnerable populations.

Our group recently proposed a mechanism of Mn neurotoxicity establishing mTOR and Wnt signaling pathway methylation, transcriptomic, and protein level dysregulation as a mechanism for how developmental Mn exposure produces a lasting hypofunctioning catecholaminergic system and ADHD-like attention deficit phenotype in rats (Chapter 2). We further determined for the first time a low dose of MPH that ameliorated attention deficits caused by developmental Mn exposure (1). Here we aimed to establish an association between attentional benefits of chronic low dose MPH treatment and lasting prefrontal cortex gene expression in developmental Mn exposed rats.

Through conducting RNA-sequencing gene expression analysis on the prefrontal cortex tissue of control and developmental Mn exposed animals with and without chronic MPH administration (C v Mn, C v MnMPH, Mn v MnMPH) we have found that developmental Mn exposure alters epigenetic regulation, inflammation, cellular development, and neuronal processes and MPH administration alters and normalizes similar functions to control animals. More specifically, we see altered genes, such as *tuberous sclerosis complex subunit 2 (Tsc2)* and *glial fibrillary acidic protein (Gfap)* are downregulated *while insulin-like growth factor binding protein (Igfbp1)* is upregulated. We also see developmental Mn exposure DEGs associated with

similar Gene Ontology terms impaired by developmental Mn exposure just as we have seen in our previous findings in PND 66 animals, such as with the upregulated GO term *(GO:0032008) positive regulation of TOR signaling* and downregulated GO term *(GO:0007417) central nervous system neuronal development* (Chapter 2). Furthermore, the IPA causal and upstream regulators that were shown to regulate developmental Mn exposure induced transcriptomic changes, include the top regulators *insulin receptor* (*Insr*) and *CAMP responsive element binding protein 1* (*Creb1*). These genes are known to be upstream regulators of mTOR and Wnt signaling through the insulin-PI3K-AKT-axis (51).

Interestingly, we found several developmental Mn altered genes that were also enhanced by MPH treatment in Mn exposed rats. These genes include solute carrier family 29 member 4 (*Slc29a4), cadherin 2 (Cdh2), LDL receptor protein 5 (Lrp5), family tyrosine kinase lyn (Lyn),* and *ring finger protein 43* (*Rnf43)* and highlight the ability of prolonged MPH treatment to rescue catecholaminergic, mTOR and Wnt pathway transcriptomic outcomes in addition to previous expected pharmacological mechanisms to ameliorate attention deficits. We additionally, see evidence that MPH normalizes further upstream pathways that regulate Wnt and mTOR signaling, such as with being associated with upregulated genes involved in the GO term *(GO:0051898) negative regulation of protein kinase B signaling cascades (Akt)* as well as normalizing the downregulated GO term *(GO:0042417) dopamine metabolic process*. This is further supported by the causal and upstream analysis showing *catechol-o-methyltransferase* (*Comt*), *histone deacetylase 7* (*Hdac7*), *DNA methyltransferase 3a*

(*Dnmt3a*), *insulin like growth factor 2* (*Igf2*), *quaking* (*Qki*), and *lymphoid enhancer binding factor* (*Lef1*), among others, as significant gene regulators of our normalization phenotype due to their role in epigenetic regulation of catecholaminergic function and/or upstream mTOR and Wnt pathway regulation (Chapter 2; 51).

Furthermore, our IPA analysis revealed several catecholamine and mTOR related genes, that were not only altered by developmental Mn exposure, but that were also enhanced by MPH treatment in Mn exposed rats, as causal and upstream regulators. These regulators include catecholamine regulator and also differentially expressed *solute carrier family 7 member 5* (*Slc7a5)*, the Wnt and mTOR related genes *insulin like growth factor 1* (*Igf1), lymphoid enhancer-binding factor 1 (Lef1),* and *vascular endothelial growth factor A* (*Vegfa),* and the epigenetic modulator *histone deacetylase 2* (*Hdac2).* IPA upstream and causal regulators of our altered MPH phenotype not normalized in MnMPH animals show *AKT serine/Threonine kinase 1* (*Akt*), *NFE2 like BZIP transcription factor 2* (*Nfe2l2*), *tyrosine 3-monoxygenease/tryptophan 5-monoxygenase activation protein zeta* (*Ywhaz*), and *catenin beta interacting protein 1* (*Ctnnbip1*) as regulators, which are also connected to mTOR, Wnt signaling, and related to our PND 66 gene expression findings (Chapter 2; 51, 57-58).

Although in general, MPH acts as a dopamine, norepinephrine, and at low-level serotonin transporter inhibitor to alter attentional function, the exact molecular pathways involved in this context remains unclear (59). Taking this notion in to context of our current findings, the literature supports our findings above that MPH can alter

insulin regulation of the PI3K-AKT axis to control Wnt and mTOR function resulting in altered catecholaminergic gene expression outcomes (51,60-65). For example, *in vitro* research has revealed that SH-SY5Y neuronal like cells when exposed to MPH and a dopamine transporter inhibitor still have activated Wnt signaling (60). In addition, levels of the Wnt pathway components, β-catenin, VEGF, and TrkB, have been shown to be elevated in juvenile male mice after a low dose of chronic MPH treatment (1 mg/kg/IP) and reduced after high dose chronic treatment (10 mg/kg/IP) (63). Additionally, 8-day MPH administration can regulate phosphorylation of GSK3 and Akt in C57Bl/6J adult mice (64). Lastly, MPH affected the mTOR pathway by decreasing the activity of Akt and mTOR substrates after short term treatment and increasing them after long-term treatment (65).

In regard to our proposed mechanism of developmental Mn creating a proinflammatory environment that leads to epigenetic and transcriptomic changes of inhibited Wnt signaling components and activated mTOR signaling to create a hypofunctioning catecholaminergic system (Chapter 2, Section 7, Figure 8) our present evidence suggests chronic MPH enhances *Lrp5, Lyn, Vegfa, Igf1,* and *Hdac2* and normalizes the important mechanism upstream and causal regulators *Comt, Hdac7, Igf2,, Dnmt3a,* and *Qki* among others. These regulators are important mediators of the Mn-induced oxidative stress and epigenetic regulation activation of inflammation response, the inhibition of Wnt signaling and activation of mTOR response, and regulation of catecholaminergic protein pathways all noted in the step by step Chapter 2 proposed mechanism. Thus, the potential of MPH treatment to normalize Wnt and

mTOR pathways dysregulated by developmental Mn exposure and ameliorate catecholaminergic hypofunction may be explained through our differential gene expression findings.

Ongoing studies in our group will be to assess the gene expression outcomes of MPH on normal control rats to discern key differences between how MPH interacts between control rats and Mn exposed rats. Additionally, based on our prior published work showing developmental Mn exposure alters DNA methylation associated with differential gene expression and attention dysfunction it will be important to integrate DNA methylation analysis to uncover a further deepened understanding of developmental Mn exposure and chronic MPH administration mechanisms of action. Overall, our findings underscore the importance to further investigate MPH's mode of action to target the insulin-PI3K-AKT-mTOR signaling pathway as further understanding of this regulation may aid therapeutic interventions for children at risk of elevated Mn exposure and the development of attention deficits.

