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MATERNAL CHOLINE SUPPLEMENTATION AS A NUTRITIONAL INTERVENTION TO PROTECT AGAINST MN-INDUCED NEUROTOXICITY

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by

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Abstract

MATERNAL CHOLINE SUPPLEMENTATION AS A NUTRITIONAL INTERVENTION TO PROTECT AGAINST MN-INDUCED NEUROTOXICITY

Shanna L. Howard

Studies in children have reported associations between elevated manganese (Mn) exposure and ADHD-related symptoms of inattention, impulsivity/hyperactivity, and psychomotor impairment. Rodent model studies have recapitulated these ADHD-like impairments and found that these deficits are associated with hypofunctioning of the catecholaminergic system in the prefrontal cortex and striatum - brain regions that in part mediate attention, impulse control, emotion regulation, and sensorimotor function – but the mechanism by which Mn causes these lasting alterations is not well understood. While therapies such as methylphenidate (Ritalin) have proven to be effective in lessening symptoms for some children diagnosed with ADHD, there are no established treatments available for children exhibiting ADHD-like symptoms that may be linked to elevated Mn exposure during development.

Ideally, rather than rely on medications to treat the ADHD-like symptoms associated with elevated Mn exposure, it would be preferred to have intervention options that might protect against the neurotoxic effects of Mn. One potential nutritional intervention to protect against Mn-induced deficits is maternal choline supplementation (MCS), which has been shown to provide cognitive benefits to typically developing children and animal models and lessen cognitive and molecular

dysfunction caused by various environmental and genetic insults. Given this, my dissertation focuses on further elucidating the lasting effects of Mn on cognition and neuronal function, and investigates whether MCS is an effective intervention to protect against Mn-induced deficits.

In Chapter 1, I present a review of Mn, including a broad overview of its properties and human usage, its role in physiological systems, as well as toxicity of elevated exposure. This chapter also provides an overview of choline, including sources, biological function, and the cognitive and neurological benefits of choline supplementation. This information will provide context for the gap in knowledge addressed in this research; namely, whether MCS is effective in protecting against Mn-induced cognitive and molecular alterations.

In Chapter 2, I demonstrate that developmental Mn exposure produces a constellation of deficits consistent with ADHD symptomology in a rat model of childhood environmental Mn exposure, including dysfunction in attention, reactivity to errors and reward omission, and deficits in learning and sensorimotor function. I also show that MCS offered some protection against the adverse Mn effects, including lessening Mn-induced attentional dysfunction and partially normalizing behavioral reactivity, but provided no protection against Mn-induced learning or sensorimotor dysfunction.

In Chapter 3, I show that developmental Mn exposure causes lasting molecular alterations in the prefrontal cortex. Using tissues from behaviorally tested animals from Chapter 2, we found that Mn-induced changes include alterations in

expression of a variety of genes, such as those related to neuronal function and inflammation, as well as changes in DNA methylation, all of which may underlie the ADHD-like behavioral phenotype described in Chapter 2. I also demonstrate that MCS is effective in protecting against some, but not all, of these Mn-induced alterations in gene expression and DNA methylation.

Finally, in Chapter 4, I summarize the findings from both data chapters, and discuss how these findings contribute to the fields of both Mn and MCS research. Altogether, the findings presented here provide further compelling evidence that developmental Mn exposure causes ADHD-like symptoms in a rat model of childhood Mn exposure, supporting the epidemiological evidence that elevated Mn exposure is a risk factor for ADHD. These findings also add to the wealth of literature demonstrating that MCS is neuroprotective for offspring and improves offspring cognitive functioning. These data provide additional support for efforts to increase choline intake during pregnancy and lactation, particularly for women at risk of environmental exposure to Mn.

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Chapter 1

DEVELOPMENTAL MANGANESE EXPOSURE AND MATERNAL CHOLINE SUPPLEMENTATION: CURRENT VIEWS

1.1 Chapter Objective

The purpose of this introduction chapter is to provide background on manganese (Mn) toxicity and choline supplementation, to frame and inform the research presented in this dissertation. The research presented here in chapters two and three discusses the lasting effects of elevated Mn exposure on behavior and neuronal function, and the efficacy of maternal choline supplementation to protect against some of these Mn-induced alterations. Therefore, this introduction will provide an overview of Mn, including a broad overview of properties and human usage, its role in physiological systems, as well as toxicity of elevated exposure. This chapter will also cover choline, including sources, biological function, and the cognitive and neurological benefits of choline supplementation. This information will provide context for the gap in knowledge addressed in this research.

1.2 Introduction

Studies in children have reported associations between elevated Mn exposure during development and ADHD-related symptoms, including inattention, impulsivity/hyperactivity, and psychomotor impairment (1–11). Rodent studies have shown that developmental Mn exposure can cause lasting ADHD-like impairments in

attention, impulse control, and sensorimotor function, and that these deficits are associated with hypofunctioning of the catecholaminergic system in the prefrontal cortex and striatum, which are brain regions critical to mediating attention, impulse control, emotion regulation, and sensorimotor function (12–19). Manganese may contribute to neurological deficits and disruption of the catecholaminergic system via mechanisms such as oxidative stress, inflammation and disruption of homeostasis of other metals (20).

Currently there are two main ways to address the potential negative effects of environmental toxicants such as Mn: 1) primary prevention, in which the toxicant is removed from the environment or the body and exposure avoided or reduced, or 2) pharmacological intervention to treat exposure symptoms (21). However, primary prevention is often not feasible because the economic and societal burden of relocating the many people in high-risk areas to "exposure-safe" spaces is challenging and expensive, notwithstanding that many toxins are ubiquitous in the environment. Also, pharmacological interventions, such as methylphenidate for treatment of ADHD symptoms, is not always completely efficacious and may cause negative side effects in some subjects (22).

However, an alternate form of intervention, such as nutritional supplementation in pregnant mothers, may hold promise for partially or fully protecting against the toxic effects of Mn exposure in their offspring, with maternal choline supplementation being an attractive option. Choline is a nutrient vital to many biochemical pathways, including those such as DNA methylation and cholinergic

signaling, that are known to be affected by excess Mn (23,24). Maternal choline supplementation has been shown to protect against deficits caused by genetic abnormalities and environmental insults such as alcohol in preclinical animal model studies (25–34). Similar studies have shown that maternal choline supplementation can improve aspects of executive function in humans and animal models in the absence of a genetic insult or stressor (26,35–41). While the evidence described above lends support to the hypothesis that the essential nutrient choline supplemented during pregnancy and lactation may protect against Mn-induced neurological deficits, this has not yet been tested. This introductory chapter will focus on the toxicity and health outcomes associated with excess Mn exposure, and provide evidence for consideration of choline supplementation as a potential therapy to protect against Mn toxicity.

1.3 Manganese Background

1.31 Manganese Properties and Usage

The transition metal Mn is the fifth most abundant metal in the Earth's crust and the twelfth most abundant of all the elements (42). It is generally found within mammalian systems in the Mn(II) or Mn(III) valence state, but can exist from Mn^{-3} to Mn^{+7} in other settings (43). Manganese is released into the environment by various natural and anthropogenic activities. Natural sources of Mn in the biosphere include weathering of Mn minerals and ores, and hydrothermal vents on the sea floor (42).

Due to its abundance in the environment and unique properties, Mn is used for many industrial purposes. Anthropogenic sources of Mn are abundant, but the largest industrial contributor to Mn release into the environment is steel production (44). Manganese is mined and used extensively in steel production because it contributes to steel strength (44). However, Mn is also found in numerous other products and industries. Manganese is or has been used in agricultural products such as fungicides, pesticides, and fertilizer, as well as in gasoline as an antiknock agent (20,45). Additionally, Mn is found in the healthcare system in products such as Mangafodipir trisodium (MnDPDP), which is used as a contrast agent in MRI imaging, and in trace element supplemented Total Parenteral Nutrition (TNP) solutions which allow for feeding through the veins to bypass the gastrointestinal tract (46,47).

1.32 The Role of Mn in Physiological Systems

Manganese is essential for proper biological function in humans, as it serves as a cofactor for metalloproteins that catalyze a diverse array of biochemical reactions, and many organ systems rely on Mn for proper functioning (48,49). For example, one enzyme in which Mn serves as a cofactor is Mn superoxide dismutase (MnSOD), which is essential for protecting cells that undergo aerobic respiration because it scavenges reactive oxygen species produced by the mitochondria that would otherwise cause oxidative cell damage (50,51). Arginase is another example of a Mn containing enzyme, and it is responsible for the conversion of arginine to ornithine and urea in the brain (49). Manganese is also a cofactor for glutamine

synthetase, the most prevalent Mn-containing enzyme in the brain (expressed in astrocytes) that produces glutamine (52,53). Humans need to ingest a moderate amount of Mn to prevent Mn deficiency and ensure proper functioning of these enzymes. Manganese deficiency is rare, but can lead to deficits in activities of the above enzymes, as well as bone growth and skeletal abnormalities, ataxia, impairments in reproductive function, lipid and carbohydrate metabolism, insulin production, and oxidant defense (54,55).

The uptake and excretion of Mn is a highly regulated process in adults. The liver is responsible for removing the majority of excess Mn absorbed in the GI tract, transporting the excess Mn from the portal blood (or plasma if excess Mn occurred via respiratory exposure) into the bile, thus leading to fecal elimination (56,57). Because the liver is responsible for the majority of Mn excretion, Mn poses a greater risk of toxicity and has been found at elevated levels in patients with hepatic disease (44,56,58,59). Additionally, the biliary excretion system is not fully developed in infants, which leads to limited fecal Mn elimination and increased body retention (48,56). This susceptibility to increased absorption and body retention of Mn in infants is of concern because infancy is also an important period of neurodevelopment (60).

There is insufficient evidence to set an Estimated Average Requirement (EAR) for daily Mn intake, because many factors affect its absorption and excretion, and there are a limited number of studies that have investigated levels of Mn sufficiency to prevent adverse health outcomes. Therefore, guidelines for an adequate

intake (AI) of Mn are generally estimated based on data of median dietary Mn intake, in part because Mn deficiency is also rare. In children, the average AI is from 1.2 mg/day (1-3 years old) to 1.5 mg/day (4-8 years old), and the AI for adult males is 2.3 mg/day and in adult females is 1.8 mg/day (55). Ingestion of excess Mn is generally seen as a more common risk than Mn deficiency. The Tolerable Upper Intake Level (UL), or the highest level of oral intake that is unlikely to cause adverse health effects in humans, was determined by human and animal studies that measured the level at which Mn begins to cause neurological deficits. This level was set at 2-6 mg/day in children, and 11 mg/day for adults (55). However, the UL is also affected by the form of Mn ingested (e.g., in water or food materials), and the nutritional and physiological state of the individual.

1.4 Manganese Toxicity

1.41 Uptake and Toxicity of Various Forms of Mn

Despite the physiological need for trace amounts of Mn, exposure to high levels can be toxic (49,61,62). Humans in certain geographical areas are at higher risk of Mn toxicity due to elevated exposure from various sources including food, water, and industrial outputs. Due to the specific properties of Mn and the way in which it is processed by the body, different routes of exposure present different risks of toxicity. The two main routes of exposure, ingestion and inhalation, vary in their likelihood to cause negative health outcomes. Oral ingestion of Mn is the most common route of

exposure. Manganese is also found in surface and groundwater supplies (20,44). Freshwater, including groundwater, levels typically range from 1 μg/L to 200 μg/L, but levels as high as 18,600 μg/L have been reported in private wells (44,63). Foods such as rice, soy, leafy greens, beans and legumes are all naturally high in Mn (61).

As noted above, the liver of adults is generally effective at removing excess Mn consumed from the diet and water before it reaches the systemic circulation (56,57,64). However, infants, who don't have a fully developed biliary system, are uniquely susceptible to ingestion of foods and water high in Mn (56). It has been shown in mice that excretion of Mn is not established until 17-18 days post-birth (65), and in rats over 40% of ingested Mn is absorbed and retained prior to PND 15 (66). However, in adult rats and humans, only around 1-5% of Mn is absorbed (64,67). Some infant foods are of particular concern because they are very high in Mn. These include soy infant formula and Total Parenteral Nutrition (TNP) (47,68). Fetal exposure to Mn over pregnancy, aside from what is known about changes in maternal blood Mn over gestation, has not been well characterized, but maternal exposure to high levels of Mn may also be a risk for the developing fetus. Several studies have shown that there is an inverted-U shaped relationship between maternal-infant blood Mn levels and infant health outcomes including birth weight and cognitive function, suggesting that both low Mn and excess levels of Mn can have detrimental effects (69–72).

Inhalation of Mn is often considered a more impactful route of exposure than ingestion, particularly in adults (73). Inhaled Mn can enter the circulation via the

lungs, and/or travel directly through the trigeminal nerve into the olfactory region of the brain, bypassing the blood brain barrier (BBB) (73). The inhalation exposure pathway also bypasses the normal first-pass hepatic regulatory pathway by which Mn is absorbed from the intestine into the portal blood, then travels to the liver where it is excreted in bile. Therefore the respiratory exposure pathway allows Mn to skirt the major hepatic physiological mechanism by which Mn homeostasis is regulated in adults (57,73). Mn is often found at high levels in the air near industrial steel production plants and those with related occupations, such as mining and welding, are at high risk for respiratory exposure (57,74). Children living near areas with industrial metal production such as ferroalloy plants can also be exposed to high levels of Mn in dust (75). This exposure is a public health concern given that children are susceptible to Mn-induced neurodevelopmental deficits and inhalation of Mn can be especially neurotoxic.

1.42 Associations Between Mn Exposure and Neurological Deficits in Children and Adults

Studies in children have reported associations between elevated Mn exposure and symptoms of inattention, impulsivity/hyperactivity, and psychomotor impairment. The attention and motor deficits associated with Mn are similar to the symptoms in children diagnosed with ADHD (76). ADHD is the most common neurobehavioral disorder in children, with approximately 6.1 million children aged 2-17 diagnosed with ADHD in the United States (Danielson et al. 2018). A meta-analysis by Shih and

colleagues found, in studies where blood and hair were analyzed, that children with ADHD had significantly higher levels of Mn (79). One set of studies reported that significantly higher levels of Mn in drinking water and hair of children 6-15 years old were associated with hyperactivity, lower IQ scores, inattention, and motor-function deficits (10,78,80). Children assessed in Bangladesh demonstrated similar findings: high levels of Mn in drinking water were shown to be associated with lower achievement test scores and impaired intellectual function as assessed by the Weschler Intelligence Scale for Children, version III (81,82). A more recent longitudinal study in Denmark by Schullehner and colleagues showed that elevated levels of Mn in drinking water were associated with an increased risk of ADHD in children, specifically the inattentive subtype of ADHD (1).

A number of studies have provided evidence that exposure timing can alter outcomes for children exposed to high levels of Mn (7,83,84). One study demonstrated that prenatally, higher levels of Mn were beneficial for adolescent cognition, but that these beneficial associations shifted towards detrimental effects when exposure occurred later in development. These findings were most pronounced in assessments of working memory, problem solving, visuospatial ability and attention (7). In a study of Italian adolescents, researchers found a beneficial association of prenatal and childhood Mn with adolescent verbal learning and memory, but these benefits were not seen in children exposed in the early postnatal period (83).