3.6. Acknowledgements

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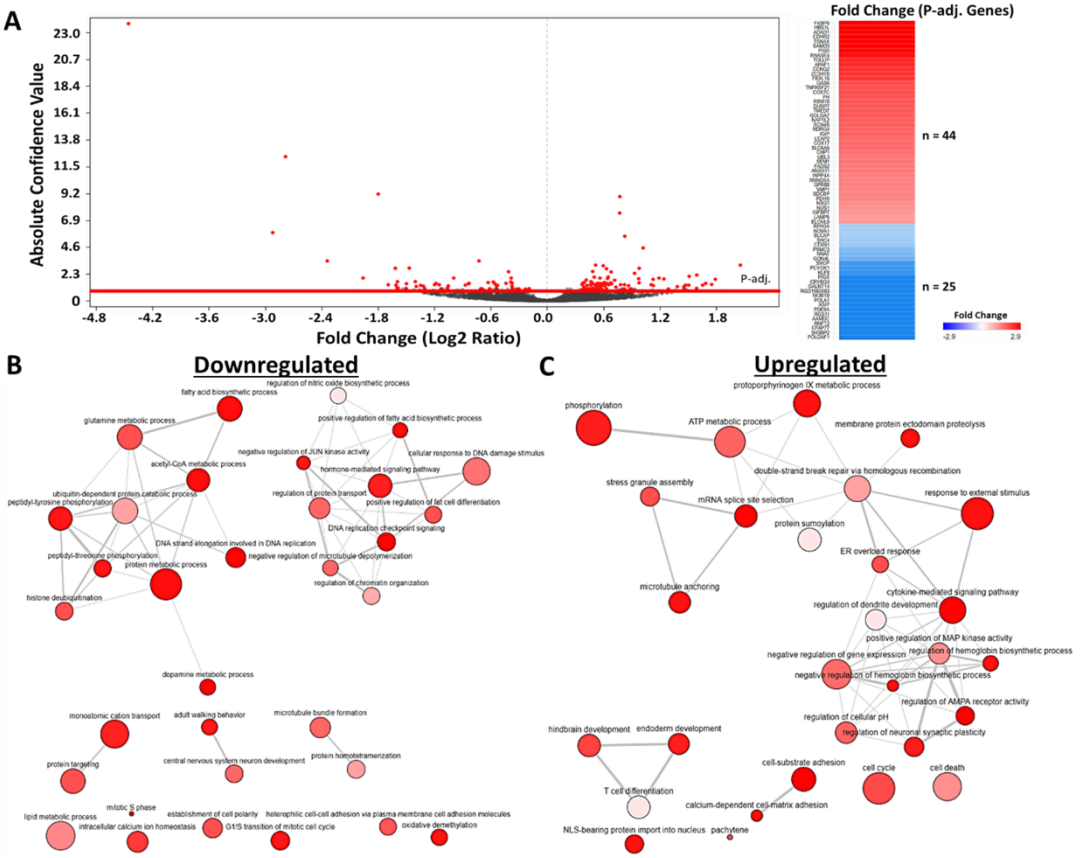
3.7 Figures

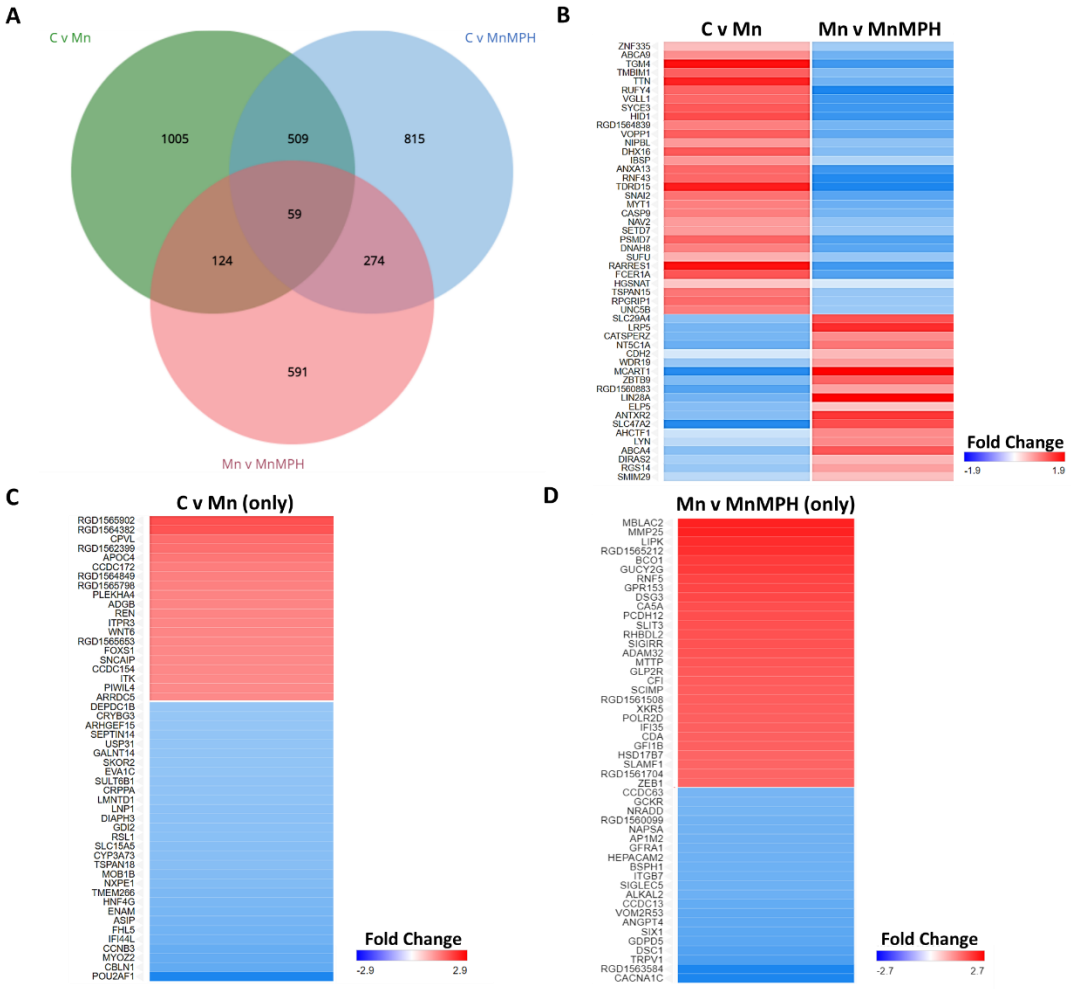
3.7.1 Figure 1: Developmental Mn exposure induces lasting gene expression changes in in the PFC of Mn rats compared to control rats A. C v Mn, differential gene expression volcano plot (left) and p-adjusted significant genes by fold change (right). B/C. Down- and upregulated Gene Ontology (GO) terms from p-unadjusted differential genes. D. List of relevant GO terms to Mn exposure *a priori* hypotheses.



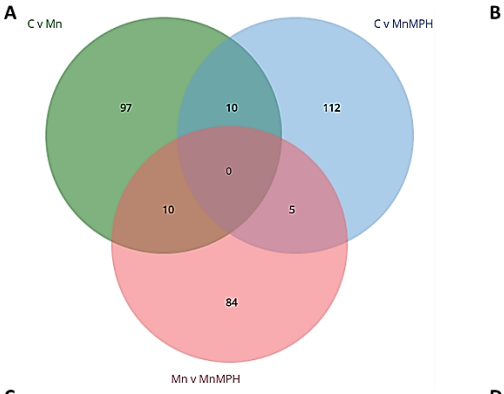
D

Gene Ontology Term	Expression Direction	Biological Function Category
Positive regulation of TOR signaling	Upregulated	Cellular Development
Negative regulation of gene expression	Upregulated	Epigenetics
Cytokine-mediated signaling pathway	Upregulated	Inflammation
Regulation of neuronal synaptic plasticity	Upregulated	Neuronal Function
Negative regulation of dendrite development	Upregulated	Neuronal Function
Dopamine metabolic process	Downregulated	Neuronal Function
Neurotransmitter secretion	Downregulated	Neuronal Function
Oxidative demethylation	Downregulated	Epigenetics
Regulation of chromatin organization	Downregulated	Epigenetics
Central nervous system neuron development	Downregulated	Neuronal Function

3.7.2 Figure 2: Differentially expressed gene analysis comparisons between treatment groups suggest MPH administration normalizes lasting developmental Mn exposure associated gene expression. A. Venn diagram comparisons of C v. Mn, C v. MnMPH, and Mn v. MnMPH DEGs, p-unadj. B. Heatmap of top 50 DEGs by foldchange in the C v Mn and Mn v MnMPH intersection group (124 genes). C. Heatmap of top 50 DEGs by foldchange only in the C v Mn group (1005 genes). D. Heatmap of top 50 DEGs by foldchange only in the Mn v MnMPH group (591 genes). Full list of genes are in Supplemental Sheet 7.