Research has also pointed to sex-specific effects of developmental Mn exposure (5,84–86). A study by Chiu et al. identified critical windows of development in which sex altered the effects of Mn on neuromotor function in adolescents. They found that higher levels of prenatal Mn were associated with better body stability in boys, assessed by various sway tests, but poorer performance in girls (84). Results from another study indicate that sex and polymorphisms in the Mn transporter gene SLC30A10 were associated with differences in sensitivity to Mn exposure and health effects, as assessed by Conners' scales. Girls had worse Conners' scoring as levels of soil Mn increased, but boys had a positive linear relationship with soil Mn only for the Conners' outcome of hyperactivity, such that exposure to higher levels of Mn was associated with greater hyperactivity. Girls with genotypes linked to higher blood Mn levels showed particularly strong positive associations between soil Mn and parent-reported Conners' scales, suggesting that girls may be genetically less efficient at regulating Mn (5). Rechtman and colleagues found that sex moderates the association between developmental metal mixture exposure and visuospatial learning; in girls, exposure was associated with slower visuospatial learning, which was driven by Mn and Cu levels in blood, urine, hair, nails, and saliva. In boys, metal exposure was associated with faster (i.e., better) visuospatial learning, but this result was driven by Cr (85).

The effects of developmental Mn exposure have been shown to be modulated by co-exposure to other metals (86–89). While measurements from a single biomarker cannot fully capture the toxicokinetic profile for all the chemicals in a

mixture, Levin-Schwartz et al. found that combining exposure data from several biomarkers into an integrated multi-media biomarker measurement can better estimate exposure effects. In their 2021 study, they found that the combined cognitive effects of the Pb and Mn multi-media biomarkers (i.e., a weighted quantile sum of metals in blood, hair, nails, urine, and saliva) were stronger than the effect of any single biomarker. Using these multi-media biomarkers, they found that increases of a Pb-Mn exposure combination better predicted decreases in Full Scale IQ and verbal IQ in adolescents (87). Another study also showed that co-exposure to Pb and Mn produces greater negative cognitive effects than exposure to either alone, such that the effect of blood Pb on IQ was more pronounced when levels of hair and toenail Mn were higher (88).

In addition to cognitive deficits, Mn exposure may also lead to psychomotor impairments, and many children with ADHD also present with co-morbid psychomotor deficits (90,91). Increased levels of soil Mn are associated with motor coordination and dexterity impairments in children ages 11-14 (2). Despite the evidence demonstrating a link between Mn and ADHD, many different genetic and environmental factors have been implicated as causes of ADHD, and there is no one factor that can predict whether a child will be diagnosed with ADHD (92). Therefore, excess Mn exposure may be one of many insults that can contribute to the deficits seen in ADHD-diagnosed children. Despite the wealth of associative research linking Mn to cognitive and psychomotor deficits, association does not imply causation. Proving causation in humans is difficult if not impossible, because exposures occur in

the context of demographic factors that may also impact neurodevelopment and behavior, such as poverty, lack of health care, etc., and these factors are often poorly measured and controlled for in epidemiological studies. Therefore, animal models are essential for establishing a causal relationship between Mn exposure and neurological deficits, and allow for investigation of underlying neurobiological mechanisms of toxicity.

1.43 Manganese is a Causative Agent of Neurological Deficits in Animal Models

Our group has done studies in animal models to determine whether Mn can cause cognitive and motor deficits and investigate potential molecular mechanisms responsible for these deficits. In the first of these studies, Kern and colleagues exposed rats orally to 0, 25 or 50 mg Mn/kg/day from postnatal day 1-21 and subsequently tested them on several cognitive tasks. They found that Mn exposed animals exhibited altered behavior in an open arena, including increased distance traveled and increased exploration of the center zone of the field/decreased thigmotaxis, indicating that they experienced behavioral disinhibition of exploratory behavior. Additionally, Mn-exposed animals were significantly impaired in spatial memory and learning (assessed using the radial arm maze), because they committed more learning errors and had a greater incidence of delay or failure to reach learning criterion (16).

Beaudin and colleagues also performed tests of cognitive and fine motor ability on rats exposed to Mn. In one study, rats were exposed to 0, 25 or 50 mg

Mn/kg/day from postnatal day 1-21 or from postnatal day 1-end of life in adulthood. Using the Montoya staircase test of fine sensorimotor function, Mn-exposed rats were shown to have significant fine-motor control impairments. Additionally, the deficits caused by the higher 50 mg/kg/day dose given in early life were not exacerbated by continued, lifelong exposure to the same dose, indicating that early developmental life was the window most sensitive to the lasting neurotoxic effects of Mn exposure (12,13). Later, this same exposure paradigm was used in rats to assess visual learning, attentional function, and impulse control using the 5-choice serial reaction time task. It was found that Mn exposure caused lasting deficits in attentional preparedness, selective attention, arousal regulation, but not impulse control, similar to symptoms seen in children with the inattentive subtype of ADHD, and as above, lifelong Mn exposure did not worsen the behavioral effects caused by exposure restricted to early life (Beaudin et al. 2017). These models demonstrate a causative link between developmental Mn exposure and deficits in functional outcomes such as attention and motor coordination. Additionally, they demonstrate the need to further investigate potential treatments or preventative measures for these diseases.

1.44 Neurobiology of Mn-induced Deficits in Executive Function

Executive function is described as the synthesis of cognitive processes required for higher order mental function (93). These cognitive processes include attention, impulse control, working memory, response inhibition, planning, judgement, decision making and cognitive flexibility. The prefrontal cortex of the

brain has been implicated in the control of these functions (93). The prefrontal cortex (PFC) is connected to sensory and motor cortices, as well as subcortical structures. These connections allow for suppression of irrelevant stimuli and increased (preferential) processing of relevant stimuli (94,95). Optimal levels of both dopamine (DA) and norepinephrine (NE) are critical for proper PFC functioning. Too much of either, such as during high stress situations, can impair PFC function and cause misguided attention and responses. Too little DA and NE can cause unguided attention, distraction, and decrease impulse control, and these symptoms are hallmarks of ADHD (95). Developmental Mn exposure has been shown to cause lasting reductions in evoked NE and DA release in the PFC, along with changes in levels of dopamine system proteins such as D1 and D2 dopaminergic receptors, dopamine and norepinephrine transporters (DAT, NET), and tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (16,18). Additionally, Santiago et al. found that Mn exposure causes lasting changes in gene transcript levels of *Th* and *Dat* (96). Therefore, Mn-induced dysregulation of the catecholaminergic system is a likely contributor to the behavioral deficits associated with Mn exposure.

1.45 Molecular Mechanisms of Mn Toxicity

Excess Mn can disrupt normal biological functions and lead to toxicity in a variety of ways. Some of these mechanisms have been well characterized, and others are less well understood. One well-accepted mechanism of Mn toxicity is oxidative stress. Redox cycling between Mn(II) and Mn(III) creates reactive radical species

which can react with and damage biomolecules and cells in many ways (97). A potentially important target of Mn oxidation in the brain is dopamine, a key neurotransmitter, and Mn can induce dopamine autoxidation (98). Manganese exposure also leads to the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and hydrogen peroxide in neurons (97,99). These free radicals can damage macromolecules including DNA and RNA, in addition to inducing the release of inflammation mediating molecules. Manganese can also accumulate in the mitochondria and interfere with ATP production and induce cytochrome C release (100), which can eventually lead to apoptosis (57,97,101).

It is particularly noteworthy that Mn may contribute to inflammatory stress. Kern and Smith demonstrated in a rodent model that astrocytes are activated in response to developental exposure to high levels of Mn and this activation increases even long after the Mn insult has ended (17,18). Inceased glial fibrillary acidic protein (GFAP) levels is a marker for astrocyte activation, which generally occurs during central nervous system trauma (102). In the Kern and Smith study, Mn exposure led to increased GFAP levels in multiple brain regions, including the prefrontal cortex, striatum and nucleus accumbens of 24 day old rats, and GFAP levels remained elevated in postnatal day 107 adults, despite Mn exposure ending at weaning (PND 21) (17). Conley et al. also found that both developmental and lifelong exposure to Mn caused a lasting increase in reactive astrocytes in the medial prefrontal cortex, and in addition, these GFAP-positive astrocytes exhibited a greater relative increase in the proinflammatory A1 phenotype compared to the anti-

inflammatory A2 phenotype (18). Astrocyte activation is associaed with production of pro-inflammatory cytokines and reactive oxygen species, either directly or along with activated microglia, resulting in neuroinflammation that may lead to neurodegeneration in cases of chronic activation (103). Additionally, Sarkar et al. (104) demonstrated that Mn exposure can lead to the activation of the NLRP3 inflammasome in vivo, and in microglial cells primed with bacterial lipopolysaacharide (LPS). This causes the cleavage of pro-IL-1β into mature IL-1β, in addition to caspase-1 cleavage, which are both pro-inflamatory signals (104). They also showed that microglia primed with LPS and exposed to Mn had signs of impaired mitochondrial function such as decreased ATP production and respiration rate, and mitochondrial dysfunction is known to contribute to NLRP3 activation (104,105). Sarkar and colleagues also demonstrated that Mn exposure to LPS-primed cells caused an increase in exosome release, and these exosomes contained ASC (an adapter protein that allows for inflammasome formation). Endocytosis of these exosomes by microglia then led to an increase in levels of pro-IL-1β and *NLRP3*. Together, these findings indicate that Mn activates inflammasome pathways and promotes the release of pro-inflammatory molecules (104)

Excess Mn can also alter the homeostatic balance between various other metals. Iron (Fe) is one example, which has some atomic properties similar to Mn and therefore is found in similar biological pathways. Iron and Mn share several transporters including divalent metal transporter-1 (DMT-1) and transferrin receptor, and deficiency in Fe leads to an increased expression of these transporters and thus

greater uptake and accumulation of Mn (106). Additionally, excess Mn has been shown to disrupt cellular Fe homeostasis in PC12 cells and animal models. Manganese increases levels of labile iron and affects iron regulatory protein binding to mRNAs that encode iron-related proteins (107,108)

Manganese also interacts with various other molecules to cause toxicity. In rats, elevated Mn exposure has been shown to impede the blood brain barrier transport of choline via cationic competition, thus reducing brain uptake of choline (109). Additionally, Mn can alter the expression of miRNAs, which are necessary for proper gene regulation and expression (110). A number of miRNAs have been found to increase in expression, and some have also been shown to decrease in human cells exposed to excess Mn. Several of these miRNAs even target Mn-transporter genes, including at least five miRNAs that are thought to target the Mn efflux transporter ATP13A2 (111).

Finally, another emerging mechanism by which Mn may cause lasting neurotoxic effects is modulation of epigenetic regulation, which may in turn cause changes in gene expression. A number of studies have demonstrated that Mn exposure causes alterations in epigenetic markers, including DNA methylation and histone modifications. Guo and colleagues demonstrated that Mn causes changes in acetylation status of histones by altering the expression of histone acetyl transferase (HAT) and histone deacetylase (HDAC) (112). In a cell model, chronic exposure to Mn caused changes in methylation at key genes that are important for neuronal cell health, including those involved in the onset of Parkinsonism, as well as genes

involved in chromatin modification and regulation of transcription (113). In a mouse model of maternal Mn exposure, male offspring had lasting promoter hypermethylation and transcript downregulation in the hippocampus, including in genes involved in neurogenesis and neuronal function (114). A study in humans showed that DNA methylation in placental samples was associated with body Mn levels (assessed via toenail Mn levels); 713 loci had methylation levels associated with Mn exposure, including several involved in neurodevelopment (115). Finally, a study from our lab demonstrated that developmental Mn exposure in male rats caused lasting alterations in DNA methylation that were associated with changes in gene expression, including in genes related to inflammation and neuronal function (96). These studies provide evidence that Mn-induced modifications of epigenetic markers, such as DNA methylation, may contribute to the lasting alterations in expression of various proteins (16,18), and genes (96,112,116–118) in Mn-exposed cells and animals.

1.5 Addressing Environmental Mn Exposure Health Outcomes

Generally, there may be two main ways to manage the potential or realized adverse health effects from an environmental toxicant such as Mn. The first is primary prevention, which involves preventing exposure to the toxicant altogether. This may involve removal of the exposure source (e.g., paint abatement for Pb, removal of the toxicant from the industrial manufacturing, etc.), removal of the subject from the contaminated environment, or some combination of both. Although

effective, preventing toxin exposure is not always feasible for a variety of reasons, such as inability to finance relocation of at-risk subjects or populations, ubiquity of a toxin in the environment, and/or the subject not even knowing the toxin is present in their environment.

Another approach may be therapeutic treatment to reduce body levels of the toxicant, or to treat the symptoms of intoxication. For example, chelation therapy is used to remove lead from tissues of lead-exposed individuals, particularly those presenting with symptoms of lead toxicity such as neurological deficits, gastrointestinal irritation, and encephalopathy (119). Because the symptoms of Mn induced deficits are similar to those seen in children with ADHD (i.e. attention, impulse control and motor deficits), animal studies have been done in our group to determine the efficacy of therapeutics in alleviating these deficits in a model of childhood Mn exposure. The most commonly prescribed drug for the treatment of ADHD is methylphenidate (120,121), which acts by blocking the dopamine and norepinephrine re-uptake transporters DAT and NET, and thereby increase synaptic levels of DA and NE in the brain (122). Therefore, work in our lab has investigated the efficacy of methylphenidate to alleviate Mn-induced cognitive deficits. Beaudin and colleagues demonstrated that a 2.5 mg/kg/day dose of methylphenidate alleviated impulse control and fine motor deficits but exacerbated focused attention deficits in the rat model of Mn exposure deficits described earlier. Additionally, methylphenidate increased the spine density on pyramidal neurons in the prefrontal cortex of Mn-dosed animals; however, treatment of non-Mn exposed animals with methylphenidate

actually reduced neuronal spine density in the same region (Beaudin et al. 2017; Beaudin et al. 2015). Finally, in a more recent study from our group, a dose-response effect of methylphenidate was investigated in animals dosed with 50 mg Mn/kg/d from postnatal day 1 to 21. In male animals, 0.5 mg/kg/d of methylphenidate ameliorated Mn attentional dysfunction, whereas 3.0 mg/kg/d of methylphenidate diminished sensorimotor deficits caused by Mn (123). In female animals, acute methylphenidate dosing with the 0.5 mg/kg/d dose attenuated Mn-induced reaching dysfunction without alleviating the grasping dysfunction (124).

These studies show that pharmacological interventions may provide mixed efficacy depending on the domain of function evaluated, and additionally, stimulant medications such as methylphenidate can cause unwanted side effects (including loss of appetite, dizziness, irritability, heart and blood vessel problems, etc.). Since primary prevention and pharmacological intervention aren't always feasible or successful, investigations are needed to find another treatment option to prevent or protect against the negative effects of excess Mn. One potentially efficacious treatment may be maternal choline supplementation, which has been shown to provide cognitive benefits in a variety of studies done in rodents and humans (Blusztajn, Slack, and Mellott 2017).

1.6 Choline Background

Choline is an essential nutrient that is critical for proper physiological function. Choline is utilized in several critical biochemical pathways, including

synthesis of the neurotransmitter acetylcholine, formation of membrane phospholipids, and one-carbon reactions involving donation of a methyl group (125). Cells can synthesize phosphatidylcholine de novo, and this can then be converted to choline via the CPD-choline pathway (126). Phosphatidylcholine synthesis occurs via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway and utilizes the membrane phospholipid phosphatidylethanolamine and methyl donor S-adenosyl methionine (SAM) (Vance 2013; Steven H Zeisel 2006). However, choline is considered an essential nutrient because this pathway alone can't supply enough choline to meet physiologic needs (128).

While choline is an essential nutrient, the adequate intake (AI) level for adults or children is not well known. In fact, current AI levels for people of all ages and genders are based on a single study by Zeisel et al. (1991) that determined a choline intake level to prevent liver dysfunction in men (Institute of Medicine (US) Food and Nutrition Board 1998; S H Zeisel et al. 1991). In this study, Zeisel and colleagues showed that after just three weeks on a choline restricted diet, healthy men developed signs of liver injury (Zeisel et al. 1991). The recommended adequate intake level for women during pregnancy (450 mg/day) and lactation (550 mg/day) was increased slightly from that of non-pregnant adult women (425 mg/day), based on studies that measured fetal, placental, and breast milk concentrations of choline, with the assumption that levels in these tissues were equivalent to the additional amount a pregnant or lactating woman would need to ingest (131). However, no human studies have been done to determine whether these "adequate intake" levels are actually

adequate for optimal physiological function, despite the knowledge that choline is crucial for fetal development. In a study utilizing patient-reported dietary intakes, consumption of increased levels of choline was inversely associated with risk of neural tube defects (132). It has also been demonstrated that an increase in choline intake does not lead to increased excretion in urine during pregnancy, further suggesting that the demand for choline increases during pregnancy and that additional dietary choline is being utilized in some way by the pregnant mother or fetus (133).

While further studies are needed to fully elucidate an appropriate choline AI level for different life stages, a nationwide survey done by the National Center for Health Statistics between 2009 and 2012 revealed that only approximately 10% of the population over two years of age even met the current AI; this level dropped to just under 7% for adults over 19 (134). Critically, only ~8.5% of pregnant women consume the adequate intake of choline (135), indicating that a large proportion of infants may not be receiving sufficient levels of choline to meet developmental needs. The foods highest in choline include liver at ~0.5 mg/g, and eggs at ~0.25 mg/g (136,137). Additionally, meats, fish, wheat germ, dairy products, peanuts, and cruciferous vegetables provide moderate amounts of choline ranging from ~0.025 mg/g to ~0.075 mg/g (136).

1.7 Benefits of Maternal Choline Supplementation

1.71 Cognitive Benefits of Choline Supplementation in the Absence of a Stressor or Disease

Both human and rodent studies have demonstrated that choline supplementation can provide cognitive benefits. Williams, Meck and colleagues have done numerous studies investigating the effects of maternal choline supplementation (MCS) on memory and attention in offspring. These studies have continuously demonstrated that prenatal choline supplementation in rats leads to better performance on impulse inhibition and spatial memory processing tasks when compared to non-supplemented or choline deficient counterparts. (38–40,138–142).

Results from a human study done by Caudill and colleagues indicate that MCS during the third trimester of pregnancy improves infant performance on tests that indicate cognitive ability. The diets of the mothers recruited for this study were tightly controlled, with the control group receiving 480 mg of choline per day and the supplemented group receiving 930 mg per day (compared to the adequate intake value of 425 mg per day). Infants were tested at approximately 4, 7, 10 and 13 months of age on two visual attention tasks: visually guided reactive saccades, which measures how abruptly an infant can shift visual fixation onto a target, and memory-guided anticipatory saccades, which measures whether an infant predicts the appearance of a picture due to patterns of past picture movement and moves their eye in anticipation of the photo appearance (143). The infants from mothers who received the choline supplemented diet had a lower mean reaction time across all ages of testing, indicating the ability to process information more rapidly. Additionally,

longer durations of elevated choline intake increased these cognitive benefits, even in the offspring of mothers receiving only the adequate intake (36). A follow-up study assessed sustained attention in the offspring at 7 years old, and found that children in the supplemented group showed superior performance on the primary endpoint (task score) compared to the control group, and a superior ability to maintain correct signal detections across sessions, indicating that maternal choline supplementation improved sustained attention (35). These findings are promising because they recapitulate results from the numerous rodent studies demonstrating that MCS confers a cognitive benefit to offspring (38–41,139,142). Therefore, positive findings from similar rodent disease models may also translate to benefits in humans.

In addition to maternal supplementation benefits, choline may even provide benefits when not maternally supplemented. Tabassum and colleagues did a study to test the motor and cognitive effects of choline supplementation in adult rats and measured different chemicals in the brain that are thought to be responsible for these functional benefits. They found that the choline supplemented rats had improved locomotor activity, increased muscular strength, and improved performance on memory tests. In addition, lipid peroxidation decreased, antioxidant enzyme activity increased, and levels of antioxidant compounds increased as well. In addition, levels of the neurotransmitter acetylcholine (ACh) and monoamines DA and NE increased (144).

1.72 Benefits of Choline Supplementation in Genetic Disease Models

In addition to studies investigating the effects of choline supplementation on "normal" animals and humans, researchers have investigated the effects of MCS on models of genetic disorders. Several studies have been done to test whether MCS can protect against deficits in a mouse model of down syndrome, and MCS from conception until weaning has been shown to improve selective attention, focused attention, emotional regulation, and spatial mapping in this model, in addition to increasing hippocampal neurogenesis (27–30). In the first of these studies by Moon et al., pregnant mouse dams were supplemented with choline from insemination to postnatal day 21, and their offspring tested on attention and emotional regulation using the 5-choice serial reaction time task. They found that offspring from supplemented down syndrome dams performed better than their unsupplemented down syndrome counterparts, and did not significantly differ from controls in performance on more difficult tasks (30). Velazquez et al. used a similar choline supplementation paradigm and tested the mice using a radial arm water maze. They found that offspring of maternally choline supplemented down syndrome dams performed significantly better than the unsupplemented down syndrome mice, indicating an improvement in spatial learning. This improvement was found in conjunction with increased hippocampal neurogenesis, indicated by increased doublecortin-positive cells in the hippocampus (doublecortin is a marker for immature neurons) (29). Ash et al. recapitulated these radial arm water maze results in the same animal model and also found that, in the medial septum, the number and

density of cholinergic neurons was normalized to control levels in the maternally choline supplemented down syndrome mice (28).

Another study by Langley et al. used a mouse model with an autism-like phenotype (BTBR model). Mice dams received MCS from the day of mating to PND 21, and offspring were assessed using a variety of tasks intended to test locomotor activity, exploratory behavior, anxiety, repetitive behavior, and social interaction. Choline supplementation in the dams normalized offspring anxiety and exploratory behavior to control levels in the open field test. Maternal choline supplementation also decreased repetitive behavior, as assessed in the marble burying task, in both control and BTBR mice. Choline supplementation also normalized the percentage of open-arm entries in the elevated plus maze, indicating decreased anxiety compared to non-supplemented counterparts. Also, a deficit in social engagement time seen in the control BTBR mice was significantly improved in supplemented BTBR mice (145).

Chin et al. (146) investigated whether choline supplementation from PND 1-21 could protect against neurological deficits in a mouse model of the genetic developmental disorder Rett syndrome. The authors found that choline supplementation of mice from PND 1-21 was able to alleviate coordination deficits, anxiety, and social preference. Immunohistochemistry of the mouse brains revealed that MCS rescued the neurite length and abundance of branching phenotype characteristic of the Rett syndrome model. Additionally, cell culture methods using shMeCP2 neurons showed that the addition of 100 μM choline to the media every two days also normalized cell body size, dendritic length and neuronal levels of

synapsin1, a protein associated with presynaptic membrane vesicles. The authors tested whether blocking each of the three main choline pathways would prevent the beneficial effects of choline supplementation in the shMeCP2 neurons. Inhibiting acetylcholine synthesis and inhibiting DNA methylation did not show any effects, and choline was still able to rescue mean total dendritic length. However, inhibiting phosphatidylcholine synthesis using geranylgeraniol prevented these morphological benefits seen from choline supplementation. Therefore, it can be hypothesized that the phosphatidylcholine synthesis pathway is crucial for mediating neuron growth and function in this model (146).

1.73 Benefits of Choline Supplementation in Environmental Insult Models

Choline and other methyl donors have been shown to provide similar benefits to those described above in models of external stressors. For example, Schneider, Thomas and colleagues did a variety of studies to assess the benefits of perinatal choline supplementation in a model of fetal alcohol syndrome. They found that MCS during pregnancy or shortly thereafter attenuated the negative effects of fetal alcohol exposure, and that offspring of choline supplemented dams performed better on discrimination, spatial learning, and working memory tasks, and showed normalized perseverative behavior and hyperactivity (33,34,147–149). In addition, results of behavioral tests indicated that direct (postnatal) choline supplementation during young adulthood (PND 40-60) is able to attenuate some working memory deficits caused by alcohol exposure, but not the hyperactivity and spatial memory deficits

(150). Shulz et al. determined that in a model of maternal stress, which has been shown to induce sex-specific changes in offspring cognition, behavior, and body mass (151), MCS through gestation and lactation normalized anxiety-related behaviors seen in females and social behavioral deficits seen in males (152). Maternal choline supplementation also attenuates seizure-induced memory impairment in rats (31,153), and recognition memory in a rat model of fetal-neonatal iron deficiency (32,154). In humans, MCS lessens deficits in cognition, including working memory, visual recognition memory, verbal memory, and eyeblink conditioning, as well as normalize postnatal growth in human infants born to alcohol-using mothers (155–157).

1.74 Epigenetic Effects of Choline Supplementation

There is evidence that supplementing the maternal diet with choline can alter the epigenetic state of offspring, particularly DNA and histone methylation, presumably because choline is a methyl donor. In a 2012 human study, Jiang and colleagues found that MCS during the third trimester altered the epigenetic status of fetal-derived tissues. In the placenta and cord venous blood, MCS caused higher DNA promoter methylation and gene expression of cortisol-regulating genes. Global DNA methylation, histone methylation, and expression of several methyltransferases were also higher in the placenta of MCS offspring (158). Studies by Kwan et al. in mice demonstrated that MCS alters DNA methylation, globally and at specific promoters, as well as expression of a variety of genes including those involved in inflammation (159,160). Roque-Jiminez et al. found that supplementing pregnant ewes throughout

gestation with an herbal choline formula increases the percentages of 5-hydroxymethylcytosine (5-hmC) in whole blood, and offspring born from ewes supplemented during the second trimester of gestation maintained the greatest percentage of 5-hmC (compared to no supplementation and supplementation during other trimesters) for several weeks post-birth (161).

A study in a high-fat diet mouse model found that prenatal MCS modified offspring DNA methylation, and interacted with the high-fat feeding status of both dams and offspring. In dams under the high-fat diet, MCS increased global DNA methylation and DNA methyltransferase 1 (*Dnmt1*) expression in fetal liver and brain. However, during the postnatal period, offspring that received MCS and remained on a high-fat diet were found to have lower global DNA methylation, and *Dnmt1* expression was unaltered in both the liver and visceral adipose tissue (162). Dolinoy et al. determined that bisphenol-A (BPA), a known estrogenic additive in plastics, leads to decreases in methylation of CpG sites in some genes, which leads to increased expression of these genes, and that this hypomethylation can be counteracted by maternally supplementing with methyl donors such as folic acid (163).

Several studies have demonstrated that choline supplementation may normalize alterations in DNA methylation caused by fetal alcohol exposure (164–166). In humans, prenatal alcohol exposure significantly elevates DNA methylation of proopiomelanocortin (*POMC*) and *PER2*, and increases levels of stress hormone in both mothers and offspring. In young children diagnosed with fetal alcohol-related

disorders, nine months of choline supplementation reduced DNA hypermethylation and increased expression of POMC and PER2 (166). Bekdash et al. demonstrated in rats that prenatal alcohol exposure alters histone and DNA methylation in *POMC*-producing neurons, as well as expression of epigenetic modulator genes including histone and DNA methyltransferases. Maternal choline supplementation normalized levels of methylation in the *POMC* gene, as well as expression of methyltransferase genes and corticosterone response to LPS, thus protecting against some adverse effects of prenatal alcohol exposure on these neurons (165). Otero and colleagues used a rat model of direct supplementation in which alcohol was given to rat pups from PND 2–10, and either choline or saline was administered subcutaneously to each subject from PND day 2 to 20. At PND 21, alcohol exposure caused DNA hypermethylation in the hippocampus and PFC, which was significantly reduced after choline supplementation. In contrast, control animals showed increases in DNA methylation in both regions after choline supplementation, suggesting that choline supplementation has different effects depending upon the initial state of the brain (164). Numerous studies describe the effect of choline supplementation to alter epigenetic states, particularly DNA and histone methylation, as well as to normalize changes in methylation caused by stressors such as fetal alcohol exposure, which may play a role in the ability of MCS to provide cognitive benefits to offspring.

1.8 Potential Mechanisms by which MCS may Protect Against Mn-Induced Toxicity

There is some evidence that MCS may interact with or affect directly various pathways that are altered upon exposure to high levels of Mn (24,61). One theoretical mechanism by which MCS may normalize or protect against the effects of elevated Mn exposure may be to normalize activity of Mn-altered enzymes. Intraperitoneal injection of choline chloride into rats has been shown to increase tyrosine hydroxylase activity, the rate limiting protein in dopamine synthesis (167). Manganese exposure in early postnatal development leads to reduced levels of tyrosine hydroxylase protein (18), and therefore choline supplementation has the potential to normalize this effect. Manganese exposure can also affect activity of acetylcholinesterase (AChE), the enzyme that hydrolyzes excess acetylcholine in the synapse (168). This Mn effect on enzyme activity can vary depending on the route and age of exposure, summarized by Peres et al. (61), but several studies in rats show that oral Mn exposure increases AChE activity (99,169,170). MCS may protect against this effect, as Cermak et al. found that prenatal choline supplementation from gestational day 11-17 reduced AChE activity in the hippocampus compared to control animals, up to five weeks postnatally (171). However, it remains unclear whether MCS can protect against the lasting effects of developmental Mn exposure that persist into adulthood.