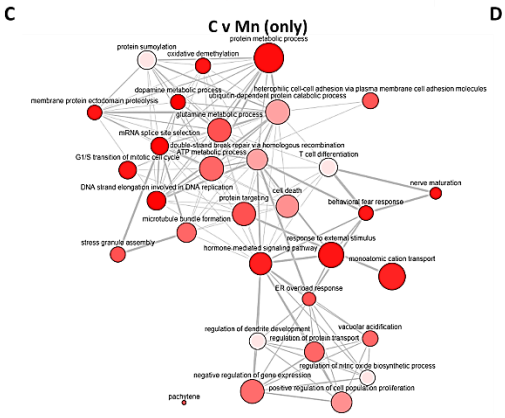


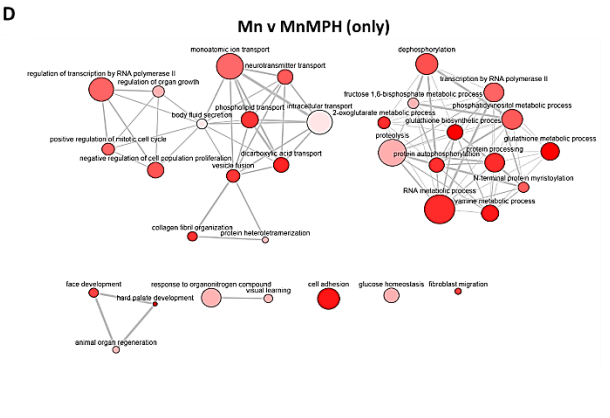
3.7.3 Figure 3: Gene Ontology term integration reveals significant epigenetic, inflammation, cell development and hypofunctioning neuronal pathway normalized and enhanced by MPH administration. A. Venn diagram comparisons of C v. Mn, C v. MnMPH, and Mn v. MnMPH GO Terms from DEGs, p-unadj. B. Gene Ontology terms shared by both C v Mn and Mn v MnMPH analysis (10 GO terms). C. Revigo Bubblemaps of p-unadj Gene Ontology terms only in the C v Mn analysis (97 GO terms). D. Revigo Bubblemaps of p-unadj Gene Ontology terms only in the Mn v MnMPH analysis (84 GO terms). Each bubblemap circle represents a $p < 0.05$ GO term, the larger the circle the more child terms within, and the thicker the line the more functional connects that GO term has with the connecting GO term. Full list of terms viewable in Supplemental Sheet 9.



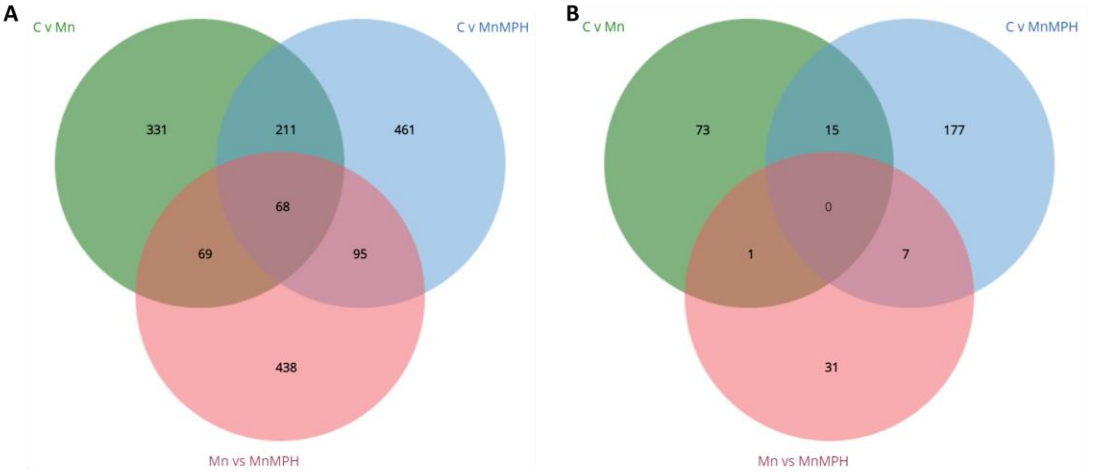
B

GO Terms	Control v Mn	Mn v MnMPH
(GO ID:0007628) adult walking behavior	Downregulated	Upregulated
(GO ID:0006874) cellular calcium ion homeostasis	Downregulated	Downregulated
(GO ID:0006629) lipid metabolic process	Downregulated	Downregulated
(GO ID:0051289) protein homotetramerization	Downregulated	Downregulated
(GO ID:0046986) negative regulation of hemoglobin biosynthetic process	Upregulated	Downregulated
(GO ID:0045947) negative regulation of translational initiation	Upregulated	Downregulated
(GO ID:0046501) protoporphyrinogen ix metabolic process	Upregulated	Downregulated
(GO ID:0030641) regulation of cellular ph	Upregulated	Upregulated
(GO ID:0010999) regulation of eif2 alpha phosphorylation by heme	Upregulated	Downregulated
(GO ID:0046986) regulation of hemoglobin biosynthetic process	Upregulated	Downregulated





3.7.4 Figure 4: Ingenuty Pathway Analysis reveals top causal and upstream regulators of our MPH administration normalize and further alteration phenotype. A/B. Venn diagram comparison of C v. Mn, C v. MnMPH, and Mn v. MnMPH IPA upstream and causal regulators with (A) incuding all $p < 0.05$ significant regulators and (B) including only regulators with activation value (+/-) 2, statistically predicted activated or inhibited. C. IPA regulators from the 69 in 'A' shown to be both altered by developmental Mn and also enhanced by prolonged MPH treatment (C v Mn and Mn v MnMPH intersection). IPA Activation Value before '/' is the value in C v Mn, while the value after '/' indicates the Mn v MnMPH value. All IPA regulators from the three group comparisons can be found in the Supplemental Sheet 10-12. Notable functions of C v Mn comparison (331 regulators) and Mn v MnMPH comparison (438 regulators) are also can be found in Supplemental Figure 3.



C

IPA Upstream/Causal Gene Name	Function	Biological Function Category	IPA Activation Value
Vascular Endothelial Growth Factor A (Vegfa)	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Can be important for motor neuron axon guidance.	Cell Development	3.59/1.80
Lymphoid Enhancer Binding Factor 1 (Lef1)	Transcription factor that binds DNA in a sequence-specific manner and participates in the Wnt signaling pathway by activating transcription of target genes in the presence of CTNNB1 and EP300.	Epigenetics	-2.31/-1.33
Histone Deacetylase 2 (Hdac2)	Catalyzes the deacetylation of lysine residues on core histones, which is epigenetic repression to control transcriptional regulation, cell cycle progression and development.	Epigenetics	-0.86/0.04
Insulin Like Growth Factor 1 (Igf1)	The protein encoded by this gene is similar to insulin in function and structure and is a member of a family of proteins involved in mediating growth and development and leads to activation of PI3K-AKT/PKB and the Ras-MAPK pathways.	Cell Development	2.15/2.22
Solute Carrier Family 7 Member 5 (Slc7a5)	High-affinity transporter that mediates uptake of large neutral amino acids such as phenylalanine, tyrosine, leucine, histidine, methionine, tryptophan, valine, isoleucine and alanine. Can mediate mTORC1 activation.	Neuronal Function	1.40/0.04
G Protein Subunit Alpha 12 (Gna12)	Predicted to enable D5 dopamine receptor binding activity and involved in TOR signaling and regulation of proteasomal ubiquitin-dependent protein catabolic process.	Neuronal Function	1.91/0.65
SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4 (Smarca4)	Involved in transcriptional activation and repression of select genes by chromatin remodeling. Mediates HDAC and CREBBP function in its transcriptional activation of genes function.	Epigenetics	2.63/0.90

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Chapter 4

CONCLUSION AND FUTURE PERSPECTIVE

4.0 Conclusion and Future Perspective

4.1.1 Conclusion

Although manganese (Mn) is an essential nutrient, excess Mn during development is associated with lasting cognitive impairments, such as attention, impulse control, and fine-motor impairments in children and animal models (1-17). Late gestation and early childhood are an important window of susceptibility to Mn toxicity as homeostatic regulation and excretion mechanisms are not fully developed (18-20). The lasting attention, impulse control, and fine-motor impairments in animal models are accompanied by lasting changes in catecholamine system proteins in the PFC and hypo-functioning of catecholaminergic neurotransmitters (21-29).

The mechanism(s) for how developmental Mn may cause these molecular and behavioral changes is complex, thus here in Chapter 1, we presented a review of the literature including how Mn works on the cellular level, the importance of studying early life development as a susceptibility period of lasting Mn neurotoxicity, and epidemiological studies of Mn exposure associations in children, and behavioral and molecular insights from animal models. We further purposed an epigenetic and neuroinflammatory hypothesis for how developmental Mn exposure may mechanistically alter brain regions and neurotransmitter circuits associated with attentional function that contributed to our understanding of Chapter 2.

In Chapter 2, we presented novel experimental data supporting the proposed mechanism that developmental Mn exposure creates a pro-oxidized environment that alters DNA methylation, leading to alterations of gene expression during neurodevelopment, such as through Wnt and mTOR signaling. Our data further suggests the proinflammatory environment generated by Mn exposure propagates activated responses from microglia and astrocytes can further alter catecholaminergic gene and receptor expression that may produce attentional impairments. The fact that we showed that these lasting molecular alterations in Wnt and mTOR signaling pathways are also associated with attention deficits in littermates further establishes the importance of further investigating therapeutic targets for these pathways.

Additionally, in Chapter 3 we have shown that chronic methylphenidate (MPH) administration can normalize Mn-induced differential gene expression in biological functions related to mTOR signaling pathways. In particular our Ingenuity Pathway Analysis causal and upstream regulator evidence suggests MPH affects pathways even further upstream of mTOR signaling, such as with the insulin-PI3-AKT signaling axis. Since, these gene expression outcomes are in animals that have also been behaviorally tested we demonstrate here that a low dose of chronic MPH administration is not only able to normalize gene expression, but is also associated with ameliorating Mn induced attentional deficits (30). Future analysis incorporating DNA methylation state of Mn and MPH altered genes will further contribute to our understanding of Mn neurobiology and efforts to prevent or protect against hypofunctioning catecholaminergic system and attention deficits.

By understanding how Mn may mechanistically drive these catecholaminergic protein changes observed in animals we will gain insight into how Mn mechanistically drives the behavioral deficits phenotype in humans. Additionally, in considering an epigenetic and inflammatory process the potential of methylphenidate in direct treatment of patients with elevated risk of Mn exposure seems promising. Further studies are necessary to elucidate their potential to ameliorate Mn-induced behavioral and biochemical deficits.

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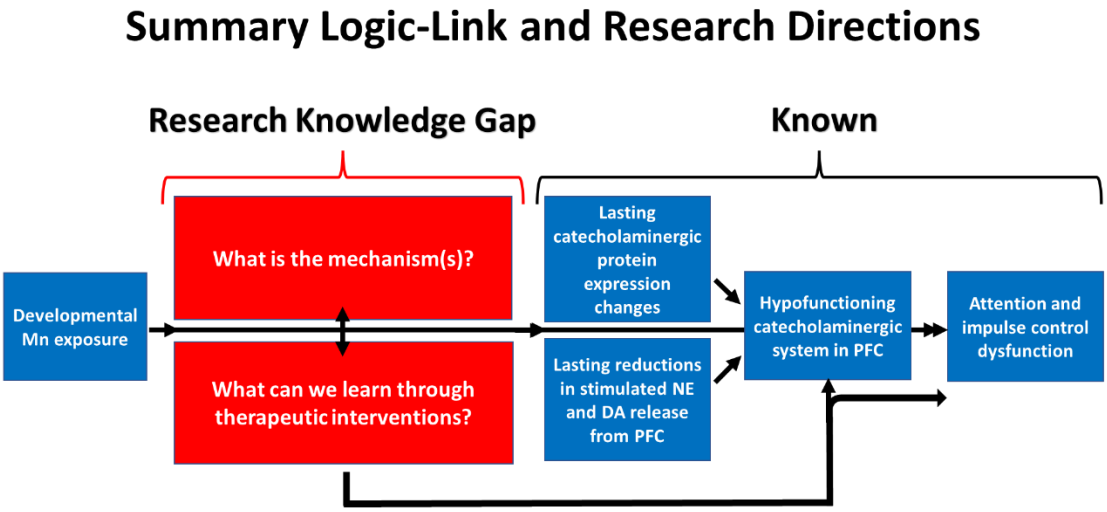
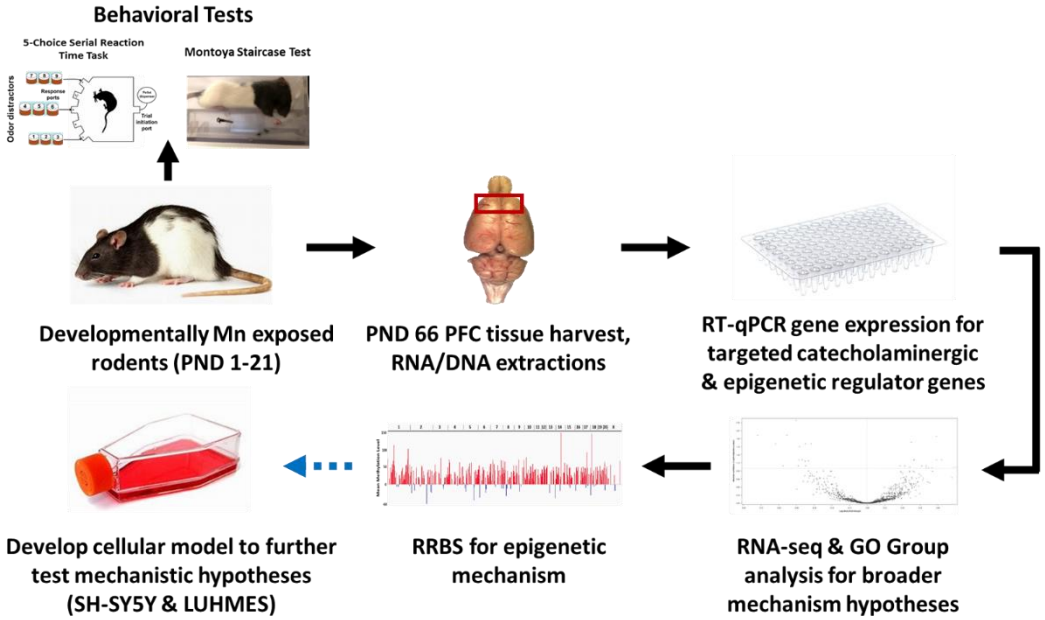
Appendix

5.1 Dissertation Overview

5.1.1 Dissertation "Logic-Link": Visual representation of the logic behind what is known about developmental Mn exposure and what knowledge my dissertation is seeking to fill. Red boxes indicate knowledge gaps, while blue boxes indicate known evidence. Arrows indicate known or predicted associations based on the box colors.