As discussed, Mn exposure causes a change in expression of various catecholaminergic system proteins such as TH, D1 receptor, D2 receptor, DAT, and NET (16,18), presumably contributing to the behavioral and motor deficits seen in children and animals exposed to excess Mn. There is evidence that expression of

many if not all of these proteins are epigenetically regulated by DNA methylation (172–176). Since choline is a methyl donor and availability during development can alter methylation status of many genes (163,177), choline supplementation in a model of Mn exposure may act to normalize these protein levels via epigenetic methylation mechanisms. Additionally, as mentioned earlier, several studies have demonstrated that choline supplementation may normalize alterations in DNA methylation caused by fetal alcohol exposure(164,165,178). Manganese has been shown to cause alterations in global DNA methylation (96), and thus MCS may protect against this broad effect as well.

Finally, there is also evidence that Mn may reduce the magnitude of the beneficial neurological and cognitive effects of MCS. As mentioned earlier, Lockman and colleagues found that Mn significantly inhibited choline uptake into the brain through the blood brain barrier by competing for the choline transporter. Manganese was tested at various doses, and increased levels of Mn tended to cause decreased brain uptake of choline, as determined by comparing the amount of choline in the perfusate versus the amount of choline in digestates of various brain regions. The authors found that elevated Mn significantly inhibited choline uptake in the frontal cortex, parietal cortex, hippocampus, and caudate putamen but not in the thalamus, hypothalamus or cerebellum (109). Therefore, in individuals receiving MCS, exposure to high levels of Mn may lessen the uptake of the supplemented choline into the brain tissue.

1.9 Gaps to be Addressed

Elevated Mn exposure, especially during early development, has been associated with cognitive and motor deficits in humans. Animal models have recapitulated these deficits, thus demonstrating causality, and have also shown that Mn exposure causes alterations in protein expression, gene expression, and DNA methylation. Maternal choline supplementation has been shown to protect against similar types of neurological deficits and confer cognitive benefits in various animal models and in healthy individuals, as well as normalize molecular changes caused by a variety of stressors and genetic abnormalities. However, few studies have been done to assess the efficacy of MCS in models of environmental toxicant exposure, especially in cases where developmental exposure produces deficits that persist into adulthood. In light of this, the research presented in this dissertation tests the specific hypotheses that Mn exposure causes alterations in behavior and molecular outcomes including gene expression and DNA methylation, and that MCS may protect against some or all of these Mn-induced alterations. If MCS proves efficacious in normalizing cognitive and molecular outcomes caused by Mn, the societal benefits could prove monumental. At-risk populations such as those living in high Mn areas could see huge benefits from something as straightforward and tractable as increased choline intake during pregnancy and lactation.

Chapter 1 References

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Chapter 2

MATERNAL CHOLINE SUPPLEMENTATION LESSENS THE BEHAVIORAL DYSFUNCTION PRODUCED BY DEVELOPMENTAL MANGANESE EXPOSURE IN A RODENT MODEL OF ADHD

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Abstract

Studies in children have reported associations between elevated manganese (Mn) exposure and ADHD-related symptoms of inattention, impulsivity/hyperactivity, and psychomotor impairment. Maternal choline supplementation (MCS) during pregnancy/lactation may hold promise as a protective strategy because it has been shown to lessen cognitive dysfunction caused by numerous early insults. Our objectives were to determine whether (1) developmental Mn exposure alters behavioral reactivity/emotion regulation, in addition to impairing learning, attention, impulse control, and sensorimotor function, and (2) MCS protects against these Mn-induced impairments**.** Pregnant Long-Evans rats were given standard diet, or a diet supplemented with additional choline throughout gestation and lactation (GD 3 - PND 21). Male offspring were exposed orally to 0 or 50 mg Mn/kg/day over PND 1–21. In adulthood, animals were tested in a series of learning, attention, impulse control, and sensorimotor tasks. Mn exposure caused lasting dysfunction in attention, reactivity to errors and reward omission, learning, and sensorimotor function, recapitulating the constellation of symptoms seen in ADHD children. MCS lessened

Mn-induced attentional dysfunction and partially normalized reactivity to committing an error or not receiving an expected reward but provided no protection against Mn-induced learning or sensorimotor dysfunction. In the absence of Mn exposure, MCS produces lasting offspring benefits in learning, attention, and reactivity to errors. To conclude, developmental Mn exposure produces a constellation of deficits consistent with ADHD symptomology, and MCS offered some protection against the adverse Mn effects, adding to the evidence that maternal choline supplementation is neuroprotective for offspring and improves offspring cognitive functioning.

1. Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental disorder among children, affecting ~5-7% of youths up through age 18 years, and ~2–3% of adults (1). ADHD is characterized by symptoms of inattention, impulsivity/hyperactivity, emotion dysregulation, and in some cases psychomotor deficits (2–4). Notably, emotion dysregulation has been shown to have a greater impact than inattention and hyperactivity on overall well-being and self-esteem, including peer relationships, academic performance and occupational attainment (5,6). While the cause(s) of ADHD and related symptoms remains poorly understood, there is an emerging body of epidemiological evidence showing that developmental environmental insults, such as exposure to elevated manganese (Mn) levels, is associated with increased risk of ADHD and related symptoms in children and adolescents (7,8,17,9–16). Although these epidemiological studies have played a

key role in raising concerns about elevated Mn exposure, they are unable to establish causality, in part because of challenges in accurately measuring Mn exposure, and quantifying and controlling for potential confounding factors.

Our prior rodent model studies have shown that developmental Mn exposure can cause lasting ADHD-like impairments in attention, impulse control, and sensorimotor function. In addition, these deficits are associated with hypofunctioning of the catecholaminergic system in the prefrontal cortex and striatum - brain regions that in part mediate attention, impulse control, emotion regulation, and sensorimotor function (18–25). While our rat model of developmental Mn exposure recapitulates many of the features of ADHD, it remains unclear whether Mn exposure also causes lasting disruption of behavioral reactivity/emotion regulation. This is a significant knowledge gap, given the central role that emotion dysregulation plays in ADHD symptomology and quality of life for children with ADHD (3–5), and the fact that Mn exposure is a risk factor for ADHD diagnosis (7).

While therapies such as methylphenidate (Ritalin) have proven to be effective for children diagnosed with ADHD, there are no established treatments available for children exposed to high levels of Mn during development. A number of nutritional interventions, including maternal supplementation with choline, folic acid, genistein, and L-methionine have been shown to mitigate the effects of a variety of environmental insults (26,27). In particular, maternal choline supplementation (MCS)

has been shown to lessen cognitive dysfunction caused by prenatal alcohol exposure (28,29), maternal stress (30), and Down syndrome (31–33) in animal models. Maternal choline supplementation has also been shown to normalize some gene expression and epigenetic molecular alterations caused by perinatal alcohol exposure (34,35), and to mitigate some of the detrimental effects of prenatal alcohol exposure in children of alcohol-using mothers (36–38). In addition, MCS has been shown to improve cognitive function of typically developing children (39–41) and animal models (42–50). These benefits of MCS may reflect, in part, the growing evidence that the amount of choline most commonly consumed by pregnant women – and pregnant laboratory animals – is insufficient to meet the increased demand for choline during fetal and neonatal development (51–53). Physiological demands for choline increase markedly during pregnancy and lactation due to choline's numerous roles in fetal development, including as a precursor biomolecule for acetylcholine, phosphatidylcholine, sphingomyelin, and betaine (39,54,55). However, over 90% of pregnant women consume less than the Adequate Intake (AI) for choline (41,51,52,56) – an amount which itself appears to be insufficient to meet the demands of pregnancy (39–41).

The present study used our rodent model of early childhood environmental Mn exposure to test two hypotheses: (1) That developmental Mn exposure causes lasting alterations in behavioral reactivity/emotion regulation (assessed via the reaction to committing an error or not receiving an expected reward) in addition to the deficits in

attention, impulse control, learning, and sensorimotor function – all areas of dysfunction seen in children with ADHD; and (2) that supplementing the maternal diet with additional choline during pregnancy and lactation will offer protection against the adverse effects of early postnatal Mn exposure on attention, impulse control, learning, sensorimotor function, and behavioral reactivity (if shown). Collectively, these findings will add to our understanding of the constellation of effects produced by developmental Mn exposure as a risk factor for ADHD, and they will provide evidence to evaluate the efficacy of MCS to protect against Mn-induced dysfunctions.

2. Materials and Methods

2.1 Subjects

Sixty-four (64) male Long-Evans rats were used for neurobehavioral assessment. Additional littermates were used for analyses of tissue Mn, choline, and choline metabolites. Males were used for several reasons. First, the focus on males reflects the evidence that attentional dysfunction is two to three times more prevalent in boys than girls (57,58). Second, human and animal studies have shown that males are more sensitive than females to developmental Mn neurotoxicity (8,22,59,60). Finally, we have previously established in male rats that developmental Mn exposure causes lasting deficits in focused and selective attention, impulse control, and sensorimotor function (18,20,21); thus, we deemed it important to build upon these findings to

investigate the potential benefits of perinatal choline supplementation to protect against those known Mn deficits.

Subjects were born in-house from 16 nulliparous timed-pregnant dams (Charles River; dams were 8-9 weeks old and pregnant at gestational age 3 days). Twelve to 24 hours after parturition (designated postnatal day (PND) 1, birth = PND 0), litters were weighed, sexed, and culled to eight pups per litter such that each litter was composed of as many males as possible (2-8) and the remainder females. Each treatment group contained n = 16 rats, and no more than 1-2 males per litter were assigned to a particular treatment group. At PND 22, all pups were weaned and pair-housed with a rat of the same treatment group and maintained on a reversed 12:12 hour light/dark cycle. Rats were housed in polycarbonate cages at a constant temperature of 21 ± 2°C. All aspects of behavioral testing and feeding were carried out during the active (dark) phase of the rats' diurnal cycle. All animal care and treatments were approved by the institutional IACUC (protocol Smitd1803) and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals (61).

2.2 Maternal choline supplementation

Pregnant dams were randomly selected to receive one of two concentrations of choline chloride in their diet (Dyets AIN-76A Purified Rodent Diet), both of which were provided *ad libitum* during gestation and lactation, from gestational day (GD) 3

until PND 21. The control purified diet (unsupplemented) contained 1.1 g choline/kg, whereas the supplemented purified diet contained 5.0 g choline/kg; both are reported by the manufacturer to contain 54 mg Mn/kg. Upon weaning on PND 21, all pups were provided Irradiated Teklad Global Soy Protein-Free Extruded Rodent Diet 2920, reported by the manufacturer to contain 80 mg Mn/kg and 1.2 g choline/kg. See Supplemental Figure S1 for a timeline of maternal choline supplementation, Mn dosing, and behavioral testing.

2.3 Manganese exposure

Neonate rats were orally exposed to 0 or 50 mg Mn/kg/day in a vehicle solution (5% w/v stevia in Milli-Q water) from PND 1–21, as in our prior studies (18–24). This Mn exposure level is not overtly toxic and does not measurably affect neonate health or nutrition, based on neonate milk intake from the lactating dam, growth rate, or blood hematocrit levels at weaning (20). Briefly, dosing was delivered once a day directly into the mouth of the pups in a volume of 15-25 µL/dose via a micropipette fitted with a flexible polyethylene gel loading tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received only the deionized water + stevia vehicle. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both, as pre-weaning exposure to 50 mg Mn/kg/day produces relative increases in Mn intake that approximates the relative increases reported in infants and young children exposed to Mn-contaminated water or soy-based formulas (or both) (20,22). A fuller

description of the Mn dosing regimen and its environmental relevance is given in the Supplementary Materials (section 1).

2.4 Testing apparatus

Sixteen identical automated 5-Choice Serial Reaction Time Task (5-CSRTT) testing chambers fitted with odor delivery systems (#MED-NP5L-OLF, Med Associates, Inc., St. Albans, VT) were used to assess specific cognitive processes, including learning, focused and selective attention, inhibitory control, and behavioral reactivity, as described previously (20,21,62). Briefly, each testing chamber contained a curved wall equipped with five 2.5 × 2.5 cm response ports fitted with light-emitting diodes that served as the visual cue, an infrared beam to register nose pokes, and pneumatic inlet and vacuum outlet ports to introduce and remove air-based odor distractors. Opposite the response wall was the food magazine wall that contained a 45 mg food pellet reward dispensing port fitted with an infrared beam to register nose pokes; each unit also contained a small house light. The entire testing chamber was enclosed in a sound attenuating cubicle. Each rat was assigned to one of the 16 testing chambers, and each chamber was balanced for the four treatment conditions.

2.5 Behavioral testing

Rats were mildly food restricted starting on PND 45 in preparation for behavioral testing, with water available *ad libitum* throughout behavioral assessment. Rats were provided food for 2 hours each day immediately after behavioral testing, so that their

body weights were maintained at ~90–95% of free-feeding weights. 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 chamber for each individual rat. A daily test session consisted of 150 trials or 50 min, whichever came first. Each trial sequence was initiated by a nose-poke in the food magazine port, which was followed by a 3 sec turnaround time to allow the rat to reorient from the food magazine wall to the response wall; trial onset began after the 3 sec turnaround time. All behavioral testing was conducted by individuals blind to the treatment condition of the subjects.

Behavioral testing began at ~ PND 50. Briefly, testing began with food magazine and nose-poke training for 11 days, followed by a 5-choice visual discrimination learning task in which the visual cue was presented immediately after trial onset (i.e., no pre-cue delay), and stayed illuminated for 15 sec (for more detail, see Beaudin et al. (20). Rats stayed on this task until they reached the learning criterion of 80% correct trials on 2 of 3 consecutive testing days. Following this task, rats were administered a second visual discrimination task with a fixed 1 sec cue duration and no pre-cue delay for 5 days. This training was followed by a series of attention and behavioral reactivity tasks as described below (see Supplemental Figure S1 for the timeline of testing).

5-CSRTT assessment is conducted via a series of progressively more difficult tasks, described in greater detail below, that challenge the ability of animals to sustain attention, inhibit premature responses, and ignore olfactory distractors while maintaining focus on the predictive visual cue. Recorded response types for all 5-CSRTT tests included the following: 1) premature responses (responses made after trial onset but before presentation of the visual cue; assessed in the first focused attention, second focused attention, and selective attention tasks); 2) correct responses (responses made to the correct port following presentation of the visual cue); 3) incorrect responses (responses made to the incorrect port following presentation of the visual cue); and 4) omissions (failure to respond within 15 sec after visual cue presentation). Premature and incorrect responses and omission errors were not rewarded and were immediately followed by a 5 sec time-out, in which the house light was turned off for 5 sec. In addition, the latency (sec) for correct and incorrect responses was recorded (response latency), as was the latency (sec) to retrieve the food pellet reward following a correct response (collection latency). Perseverative responses, defined as additional nose pokes into the response port after a correct response, were also recorded. The calculated response outcomes were: (1) %Correct, calculated as number of correct responses/(correct + incorrect + premature + omissions) × 100; (2) %Incorrect, calculated as above but with incorrect responses in the numerator; (3) %Premature, calculated as above but with premature responses in the numerator; and (4) %Omissions, calculated as above but with omissions in the numerator. Additionally, we assessed attentional accuracy via a %Accurate outcome,

calculated as number of correct responses/(correct + incorrect) × 100. The number of perseverative responses per correct response was calculated simply as #perseverative responses/#correct responses.