5.1.2 Dissertation Research Aims Visual Overview: Visual of the main experiment components of Chapter 2 and the future directions that the following Appendix figures aims to support. Black arrows indicate completed, while blue signifies future experiments that may be completed based on this work.



5.2. Mn and Cell Culture Studies

5.2.1 Mn and cell culture overview

The etiology for attention deficit disorders in children is multi-factorial, involving both genetics and environmental factors (1-8). Elevated environmental exposure to manganese (Mn) from contaminated well-water, ferroalloy industry emissions, mining, and fungicide/pesticide use is associated with features of ADHD, such as inattention, impulsivity, hyperactivity, and fine-motor impairments in children (8-27). Epidemiological studies, however, are not able to establish causation. To address this, we developed a rat model that reflects late gestation to early childhood and adolecence brain development and Mn levels children may consume through contaminated ground water and/or soy based infant formula; Long-Evans rats are given a 50 mg/kg/day oral Mn dose from postnatal day 1-21 (28-30). We found that developmental Mn exposure causes lasting attentional and fine-motor dysfunction, and that these impairments were accompanied by lasting disruption of the PFC and striatal catecholaminergic (CA) system (28-30). These CA system changes include reduced dopamine and norepinephrine release, and altered expression levels of dopamine D1 and D2 receptors, dopamine and norepinephrine transporters (DAT, NET), and tyrosine hydroxylase (TH) protein (28-30). A significant knowledge gap remains, however, in how developmental Mn causes these lasting CA system changes, and whether the use of therapeutic interventions can normalize these behavioral and CA system deficits.

In order to address this knowledge gap, I first need to establish a cell model that recapitulates the catecholaminergic system protein expression dysfunction (TH, DAT,

D2) in our rat model. A cell model provides a more tractable and easily manipulated system compared to the rat model to establish direct causality of Mn-induced changes on neuronal function for therapeutic approaches.

Here, I differentiated SH-SY5Y human neuroblastoma cells and LUHMES cells to a catecholaminergic phenotype (confirmed by growth pattern, morphology, differentiation marker synaptophysin expression) and exposed them to environmentally relevant Mn concentrations based on prior literature (Figure 1-2) (31-34). MTT cytotoxicity assay showed that Mn levels used here were not overtly cytotoxic (Figure 3). Oxidative stress levels, measured using a standard DCFDA kit, were increased by Mn exposure and is relevant to a potential Mn-induced oxidative stress mechanism for hypofunctioning catecholaminergic systems discussed in more detail in Chapter 2 (Fig. 4). I used quantitative immunofluorescent microscopy to show that Mn exposure caused decreased TH and DAT, and increased D2 protein levels, with magnitude changes that recapitulated the changes observed in our Mn-exposed rodent model previously published (30) (Fig. 5-7).

Overall, these findings suggest that developmental Mn exposure can induce similar catecholaminergic hypofunction at the protein level as in rats. Future studies are required to determine whether differentiated LUHMES cells also have similar catecholaminergic protein changes, and to assess global differential gene expression and differential DNA methylation (in particular Wnt and mTOR signaling pathway dysregulation) in both cell models. If these outcomes replicate the Chapter 2 and

Chapter 3 developmental Mn rat prefrontal cortex findings presented here, these cell models may be used to assess potential therapeutic interventions for Mn neurotoxicity.

5.2.2 Cell Culture Growth and Mn Exposure Paradigm

SH-SY5Y and LUHMES cells were grown and differentiated into mature catecholaminergic expressing cells as described (SH-SY5Y: 31/32 and LUHMES: 33/34). The specific timeline of Mn exposure is detailed visually for each cell type (5.4.1). Briefly, the cells were exposed to Mn (SH-SY5Y: 0-300 uM and LUHMES 0-100 uM) in DMEM/F12 media for one doubling time (SH-SY5Y: 48 hours and LUHMES: Overnight). This was done to allow for any potential pre-differentiation mechanisms of Mn exposure to occur, reflecting a more "developmental period." Once the doubling time was completed, the cells were exposed to their respective differentiation protocol (SH-SY5Y: 31/32 and LUHMES: 33/34) while also given their respective Mn treatment. Prior to the last day of differentiation, cells were passaged with their respective Mn treatment to carryout each of the analyses detailed below.

5.2.3 Cytotoxicity

On the last day of differentiation 5,000 SH-SY5Y and 2,000 LUHMES cells were plated in 96-well plates with their respective Mn treatments (0-300 uM; n=13-16/treatment). Next, the MTT assay colorimetric assay for assessing cell metabolic activity as a proxy for cytotoxicity was used under standard manufacturer instructions. Briefly, differentiation media is discarded and 50 uL serum/phenol free media is used with equal MTT reagent. Plates are incubated in the dark for three hours at 37 degrees

Celsius, then 150 uL of MTT Solvent was added into each well, foil-covered, shaken for 15 minutes on a shaker, then measured OD=590 nm in colormetric platereader.

5.2.4 Oxidative Stress Levels

On the last day of differentiation cells were plated in 96 wells at 5,000 cells SH-SY5Y and 2,000 cells LUHMES per well in respective treatments (SH-SY5Y: 0-300 uM and LUHMES: 0-100 uM; n=8 wells/treatment). For positive controls of increased and decreased oxidative stress levels, 100 uM hydrogen peroxide exposed cells and 1mM N-acetyl-cysteine antioxidant exposed cells, respectively were assessed (n=8 wells/treatment). Oxidative stress levels were measured using standard DCFDA kit instructions. Briefly, differentiation media was removed and cells were rinsed in 1x PBS. After rinse, DCFDA reagent was added to the wells and allowed to incubate for 45 minutes in the dark. DCFDA reagent is then removed and respective Mn and antioxidant treatments (in serum and phenol free media) were once again added as we concluded from a pilot experiment (data not shown) that Mn may act more immediately to increase oxidative stress rather than overtime. After 15 minutes of incubation in Mn and/or antioxidant media oxidative stress levels were measured in a fluorescent plate reader at 485/535 nm every 30 minutes for up to 6 hours.

5.2.5 Catecholaminergic Protein Expression

SH-SY5Y cells were grown, exposed to 0-300 uM Mn, and differentiated into mature catecholaminergic protein expressing cells as described above. Prior to the last day of differentiation cells were seeded at 50,000 cells/well into chamberslides with their perspective Mn treatment (n=2/treatment). After differentiation completion,

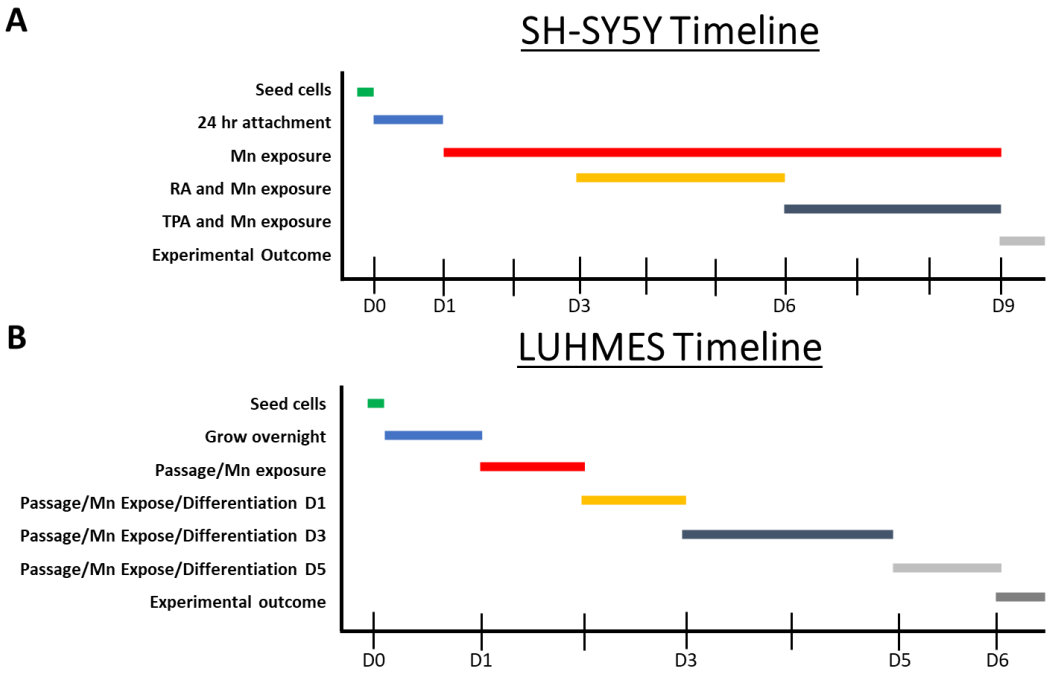
media was discarded and cells were fixed in 4% paraformaldehyde/PBS for 10 minutes room temperature on a shaker. Cells were then washed in 0.1 M PBS on a shaker for three times for five minutes each. After washing, cells were permeabilized with 0.1% Triton X-100/PBS for 30 minutes on a shaker at room temperature. Cells were once again washed with 0.1 M PBS for three times for five minutes each. Next cells were blocked with 1% BSA/1% Normal Donkey Serum/PBS for 1 hour room temperature on a shaker. After blocking, the solution was discarded and cells were incubated in their respective primary antibodies (sheep polyclonal anti-tyrosine hydroxylase (TH): 1:100, Pelfreez-bio P60101; rabbit polyclonal anti-dopamine receptor D2 (D2): 1:250, EMD-Millipore AB5084P; and rabbit polyclonal anti-dopamine transporter (DAT): 1:250, EMD Millipore AB1591P) overnight at 4 degrees Celsius. The following day cells were washed with 0.1 M PBS on a shaker for three times for 5 minutes each. Secondary antibodies at 1:1000 were applied for 1 hour at room temperature foil-covered on a shaker (TH: donkey anti-sheep Alexa 488, Abcam ab150177; D2 and DAT: donkey anti-rabbit Alexa 594, Thermofisher A-21203). Cells were then washed in 0.1 M PBS for three times at five minutes each. DAPI nuclear staining was then applied at 1:1000 in PBS for 10 minutes room temperature. Cells were washed one last time in 0.1 M PBS for three times at five minutes each. PBS was removed, and Fluoromount G mounting media was applied. Chamberslide was coverslipped and allowed to dry overnight, room temperature, in the dark.

Fluorescent microscopy was then conducted at 40 x magnification using the Zeiss Axioimager (n=2 chamberslide wells with 30-37 images per treatment). Images were

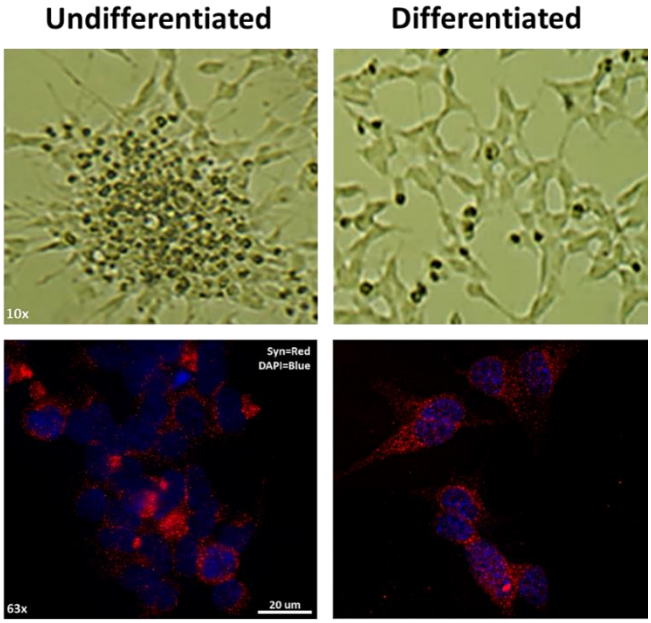
deconvolved using Autoquant software and quantification was processed using the Imaris "Surfaces" tool, with unique quantification algorithms applied to each fluorescence channel and protein. Algorithms were customized to each protein, first by applying automated thresholds for absolute fluorescence intensity and using the "Background Subtraction" tool 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.

5.3 Results

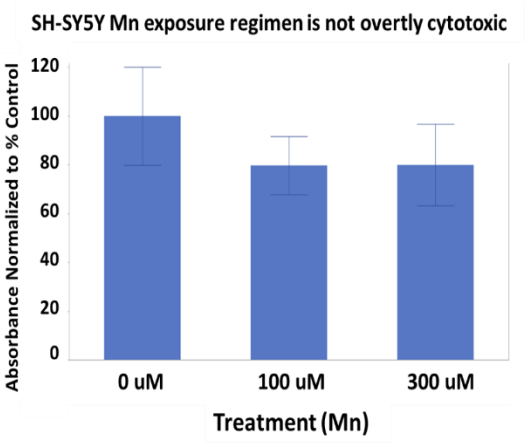
5.3.1 Mn exposure and differentiation timelines: The experimental timelines of Mn exposure and differentiation procedures are displayed below for (A) SH-SY5Y cells and (B) LUHMES cells. Briefly, both cell types were exposed to Mn in efforts to establish a cell culture model of the developmental Mn molecular phenotype (alteration of catecholaminergic system) as chemical administration was done to differentiate them into more adult-like catecholaminergic protein expressing neurons.



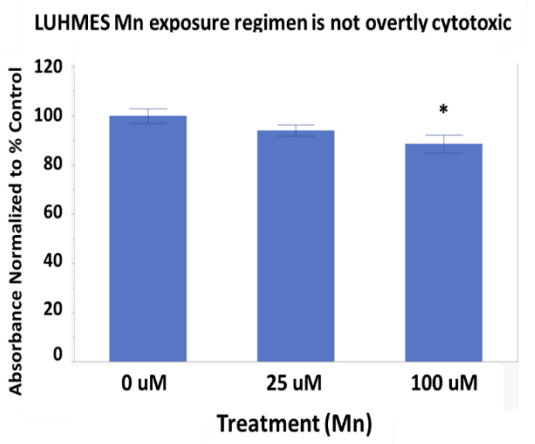
5.3.2 Differentiated SH-SY5Y cells display appropriate morphology and growth patterns: Microscope images of undifferentiated SH-SY5Y and differentiated SH-SY5Y cells are seen below. Top: Undifferentiated cells grow in clusters, while differentiated cells grow in a more spread out distribution with more pyramidal morphology. Bottom: Differentiation marker synaptophysin (red coloration) is visually increased in differentiated cells over undifferentaiated. DAPI nuclear staining of each cell nuclei (blue coloration).



5.3.3 Mn dosing regimen is not overtly cytotoxic in differentiated SH-SY5Y and LUHMES cells as confirmed by MTT cytotoxicity assay. Differentiated SH-SY5Y and LUHMES cells exposed to Mn were assessed for cytotoxicity under standard MTT kit instructions. * indicated $p < 0.05$ compared to control; ANOVA, Tukey HSD Posthoc Test.