2.6 Focused attention tasks

Focused attention can be defined as the ability to maintain attentional focus on a specific task or stimulus (e.g., a visual cue). Two successive focused attention tasks (FA1 and FA2) were administered over ~PND 85-103, and PND 104-121, respectively, following completion of the visual learning discrimination task. Both focused attention tasks assessed the ability of rats to detect and respond to a visual cue presented unpredictably in time and location (one of the five response ports). The first focused attention task used variable pre-cue delays of 0, 1, 2, or 3 sec and a fixed visual cue duration of 0.7 sec and was administered for 15 daily sessions. The second focused attention task included variable pre-cue delays of 0, 3, 4, or 5 sec and variable visual cue durations of 0.5 or 0.7 sec and was administered for 15 sessions.

2.7 Selective attention task with olfactory distractors

Selective attention can be defined as the ability to maintain a behavioral or cognitive set in the face of distracting or competing stimuli. As described in our prior studies (20), the selective attention task (SAT) with olfactory distractors was administered across 12 sessions, from PND 129-140. In this task, the pre-cue delay varied between 3 and 4 sec, with the two delays balanced across the trials within each test daily

session, and the cue duration was 0.5 sec. On one third of the trials in each session, an olfactory distractor was presented 1 or 2 sec after trial onset (i.e., 1–3 sec before the visual cue, depending on the pre-cue delay). The delay between odor distractor presentation and visual cue presentation (1-3 sec) is referred to as the odor-to-cue interval (OtoC) and was included in statistical analysis. The nine different olfactory distractors were made from liquid odorants (McCormick & Company, Inc.) diluted in propylene glycol, and delivered into an incorrect response port as scented air. The SAT was preceded by a baseline task (three sessions), administered from PND 126-128. This task was identical to the SAT, except it did not include the presentation of olfactory distractors.

2.8 Assessment of behavioral reactivity and compulsiveness in the reward omission task

Behavioral reactivity/emotion regulation was assessed by measuring the rat's performance on trials following an error or not receiving an expected reward, areas where children with ADHD have been shown to differ from typically developing children (3,4). Reactivity to committing an error was assessed in two tasks: (1) The second focused attention task, and (2) a reward omission task. In this latter task, a 0.7 sec visual cue was presented immediately at trial onset, and the food pellet reward was randomly omitted on three out of 10 correct trials. It is notable that the consequences of committing an error versus a reward omission are very different for the animal. Upon committing an error (in all tasks), the rat receives a 5 sec 'time-out'

during which the house light turns off and the rat sits in darkness, and no pellet reward is provided. In contrast, on a reward omission trial the house light remains on and all other trial conditions/cues remain unchanged from a normal rewarded correct trail. Attentional performance on the reward omission task was assessed as a function of whether the preceding trial was either (1) correct (and rewarded), (2) incorrect (including omission errors), or (3) correct but food reward omitted. We also assessed perseverative responses in the reward omission task as a measure of compulsivity, consistent with prior studies (63,64). Perseverative responses are additional nose-pokes into the correct port following a correct response. Rats were tested in the reward omission task for 12 sessions from PND 175-187.

2.9 Montoya staircase task

The Montoya staircase test was used to assess sensorimotor function, as described previously by us (19). Performance on the staircase test is a sensitive measure of skilled forelimb function directly relevant to pediatric Mn studies. This test taps essential sensorimotor functions required to successfully reach and grasp small food pellets from the descending steps of the staircase, including advancing the limb over the food, opening the digits in preparation for grasping, grasping and manipulating the food, and withdrawing the paw to place the food in the mouth (65). Optimal performance on this test requires several sensorimotor skills that are similarly assessed in human studies, including forelimb movements and digit flexion for manipulating objects. Thus, the staircase test measures aspects of fine psychomotor

skills that are known to be disrupted in children with a history of developmental Mn exposure (8,60).

Eight identical Plexiglas staircase devices were used, modeled after the original design of Montoya et al. (66). Each device contained a platform and a left-and-right side staircase. The staircases contained 6 descending steps on each side, and each step was baited with three 45 mg food pellets for a total of 36 pellets available at the start of a session. To determine the step of origin and final location of each pellet, pellets were color coded with a unique color per step level (67).

The staircase study was conducted over 1 month, beginning on PND 85. Each staircase session occurred immediately following operant testing every day. Staircase training occurred for the first 11 days. During training, rats retrieved the food pellets from the descending steps of the staircases until all subjects reached asymptotic performance of total pellets retrieved. The next 19 days comprised the testing phase of the task. Throughout training and testing, each subject was given one 10-min trial per day, 6 days per week. After each trial, the rat was removed, and the final distribution of colored pellets was quantified per step and side. The order and time of testing were balanced by treatment and remained the same each day for each rat.

Skilled forelimb performance was evaluated step-by-step for (1) the number of pellets taken (i.e., pellets that were grasped and removed from their step of origin), (2) the

number of pellets eaten (pellets that were taken and consumed), (3) the corresponding percent of success (the ratio of the number of pellets eaten/pellets taken x 100), (4) the number of pellets misplaced (all the pellets that were grasped but ended up on a different step level than their step of origin), and (5) the number of pellets lost (pellets that ended up on the floor of the apparatus, out of reach of the rats).

2.10 Tissue Mn and choline/choline metabolite levels

For blood and brain Mn levels, tissue samples were collected from weaned pups on PND 24, and from the behaviorally tested rats on PND 202. Briefly, rats were euthanized via CO_2 asphyxiation, and whole blood was immediately collected from the left ventricle of the surgically-exposed heart and stored in EDTA Vacutainers at −20 °C for analyses. The whole brain was immediately removed, and the hind-brain region collected and stored at −80 °C for Mn concentration determinations. Blood and brain Mn concentrations were determined by inductively coupled plasma–mass spectrometry (Thermo Element XR), as previously described (18,20). The analytical detection limit for Mn was 0.01 ng/mL.

Choline/choline metabolite levels were measured in plasma, brain, and/or liver tissues from dams at weaning (plasma and liver), and/or from the same PND 24 rats (liver and hindbrain) used for Mn analyses described above. Briefly, concentrations of free choline, betaine, dimethylglycine, methionine, and trimethylamine N-oxide were measured by liquid chromatography tandem mass spectrometry according to the

method of Holm et al. (68), while phosphocholine, glycerophosphocholine, phosphatidylcholine, acetylcholine, and lysophosphatidylcholine were measured by liquid chromatography/mass spectroscopy according to the method of Koc et al. (69) with modifications (70).

2.11 Statistical methods

The behavioral data were modeled using structured covariance mixed models. Fixed effects included in the models were the between-subject factors MCS (two levels corresponding to the unsupplemented and MCS groups), and Mn exposure treatment (two levels corresponding to the 0 and 50 mg/kg/d groups), and the within-subject factors pre-cue delay, cue duration, session block, distraction condition, and/or previous trial outcome, depending on the outcome analyzed. In all models, rat was included as a random effect to account for correlations within observations from the same animal. Statistical tests used the Kenward-Roger correction to improve estimation of the variance-covariance of the fixed effects parameters. Plots of residuals by experimental condition were used to examine the assumption of homogeneity. Random effects with high variance in the residuals across the levels of the within-subject factor (e.g., distraction condition) were added to the model to achieve homogeneity if necessary. The distribution of each random effect was inspected for approximate normality and presence of influential outliers. The significance level was set at $p \leq 0.05$, and p-values between 0.05 and 0.10 were considered to be trending towards significance; trending effects are presented if they

aid in clarifying the nature of the Mn and/or MCS effects. Significant main effects or interaction effects were followed by single-degree of freedom contrasts to better understand the nature of the interactions, using the Student's t-test for pairwise comparisons of least squared means. Analyses were conducted using SAS (version 9.4) for Windows. For tissue Mn and choline/metabolite data, analyses were performed using one-way (for dams) or two-way (for offspring) analysis of variance and Tukey's post hoc test for pairwise comparisons, using (version 16.0; SAS Institute, Inc.).

No rats were excluded from the study for failing to reach learning criterion. However, several rats were removed from statistical analysis due to low motivation to perform the task(s), as evidenced by an anomalously low number of response trials on multiple days on multiple tasks, as follows: Two rats omitted from the Control+Unsupplemented group, one rat omitted from the Control+MCS group, and two rats each omitted from the Mn+Unsupplemented and Mn+MCS groups.

2.12 Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request. The data are not publicly available due to ethical restrictions.

3. Results

For the 5-CSRTT measures, we focus primarily on outcomes of %Premature, %Correct, and %Accurate responses because our prior studies have shown that these measures best differentiated performance of the control vs Mn rats (20,21). Note that %Correct refers to the proportion of all responses that were correct, whereas %Accurate refers to the proportion of timely responses (i.e., responses made within 10 s of cue onset) that were correct; thus for %Accurate, premature responses and omission errors are excluded from the denominator. In general, we graphically depict the data for all four treatment groups (defined by diet and Mn exposure), regardless of whether the interaction of Mn and MCS was significant, due to our *a priori* hypotheses relating to the magnitude of the Mn effect under each of the two diet conditions.

3.1 Visual learning

Visual learning in the 5-choice task is not altered by Mn or MCS

Visual learning (i.e., learning to make a nosepoke into the illuminated port) is foundational to the subsequent, more challenging 5-CSRTT tasks. Here, there was no effect of early postnatal Mn exposure or MCS on visual learning. Specifically, there was no main effect of Mn or MCS on trials to criterion or days to criterion, and no Mn x MCS interaction for these two outcomes (p's >0.295). Moreover, there also was no effect of Mn or MCS on the percentage of rats per treatment group failing to reach the learning criterion. The lack of treatment group differences in learning the task

criterion indicates that visual acuity and motivation did not differ between groups and therefore cannot account for group differences seen in subsequent 5-CSRTT tasks.

3.2 Impulsivity

Developmental Mn exposure did not affect impulse control, but did impair the rats in learning to wait for the visual cue, and this effect is not rescued by MCS

We evaluated %Premature responses as a measure of inhibitory control in the two focused attention tasks and the selective attention task.

In the first focused attention task, Mn exposure impairs learning to withhold premature responses, and MCS does not alter this effect.

In the first focused attention task, there is a significant Mn x session block interaction [$F(2, 435.8) = 11.07$, $p<0.0001$] for %Premature responses. Both control and Mn groups commit a relatively high percentage of premature responses at the start of the task (~50%), since this was the first task in which rats faced longer pre-cue delays. However, over the task the Mn rats improve less rapidly than controls and, as a result, commit significantly more premature responses than controls in session block 3 ($p=0.0084$; Figure 1a1). There is also a significant MCS x session block interaction [$F(2, 435.8) = 17.06$, $p<0.0001$], but none of the contrasts achieved significance. The three-way interaction of MCS, Mn and block was not significant ($p = 0.69$).

In the second focused attention task, neither Mn nor MCS significantly alter premature responses.

In the subsequent second focused attention task, there is neither a main effect of Mn ($p = 0.75$) nor MCS ($p = 0.39$). However, there is a Mn x session block interaction [$F(2, 933.7) = 5.53$, $p = 0.0041$], but no significant contrasts between control and Mn groups in any session block (p's >0.40; Figure 1b1; see also Figure 1B). The three-way interaction of Mn x MCS x block is not significant ($p = 0.40$).

There is also a MCS x pre-cue delay interaction [$F(2, 932.8) = 3.18$, $p = 0.042$], though again, none of the specific contrasts between unsupplemented and MCS rats reached significance at any pre-cue delay (p's > 0.21, Figure 1c1). Differences between Mn and controls under each of the two diet conditions is presented in Figure 1C. The three-way interaction of MCS, Mn and pre-cue delay is not significant ($p = 0.24$).

In the selective attention task, MCS lessens the difference in premature responses between treatment groups in the early session blocks.

In the selective attention task there is a Mn x MCS x session block interaction for %Premature responses [$F(3, 1665) = 4.44$, $p = 0.004$], although none of the contrasts between treatment groups or diet conditions reached significance for any session block (p's > 0.12, Figure 1D). The significant three-way interaction appears to reflect the fact that, for the first two session blocks, the difference in premature responses

between Mn and control groups is greater for the unsupplemented rats than for the MCS rats.

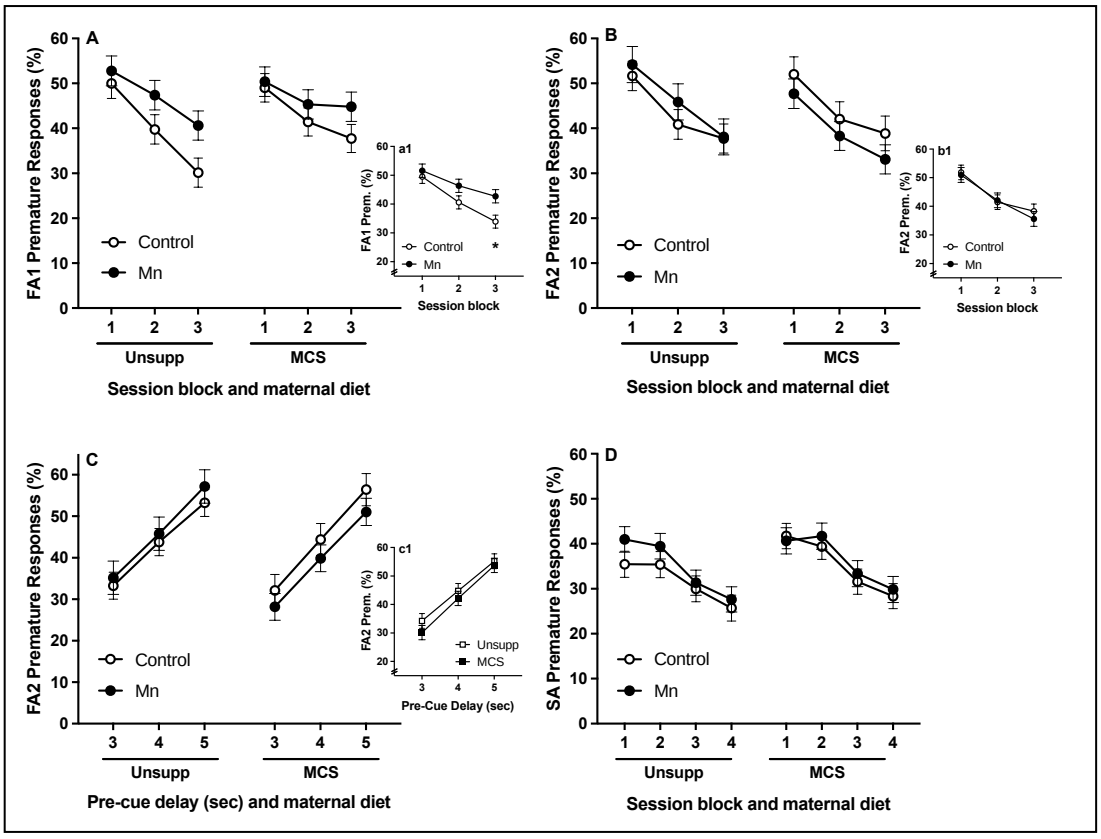

Figure 1. Effects of Mn and MCS on premature responses (impulsivity) in the focused attention 1 task (panel A), the focused attention 2 task (panels B and C), and the selective attention task (panel D), as a function of session block or pre-cue delay (sec). Sub-panels a1, b1, and c1 depict significant two-way interactions of Mn x session block or MCS x pre-cue delay, respectively. Mn exposure impaired the rate of learning to wait for the cue. MCS did not alter premature responses generally,

although there is a suggestion that MCS lessened group differences in the selective attention task. * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-15/group).