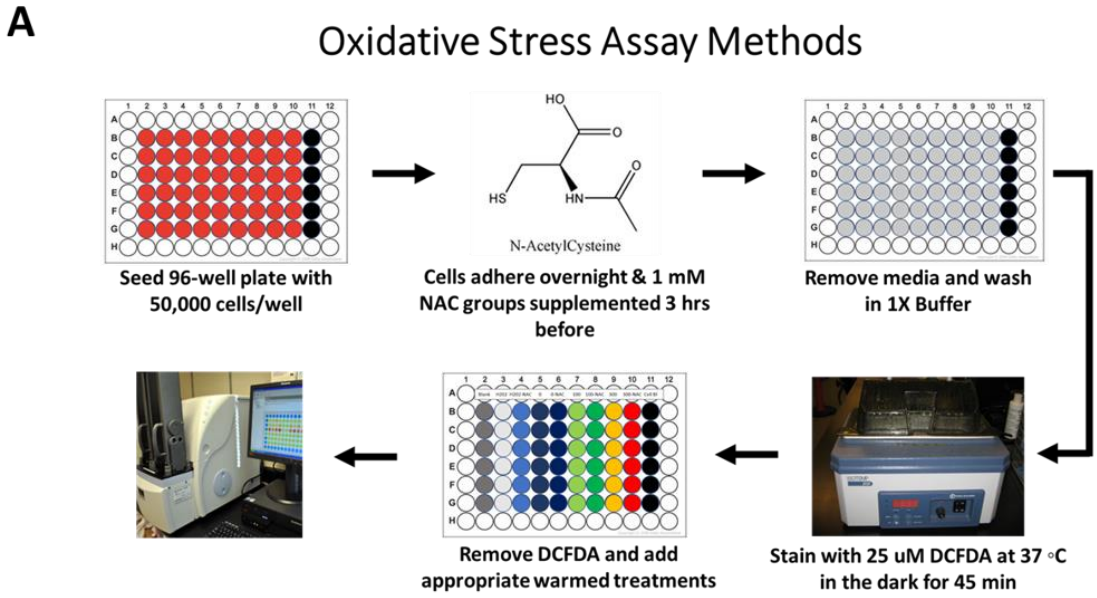


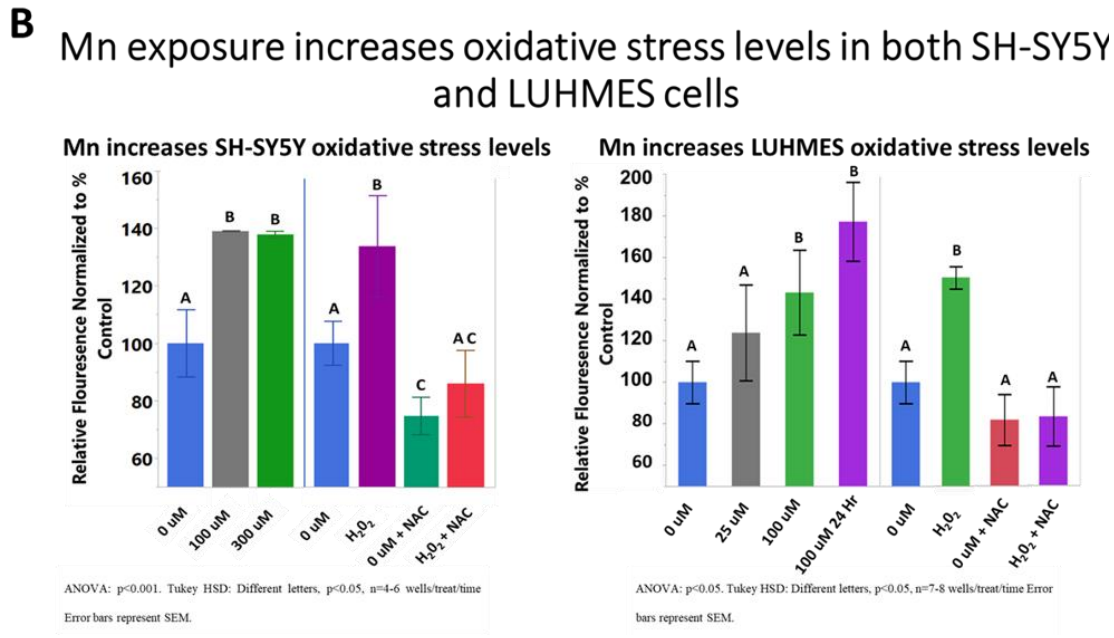
ANOVA: p=0.610, n=15-16 wells/treatment



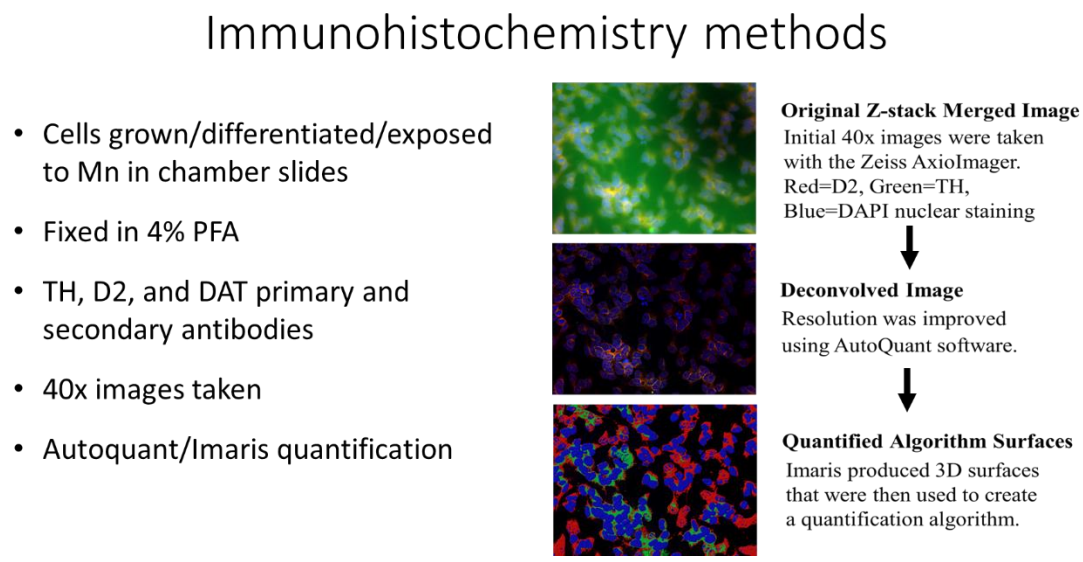
ANOVA: p=0.045; 0 uM-100 uM p=0.035 , n=13-16 wells/treatment

5.3.4 Mn exposure increases oxidative stress levels in SH-SY5Y and LUHMES cells. (A) Shows visual of oxidative stress assessment experimental timeline and plan while (B) shows the measured oxidative stress levels for differentiated SH-SY5Y and LUHMES cells. Both SH-SY5Y and LUHMES cells have elevated oxidative stress levels that are also attenuated by antioxidant response of N-acetyl-cysteine treatment.