3.3 Attention

Mn impairs focused attention and MCS lessens this effect

As described earlier, the two focused attention tasks were used to assess whether Mn exposure alters the ability of animals to maintain attentional focus, and whether the Mn impairment was lessened by MCS.

In the first focused attention task, Mn exposure impairs attention, and MCS improves learning of the control group.

In the first focused attention task, there is a significant Mn x session block interaction for %Correct responses [$F(2, 595.6) = 4.12$, $p = 0.017$]. This reflects that performance in the Mn animals improves more slowly than control animals across session blocks, such that the Mn animals have significantly fewer %Correct responses than the controls in session blocks two and three ($p=0.007$ and 0.002, respectively, Figure 2a1). The three-way interaction of Mn, MCS and session block was not significant ($p = 0.22$). Figure 2A depicts differences between Mn and controls under each of the two diet conditions.

Consistent with the %Correct outcome noted above, there is a significant Mn x session block interaction for %Accurate responses [$F(2, 601.3) = 3.33$, $p = 0.036$; not shown], and a trending three-way interaction of Mn x MCS x session block [$F(2, 601.3) = 2.58$, $p = 0.078$] (Figure 2B). This borderline interaction appears to be driven by the fact that, although all groups improve in attentional accuracy across session blocks (p's<0.0014), a Mn deficit emerges in the final two session blocks for the MCS animals (p's $= 0.018$ and 0.049, respectively; Figure 2B), but not for the unsupplemented animals. This pattern appears to reflect that the controls benefitted more from MCS than the Mn animals in terms of improvement in %Accuracy across session blocks.

In the second focused attention task, MCS improves performance of both the Mn and control animals, but in different functional domains.

In the second focused attention task there is a significant Mn x MCS x pre-cue delay interaction for %Correct responses [$F(3, 1277) = 7.32$, $p < 0.0001$] (Figure 2C). This interaction appears to be driven by different effects of MCS for the Mn and control animals, which vary as a function of the pre-cue delay. For trials with longer pre-cue delays (delays of 3, 4, or 5 sec), MCS reduces the Mn attentional impairment, relative to that seen in the unsupplemented animals. Specifically, for animals born to dams on the unsupplemented diet, Mn exposure causes a significant reduction in percent correct at the 3 sec delay ($p = 0.049$), a trend that is also seen at the 4 and 5 sec delays (p's $= 0.052$ and 0.069, respectively). In contrast, for animals born to dams on the

choline supplemented diet, no significant impairment of Mn exposure is seen at any delay, although a trending impairment is seen at the 3 sec delay ($p = 0.076$). This 3-way interaction also appears to be driven in part by group differences at the 0 sec delay condition (trials in which the cue is presented at trial onset). For these trials, the controls derive a significant benefit of choline supplementation ($p = 0.011$), whereas the Mn animals do not (Figure 2C).

We also assessed attentional accuracy (as %Accurate responses) for the second focused attention task, and again observed a significant Mn x MCS x pre-cue delay interaction [$F(3, 1271) = 3.95$, $p = 0.0081$] (Figure 2D). As with the %Correct outcome, this interaction seems to reflect a differential effect of MCS on control versus Mn animals that varies as a function of pre-cue delay. For trials with longer pre-cue delays (e.g., delays of 4 or 5 sec), MCS appears to lessen the difference in performance between the control and Mn groups. Specifically, for the unsupplemented animals there is a trending (or near-trending) Mn deficit at the 3, 4, and 5 sec pre-cue delays (p's = 0.058, 0.067 and 0.011, respectively), while for the MCS animals the Mn deficit is trending/present at the 0 and 3 sec pre-cue delays (p's = 0.090 and 0.043, respectively), but not at the 4 or 5 sec delays (p's = 0.23 and 0.53, respectively). Collectively, this evidence suggests that the lasting attentional deficits caused by developmental Mn exposure persist into the second focused attention task, and that MCS improves performance of the controls but not the Mn animals for trials

with a 0 sec pre-cue delay, whereas it appears to reduce the difference between Mn and control animals at the longer (more challenging) delays.

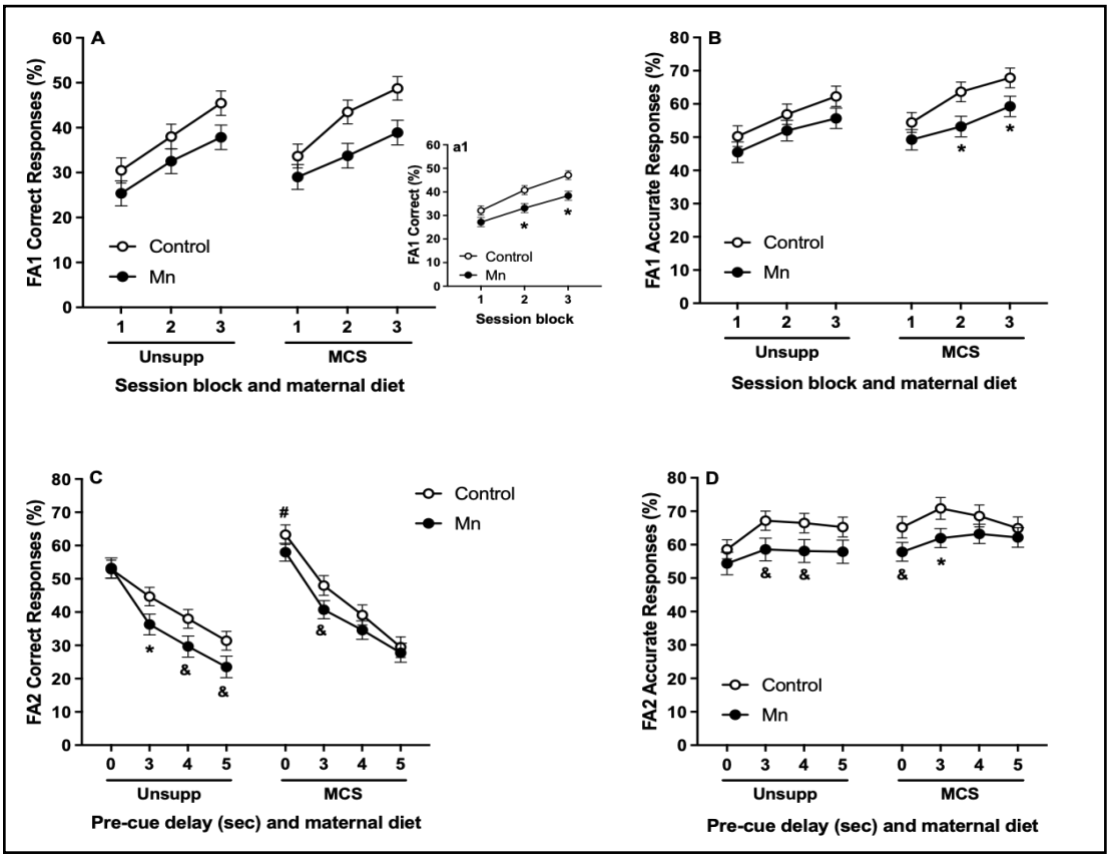

Figure 2. Effects of Mn and MCS on %Correct and %Accurate responding in the two focused attention tasks. Panels A and B depict performance in the focused attention 1 task as a function of session blocks; sub-panel a1 depicts the significant two-way interaction of Mn x session block. Panels C and D depict performance in the focused attention task 2, as a function of the pre-cue delay (sec). Mn animals exhibit an impairment in focused attention, and MCS affects attention in both groups, but in different ways. * and & indicate $p \leq 0.05$ and $p \leq 0.10$ versus controls, respectively; #

indicates $p \leq 0.05$ versus unsupplemented animals. Data are lsmeans ± SEM (n=14-15/group).

MCS normalizes the performance of the Mn rats on distraction trials in the selective attention task

To determine the effects of Mn exposure and MCS on selective attention, we assessed animal performance on a selective attention task with olfactory distractors randomly presented either 1, 2, or 3 sec prior to the onset of the visual cue (referred to as the odor-to-cue interval, 'OtoC').

Mn causes attentional dysfunction and MCS reduces the difference between Mn and control animals on trials with olfactory distractors.

For %Correct responses in the selective attention task, there was a significant Mn x OtoC interaction [$F(3, 239.2) = 2.94$, $p = 0.034$], where the Mn animals were more impaired relative to controls for trials without a distractor ($p = 0.0096$; Figure 3a1). Although the Mn x MCS x OtoC interaction was not significant ($p = 0.27$) (Figure 3A), the pattern described by the Mn x OtoC two-way interaction seems to be driven by the fact that MCS diminished the differences between the Mn and control group for distraction trials (Figure 3A); this pattern is not seen in the unsupplemented groups. In light of this, we conducted follow-up analyses where the three trial conditions with olfactory distractors (i.e., OtoC of -1, -2, -3 sec) were collapsed into a single "distraction" trial condition, thus creating only two distraction conditions

(distractor and no-distractor trials; Figure 3B). In this follow-up analysis, there was a trending interaction of Mn x MCS x distractor [$F(1, 52.16) = 3.29$, $p = 0.075$]. This trend reflects that, for non-distraction trials, the Mn animals perform more poorly than controls under both diets, but for distraction trials the Mn deficit emerges only for unsupplemented animals (Figure 3B); for the MCS condition, the Mn and controls perform similarly for trials with distractors.

For %Accurate responses, there was a significant Mn x OtoC interaction [$F(3, 235.7) = 3.14$, $p = 0.026$], where the Mn deficit was significant for trials without a distractor and trending for trials with the -3 sec distractor condition (p's = 0.0084 and 0.098; Figure 3c1); the groups were not different for trials with a -1 or -2 distractor. The Mn x MCS x OtoC three-way interaction was not significant [$F(3, 235.8) = 0.8$, $p = 0.49$]; however, there is a similar pattern of group differences as noted above for %Correct, where MCS reduces the difference in %Accuracy between control and Mn animals under the distractor conditions (Figure 3C). However, neither the full (Figure 3C) nor distractor-simplified (not shown) %Accurate models revealed a significant Mn x MCS x odor interaction (p's > 0.31). Collectively, these %Correct and %Accurate findings suggest that MCS lessens the difference between control and Mn animals for trials with olfactory distractors.

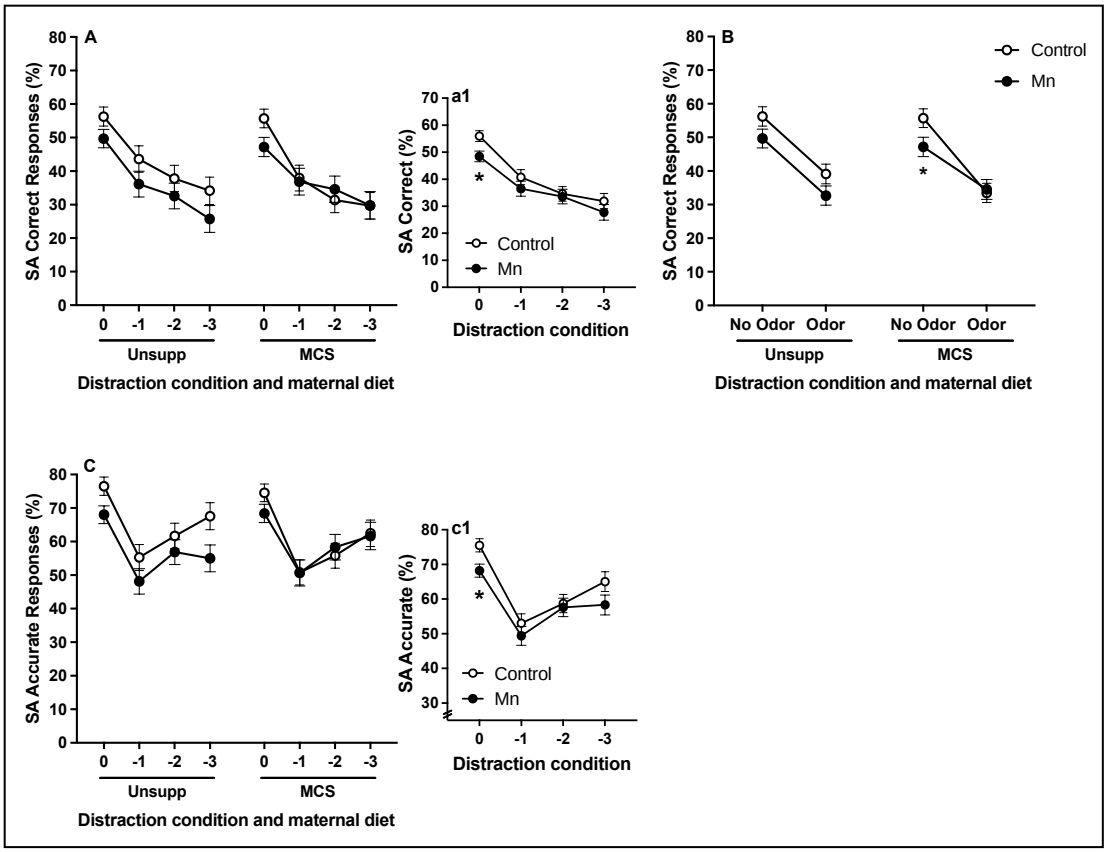

Figure 3: Effects of Mn and MCS on performance in the selective attention task. Panel A depicts %Correct for the analysis in which all four odor distraction conditions were included separately, whereas Panel B depicts %Correct responses for the analysis in which the four odor distraction conditions were collapsed into distractor (odor) vs no distractor (no odor) conditions. Panel C depicts %Accurate responses for all four distraction conditions. Sub-panels a1 and c1 depict significant two-way interactions of Mn x odor distractor condition. Mn causes a deficit in attention, but not selective attention specifically, and MCS reduces the difference between Mn and control animals under distraction conditions. * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-15/group).

3.4 Behavioral reactivity

Mn alters reactivity to errors and reward omission, effects which are partially normalized by MCS

In the second focused attention task, Mn animals born to dams on the unsupplemented diet do not exhibit behavioral reactivity to an error, and MCS improves performance in control animals following an error.

In the second focused attention task, control animals that received MCS had significantly more correct responses than their unsupplemented counterparts at the 0 sec pre-cue delay (reported above in Figure 2C). We hypothesized that this might be due to MCS reducing the disruptive effect of committing an error on the prior trial, as trials with a 0 sec pre-cue delay are closest temporally to the prior trial (i.e., if the outcome of the prior trial was disruptive to the animal, it would be most apparent for trials for which the cue was presented immediately after trial onset). Therefore, we assessed %Correct responses on trials with a 0 sec pre-cue delay, as a function of the outcome of the prior trial (correct or error).

The analysis of %Correct for trials with a 0 sec pre-cue delay revealed a significant Mn x MCS x previous trial outcome interaction [$F(1, 273.2) = 6.13$, $p = 0.014$]. This interaction reflects that the reaction to committing an error varied as a function of both Mn exposure and maternal diet. Specifically, under the unsupplemented diet,

only the controls exhibited fewer correct responses on trials following an error (relative to after a correct response); the Mn animals showed no decline in performance due to making an error on the prior trial ($p < 0.0001$ for control and $p = 0.79$ for Mn animals comparing error vs. correct; Figure 4). In contrast, under the MCS condition, the magnitude of the disruptive effect of making an error was similar in the control and Mn treatment groups, with the reduction in %Correct for trials following an error (relative to after a correct response) trending for the control animals (p's = 0.074 and 0.17 for control and Mn groups, respectively; Figure 4). Moreover, in the control animals, MCS (vs. the unsupplemented condition) significantly increased correct responses after an error, but not following a correct response (p's=0.05 and 0.50, respectively), while in the Mn animals, MCS did not improve performance following either a correct response ($p = 0.34$) or error ($p = 0.73$), compared to unsupplemented animals (Figure 4).

In contrast to performance on trials with a 0 sec pre-cue delay, there was no Mn x MCS x previous trial outcome interaction for performance on trials with a 3 sec pre-cue delay [$F(1, 273.8) = 1.72$, $p = 0.19$] (not shown). The absence of this three-way interaction for trials with a longer pre-cue delay supports the inference that the group differences seen at the 0 sec pre-cue delay (described above) reflects reactivity to the outcome of the prior trial.

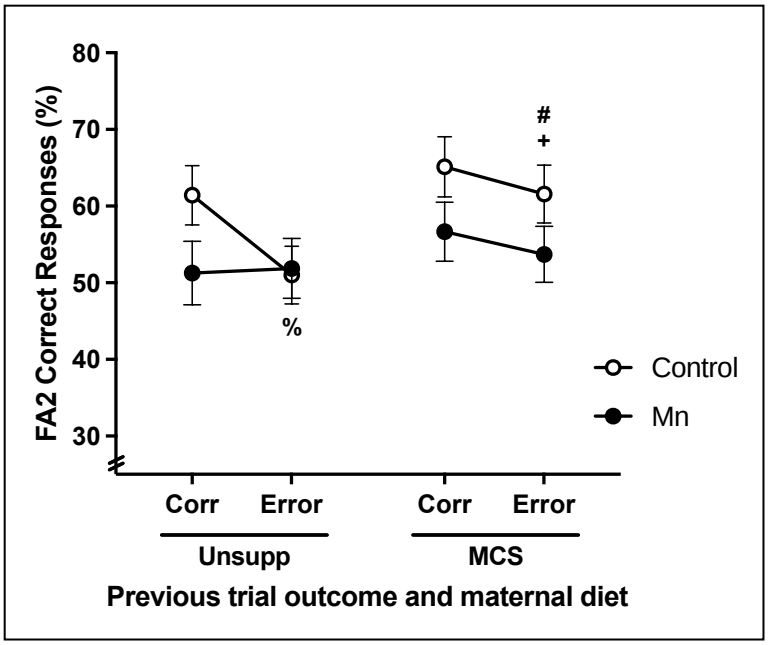

Figure 4: Effects of Mn and MCS on performance in the current trial as a function of the prior trial outcome (i.e., correct or incorrect) in the second focused attention task for trials with a 0 sec pre-cue delay. For animals born to dams on the unsupplemented diet (left side), Mn blunts reactivity to a prior error. Maternal choline supplementation (right) normalizes error reactivity of the Mn animals. Prior trial outcomes are Corr (correct response) or Error (incorrect, premature, or omission error). % and + indicate $p \leq 0.05$ and $p \leq 0.10$ versus following a correct response, respectively; # indicates $p \leq 0.05$ versus unsupplemented animals. Data are lsmeans ± SEM (n=14-15/group).

Developmental Mn exposure and MCS both alter reactivity to committing an error and reward omission in the reward omission task

In the reward omission task, we introduced a novel trial condition in which the food pellet reward is unexpectedly omitted on a subset (30%) of correct response trials and

assessed how this affects performance on the subsequent trial. Both errors and reward omission have been shown to be disruptive to performance in our prior studies with similar operant tasks (33,71), and both are often altered in children with ADHD compared to typically developed children (3,4,72–74). Specifically, we assessed performance on each trial as a function of whether the preceding trial response was: (1) correct with a food pellet reward, (2) an error (incorrect, no food pellet reward + 5 sec time out), or (3) correct but with the food pellet reward omitted (RO).

In the unsupplemented animals, Mn lessens reactivity to an error but increases reactivity to the omission of an expected reward

For %Correct responses, there was a significant three-way interaction of Mn x MCS x previous trial outcome [$F(2, 410.9) = 4.13$, $p = 0.017$] (Figure 5A). This interaction reflects that for the unsupplemented controls, committing an error on the prior trial caused a reduction in %Correct on the subsequent trial (versus a correct response on the prior trial; $p < 0.0001$), whereas the unexpected omission of the food reward on the prior (correct) trial had no effect on performance ($p = 0.17$). A markedly different pattern was seen in the unsupplemented Mn animals; for these animals, committing an error on the prior trial did not affect performance compared to trials following a correct response ($p = 0.15$) (Figure 5A). In contrast, performance of the unsupplemented Mn animals was significantly decreased on trials following the omission of the expected food reward relative to after a correct response ($p = 0.0032$). These findings demonstrate that the unsupplemented animals exposed to Mn early in

life respond differently than unexposed controls (no Mn) to both committing an error and not receiving an expected reward.

MCS normalizes behavioral reactivity to an omitted reward in the Mn animals

Notably, a different pattern of performance was seen for the MCS animals. Specifically, for the Mn-exposed animals, MCS normalized performance on trials following a reward omission, such that performance was the same as for trials following a correct response – a pattern matching that of the unsupplemented controls (no Mn) (Figure 5A). However, MCS did not alter how the Mn animals perform following an error (vs a correct response), compared to their unsupplemented Mn-exposed counterparts (Figure 5A). Finally, under the MCS diet, performance on trials following a correct response was significantly worse in the Mn animals than controls ($p = 0.019$), a deficit that was not present in the unsupplemented groups; this appears to be because the controls benefited from MCS with improved performance on trials following a correct response, but the Mn animals did not (Figure 5A).

For unexposed controls, MCS lessens the disruptive effect of committing an error

For the unexposed controls, there was a trend for MCS to lessen the disruptive effect of committing an error on the prior trial; i.e., to improve performance on trials that followed an error (relative to post-error trials for the unsupplemented controls) ($p = 0.076$, Figure 5A). However, for the MCS controls, %Correct responses on trials following an error was still lower than on trials that followed a correct response ($p <$

0.0001). Furthermore, for the MCS controls, performance on trials following a reward omission was lower than that on trials following a correct response ($p = 0.0015$), an effect that was not seen in the unsupplemented control animals. However, it is notable that performance on trials following a reward omission did not differ between the unsupplemented and supplemented controls; thus, the relative drop in performance by the MCS controls on trials following a reward omission (versus performance on trials after a correct response) was due to an *increase* in performance on trials following a correct response, not a decline in performance following reward omission.

Mn animals exhibit less post-error slowing than controls, and MCS normalizes this effect.

For response latency, there was a three-way interaction of Mn x MCS x previous trial outcome [$F(2, 121.4) = 5.11$, $p = 0.0074$] (Figure 5B). This reflects that for animals born to dams on the unsupplemented diet, both control and Mn groups exhibit post-error slowing (i.e., increased response latency following an error vs. a correct response; $p < 0.0001$); however, for the unsupplemented groups, the post-error slowing was significantly less pronounced in Mn animals than controls ($p = 0.0046$), and the magnitude of the difference in response latency following a correct response compared to an error is smaller in the Mn versus control animals (Figure 5B). Maternal choline supplementation normalizes post-error slowing in the Mn animals, such that the response latency following an error is the same as in controls, and the magnitude of the slowing (i.e., increase in response latency following an error versus

a correct response) is comparable in the Mn and control animals. Finally, trials following a reward omission have a comparable response latency to trials following a correct response across treatment groups, except in control animals that received MCS; these animals exhibit a slight but statistically significant increase in response latency following a reward omission compared to a correct response ($p = 0.044$) (Figure 5B).

Mn increases perseverative responding following a correct response in the unsupplemented animals, but this abnormality is not seen in the Mn animals born to supplemented dams.

The analysis of perseverative responses per correct response revealed a three-way interaction of Mn x MCS x previous trial outcome [$F(2, 111.2) = 3.54$, $p = 0.032$] (Figure 5C). All animals make more perseverative responses on trials following a correct response than on trials following an error or reward omission (p's < 0.0001), and they make the fewest perseverative responses on trials following a reward omission (compared to trials following either a correct response or error; p's < 0.0001). For groups in the unsupplemented diet condition, the Mn animals tend to make more perseverative responses following a correct trial than controls ($p = 0.066$). However, for the MCS groups, Mn animals trended to have fewer perseverative responses than controls following a correct response ($p = 0.053$), and there is a significant decrease in perseverative responses following a correct response in the

MCS+Mn animals compared to their unsupplemented counterparts ($p = 0.0019$) (Figure 5C).

Mn increases food pellet collection latency; MCS reduces pellet collection latency in Mn animals and increases collection latency in controls, mirroring the effect seen for perseverative responses.

For food pellet collection latency (i.e., the time to collect the food pellet reward following a correct response), there was a Mn x MCS x previous trial outcome interaction [$F(2, 410.3) = 6.69$, $p = 0.0014$] (Figure 5D). The pattern of effects seen for pellet collection latency was similar to that seen for perseverative responses, as expected, because committing more perseverative responses delays collecting the pellet reward. In general, pellet collection latency is longest on trials following a correct response, and shortest for trials following a correct response with an omitted reward. Unsupplemented Mn animals have a significantly longer collection latency following a correct response compared to controls ($p = 0.026$), while under MCS, Mn animals have a significant decrease in collection latency compared to unsupplemented Mn animals following a correct response ($p = 0.0005$). In addition, Mn animals that received MCS have significantly shorter collection latency than controls following a correct response ($p = 0.0006$) (Figure 5D). This effect is enhanced by the fact that MCS also increases collection latency in control animals following a correct response, compared to unsupplemented animals ($p = 0.039$).

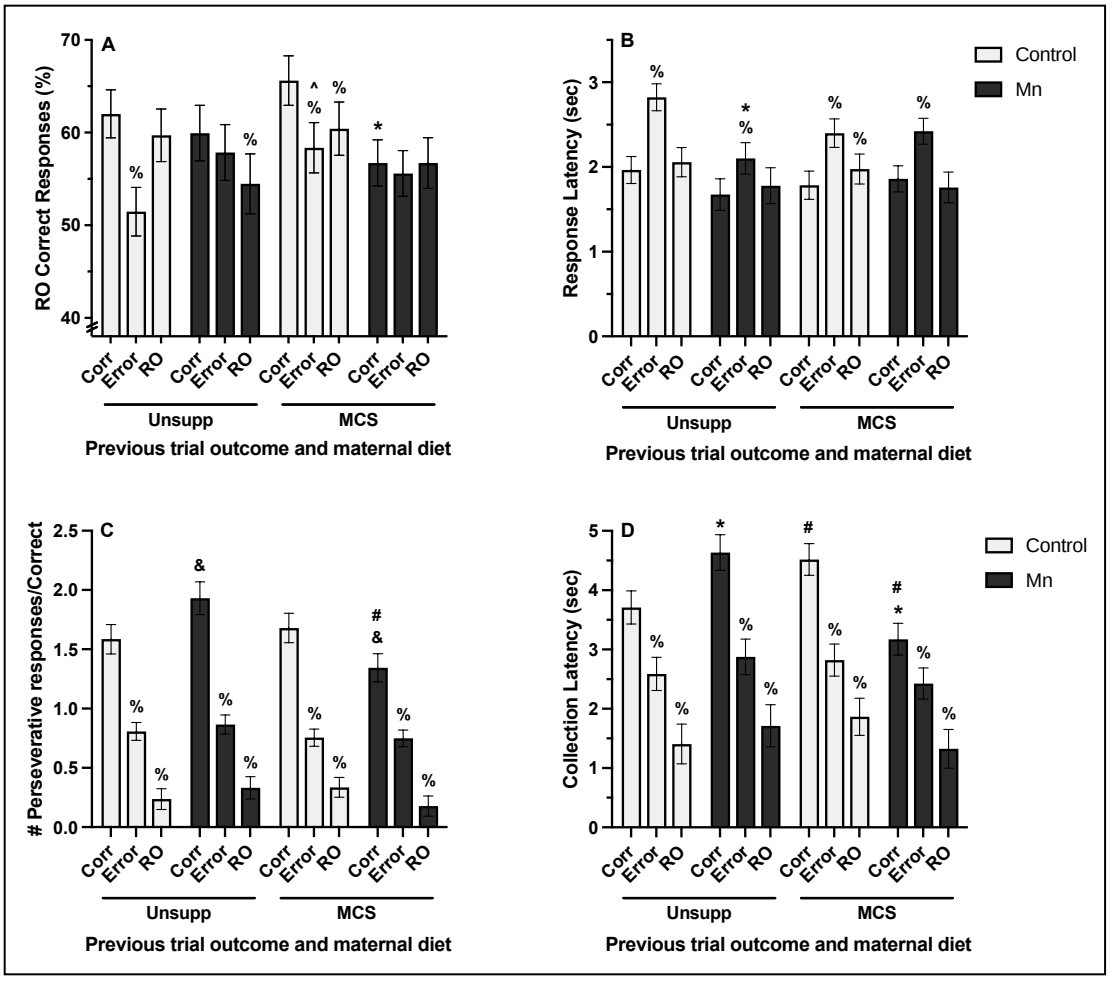

Figure 5: Effects of Mn and MCS on performance in the current trial as a function of the prior trial outcome in the reward omission task. Panels A, B, C and D depict, respectively, %Correct, response latency, perseverative responses, and food pellet collection latency. For the unsupplemented animals, Mn blunts error reactivity, but heightens reactivity to an omitted reward. Maternal choline supplementation (MCS) partially normalizes reactivity to both errors and reward omissions of the Mn animals. Prior trial outcomes are Corr (correct response), Error (incorrect or omission error), or RO (correct with reward omitted). % indicates $p \leq 0.05$ versus following a correct

response; * and & indicate $p \leq 0.05$ and $p \leq 0.10$ versus controls, respectively; # and ^ indicates $p \leq 0.05$ and $p \leq 0.10$ versus unsupplemented animals, respectively. Data are lsmeans ± SEM (n=14-15/group).