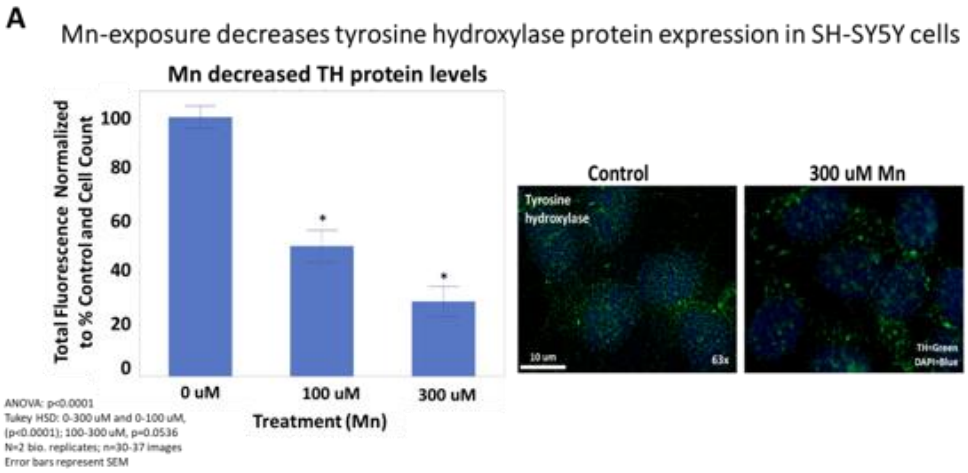


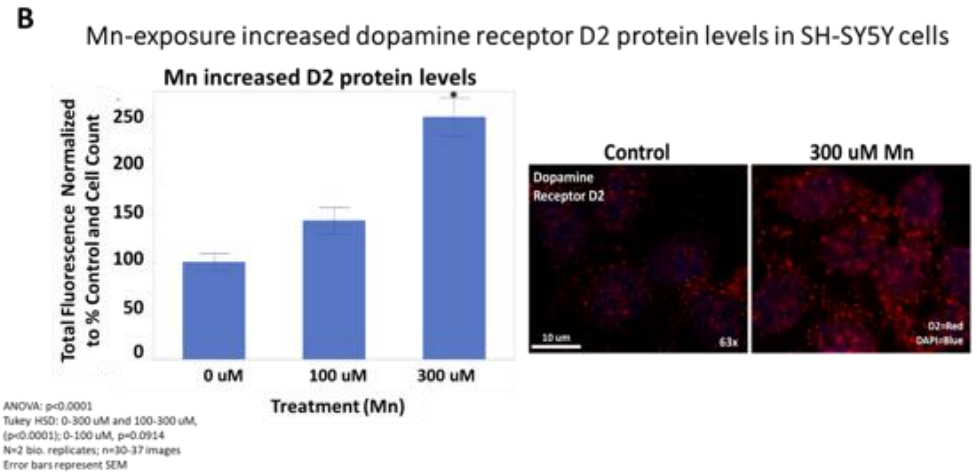


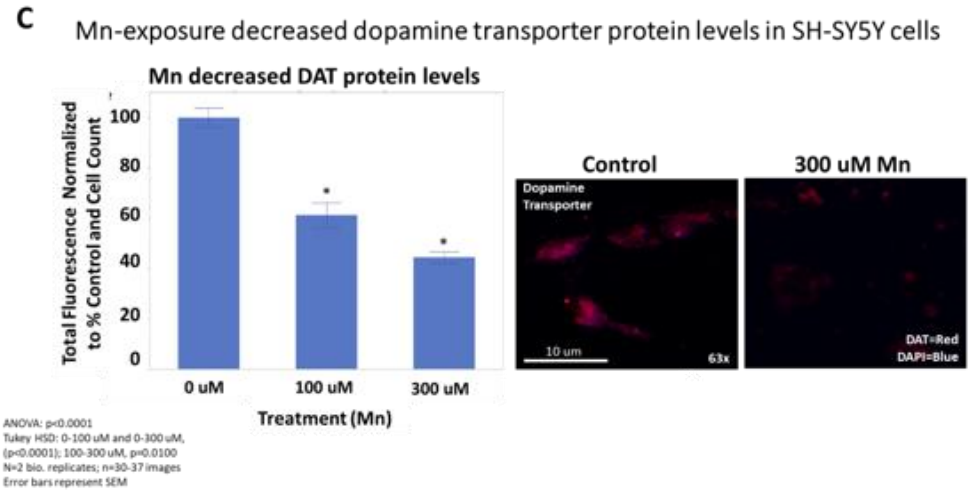
5.3.5 Immunohistochemistry methods to assess developmental Mn exposure alteration of protein levels. A visual graphic of the immunohistochemistry methods for assessing catecholaminergic protein expression levels in SH-SY5Y cells from when they were grown, Mn treated, PFA fixed, and undergone immunocytochemistry and immunofluorescence microscopy analysis.



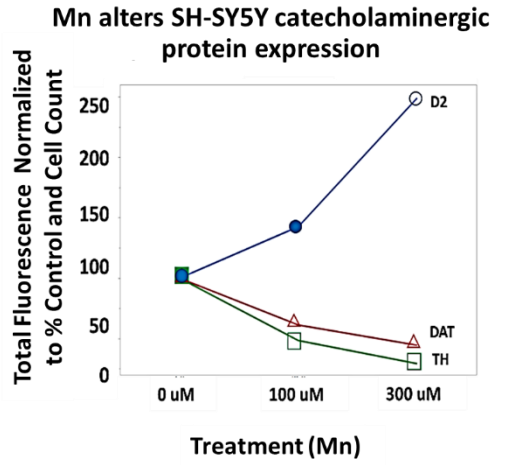
5.3.6 Mn exposure alters tyrosine hydroxylase, dopamine receptor D2, and dopamine transporter protein levels in SH-SY5Y cells (A) Mn exposure decreases tyrosine hydroxylase, (B) increases dopamine receptor D2, (C) and decreases dopamine transporter protein expression levels. * indicated $p < 0.05$ compared to control.



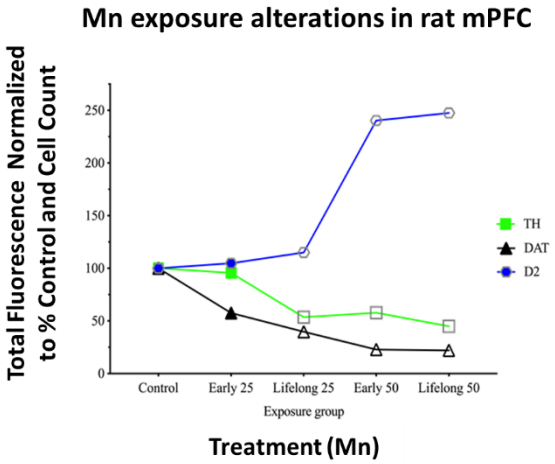




5.3.7 Mn exposure alters catecholamine protein expression levels in SH-SY5Y cells similar to our published findings in rat prefrontal cortex (Conley et al., 2020). Left figure represents the data shown in Appendix Figure 6 of SH-SY5Y catecholaminergic protein expression by percent control/doses of Mn exposure. The right figure represents data from our prior study (Conley et al., 2020) that shows similar developmental Mn exposure induced magnitude of catecholaminergic protein level changes.



Mean levels of differentiated SH-SY5Y cells normalized to % Control and Cell Count. Open symbols indicate significantly different from respective control ($p < 0.0001$). Error bars reflector SEM.



Mean levels of mPFC proteins normalized to respective control group. Open symbols indicate significantly different from respective control ($p < 0.05$).

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LIST OF SUPPLEMENTAL MATERIAL

6.1 CHAPTER 2 SUPPLENTAL MATERIAL

6.1.1 Supplemental Methods and Figure PDF

Supplemental methods, figures, and charts that support the behavioral, differential methylation, gene expression, and protein level findings of the main manuscript.

6.1.2 Supplemental Excel Sheet

Supplemental raw data of differential methylation and gene expression data. Including Mn exposure individually altered genes, Gene Ontology terms, and Ingenuity Pathway Analysis causal and upstream regulator findings.

6.2 CHAPTER 3 SUPPLEMENTAL MATERIAL

6.2.1 Supplemental Methods and Figure PDF

Supplemental figures that support the differential gene expression findings of the main manuscript.

6.2.2 Supplemental Excel Sheet

Supplemental raw data of differential gene expression analysis. Including individually altered and integrated genes, Gene Ontology terms, and Ingenuity Pathway analysis findings of the C v Mn, C v MnMPH, and Mn v MnMPH two way comparisons.