3.5 Sensorimotor function

Early life Mn exposure causes lasting impairment in sensorimotor function that is not altered by MCS.

To determine whether MCS can mitigate the lasting sensorimotor impairments caused by developmental Mn exposure, animals were assessed using the Montoya staircase test of skilled forelimb performance. Mn exposure caused lasting impairment in sensorimotor function, as demonstrated by a significant Mn x step level interaction for the number of pellets taken [$F(5, 229.3) = 10.66$, $p < 0.0001$], number of pellets eaten [$F(5, 232.6) = 4.57$, $p = 0.0005$], %Success [$F(5, 231.4) = 3.26$, $p = 0.0073$], and the number of pellets misplaced [$F(5, 356.7) = 5.76$, $p < 0.0001$]. Specifically, there was a significant Mn deficit on steps 4 and 5 of the staircase, where the unsupplemented Mn animals ate significantly fewer pellets and misplaced more pellets than their unsupplemented control counterparts (pellets eaten, p's = 0.0017 and 0.0002 for steps 4 and 5 respectively; pellets misplaced, p's = 0.0005 and 0.002) (Figure 6a1 and 6b1, respectively). MCS did not affect this Mn-induced impairment in reaching and grasping, based on findings that neither the main effect of MCS (p's >0.16), nor any interactions involving MCS and Mn were significant for any of the

outcomes (e.g., Mn x MCS, P's >0.32; Mn x MCS x step level, p's >0.72); see figure panels 6A and 6B for the Mn x MCS x step plots for pellets eaten and misplaced).

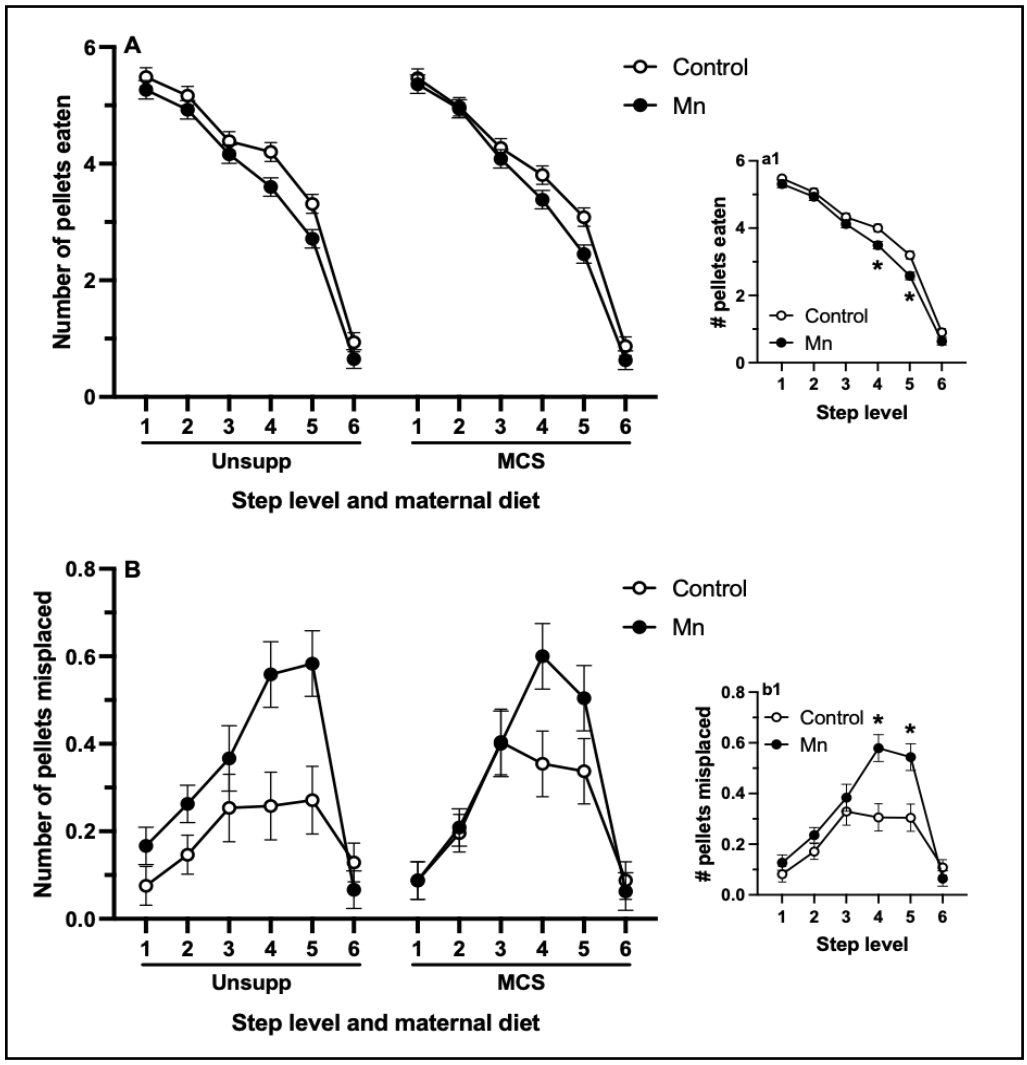

Figure 6: Effects of Mn exposure and MCS on sensorimotor function on the Montoya staircase task. Panel A depicts the number of pellets eaten, whereas Panel B depicts number of pellets misplaced, both as a function of step level. The insert panels show performance collapsed across the two diet conditions. Mn exposure causes a lasting

deficit in fine motor function that is not rescued by MCS. * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-15/group).

3.6 Choline and choline metabolite levels

MCS measurably altered choline and choline metabolite levels in dams and offspring.

To confirm that oral MCS over gestation and lactation altered levels of choline and/or its metabolites in dams and their offspring as expected, and whether oral Mn exposure altered choline/metabolite status in the offspring of MCS-treated dams, we measured levels of choline and selected metabolites in dam and offspring tissue (dam plasma and liver upon completion of MCS at PND 21, offspring liver and brain at PND 24). As expected, MCS produces measurable changes in choline and some of its metabolites in dam plasma and liver, and PND 24 pup liver and brain (see Supplementary Table 1 for choline and metabolite measurements in dams, and Supplementary Table 2 for measurements in pups).

3.7 Body weight; blood and brain Mn levels

Oral Mn dosing and MCS decreased body weight in weanlings, which returned to control levels by the start of testing.

At the end of Mn dosing and MCS on PND 21, there was a significant effect of Mn [$F(1, 87) = 26.6$, $p < 0.0001$] and MCS [$F(1, 87) = 7.5$, $p = 0.0075$] to decrease body weight (Supplementary Table 3), but there was no interaction of Mn and MCS on body weight ($p = 0.51$). By the start of behavioral testing on PND 49, there was still a main effect of Mn to decrease body weight [$F(1, 60) = 4.68$, $p = 0.034$]; however, Tukey's contrasts revealed no significant differences in body weights across the four treatment groups (p's > 0.10). Body weights of four treatment groups over the course of the study are shown in Supplemental Figure S2.

Oral Mn dosing increased blood and brain Mn levels in weanlings, and returned to control levels in adulthood.

Oral Mn exposure over PND 1-21 increased Mn levels in both blood and brain tissue in PND 24 weanlings. Specifically, Mn exposure significantly increases blood Mn levels ~six-fold, from 34 to 209 ng/mL in control vs. Mn exposed unsupplemented groups [$F(1, 45) = 398.4$, $p < 0.0001$] (Supplemental Figure S4A). There was also a significant interaction of Mn x MCS on blood Mn levels [$F(1, 42) = 8.37$, $p = 0.0059$]; however, Tukey's contrasts revealed no significant difference between blood Mn levels in unsupplemented versus MCS animals exposed to Mn. The mean blood Mn levels of MCS animals are 26 ng/mL in control and 304 ng/mL in Mn animals.

Mn exposure also causes an approximately three-fold increase in brain Mn levels in PND 24 offspring, from approximately 3.8 to 9.3 μg/g in control versus Mn-dosed

unsupplemented animals, as demonstrated by a significant main effect of Mn [$F(1, 36) = 129.7$, $p < 0.0001$] (Supplemental Figure S4B). There is also a trending main effect of MCS on brain Mn levels in offspring [$F(1, 36) = 3.96$, $p = 0.054$], as well as a trending Mn x MCS interaction [$F(1, 36) = 3.13$, $p = 0.085$]. Tukey's pairwise comparisons revealed that MCS animals treated with Mn had significantly higher brain Mn levels than their unsupplemented counterparts. The mean brain Mn levels of MCS animals are 3.8 µg/g in control and 14.3 µg/g in Mn animals.

Finally, while blood and brain Mn levels in the PND 200 behaviorally-tested animals return to baseline levels, there remained a statistically significant but biologically unremarkable effect of MCS [$F(1, 27) = 7.87$, $p = 0.0092$] to reduce blood Mn levels compared to the unsupplemented group, and a similar trending effect of MCS to reduce brain Mn levels in the Mn-exposed group [$F(1, 20) = 3.19$, $p = 0.089$] (Supplemental Figure S4D). However, Tukey's pairwise comparisons revealed no significant difference between any groups.

4. Discussion

Studies in children and adolescents have revealed associations between developmental Mn exposure and inattention, impulsivity/hyperactivity, emotion dysregulation, and sensorimotor dysfunction (7,8,17,9–16). However, there are currently no recognized interventions, other than exposure prevention, to mitigate the neurotoxic effects of elevated developmental Mn exposure. Here, using a rodent

model of environmental Mn exposure, we demonstrate that developmental Mn exposure causes lasting dysfunction in attention, behavioral reactivity, learning, and sensorimotor function, and that increasing maternal choline intake during pregnancy and lactation was effective in protecting against some of these Mn-induced impairments, particularly in the domains of attentional function and behavioral reactivity in male rats. MCS also was beneficial for the unexposed animals in the domains of learning, attention, and behavioral reactivity. Collectively, these findings further establish developmental Mn exposure as a significant risk factor for environmentally-induced ADHD and related symptoms. They also demonstrate that MCS exerts lifelong cognitive benefits to offspring, including providing some protection against the neurotoxic impacts of elevated Mn exposure. Given recent evidence that 90% of pregnant women consume less choline than the recommended Adequate Intake, these data provide additional support for efforts to increase choline intake during pregnancy and lactation, particularly for women at risk of environmental exposure to Mn. These findings and inferences are discussed in detail below.

4.1 Developmental Mn exposure causes lasting dysfunction in attention, learning, behavioral reactivity, and sensorimotor function, recapitulating the pattern of symptoms reported in children with ADHD.

Our findings demonstrate that developmental Mn exposure causes lasting dysfunction in attention, impulse control, sensorimotor function, and behavioral reactivity (i.e.,

emotion regulation) – a constellation of impairments commonly seen in children diagnosed with ADHD (2–4). For example, across the three attention tasks, the Mn-exposed animals are particularly impaired in the later session blocks of testing (FA 1 task), and/or under trial conditions with the longest pre-cue delays (FA 2 task). The former finding reflects that the Mn animals improve in attentional accuracy more slowly over testing than controls, while the latter likely reflects deficits in sustained/focused attention at the most challenging (longer) pre-cue delays. Similarly, the Mn exposed animals appear to be more impulsive than controls, based on higher rates of premature responses across all three attention tasks (Figure 1). We should note, however, that the pattern of %Premature responses across session blocks within each attention task for the offspring of unsupplemented dams suggests that the higher rates of premature responses in the Mn animals in some session blocks may be a result of Mn animals learning to withhold premature responses more slowly than controls, rather than inherently greater impulsivity per se. Finally, developmental Mn exposure also causes lasting impairments in sensorimotor function in the Montoya staircase test – impairment that is most pronounced at the steps that are the most challenging to reach, based on significant reductions in the number of pellets taken, pellets eaten, %Success, and increased number of pellets misplaced (Figure 6). Collectively, these findings are fully consistent with our multiple prior studies (18–24), and they further implicate elevated developmental Mn exposure as a significant risk factor for environmentally-based ADHD and related symptoms (7,8,17,9–16).

Notably, we also report new findings showing that developmental Mn exposure leads to lasting dysregulation in behavioral reactivity, an essential functional domain comparable to emotion regulation in children (3,4). Here, we evaluated behavioral reactivity by assessing how attentional performance was affected by either (a) committing an error on the prior trial, or (b) not receiving an expected food pellet reward. As expected from prior studies (33,71,75–78), we found that control animals perform significantly more poorly on trials following an error (relative to after a correct response), and that they slow their response time on trials following an error (Figure 4, 5A, B), a phenomenon called "post-error slowing" (33,71,75–81). This pattern of changes likely reflects the fact that processing an error diverts attentional resources, leading to both impaired performance and longer response latencies on post-error trials (82). In contrast, for the Mn animals, committing an error on the prior trial did not affect their %Correct responses, and had only a small effect on their correct response latency (Figure 4, 5A, B). These findings indicate that the Mn-exposed animals may be less attentive to performance errors (i.e., negative feedback), and have diminished capacity to learn from trial history and adjust their behavioral strategy accordingly, suggesting impairment in performance monitoring and reactive control (79). The pattern seen in the Mn exposed animals on trials following an error – less performance disruption and less post-error slowing than seen in controls – mirrors findings showing that ADHD children are less responsive to committing errors than typically developing children (79), and exhibit diminished post-error slowing (83,84). Consistent with these findings, neuroimaging results have revealed

that children with ADHD have significantly weaker error-related activation in the salience network than typically developing children (85), suggesting that ADHD children may be less sensitive to detecting the saliency of their errors.

Interestingly, in contrast to their reduced sensitivity to committing an error, the unsupplemented Mn exposed rats exhibit a heightened reaction to the omission of an expected food reward on the prior trial, relative to that seen in controls (Figure 5A, B). The unsupplemented Mn animals exhibit a significant drop in %Correct performance on trials following a reward omission, compared to their performance after a correct response (Figure 5A, B), a pattern not seen in controls. This finding suggests that the Mn animals are more sensitive to reward (and its omission) than controls, a pattern which is again reminiscent of findings with ADHD children. Specifically, studies show that ADHD children often prefer smaller, more immediate rewards over larger, long-term rewards, are more sensitive than typically-developing children to the positive effects of a reward (72,86); and are more affected by the loss of an anticipated reward (74). This pattern thus shows clear parallels with our current findings that the Mn rats exhibit greater drops in performance on trials following a reward omission than do controls.

Consistent with these findings, the Mn-exposed animals commit a higher incidence of perseverative responses on trials following a correct response than do controls. Specifically, while animals across all treatment groups commit more perseverative

responses on trials following a correct rewarded response than on trials following an error or omitted reward, this increase is more pronounced for the Mn animals than controls (Figure 5C), a pattern also seen for food pellet reward collection latency (Figure 5D). These findings provide additional support for the inference that the Mn exposed animals are more sensitive to reward/reinforcement than controls, a pattern also seen in ADHD children (noted above). The findings also support that the Mn animals are more compulsive than controls, a suggestion that is again consistent with the suite of symptoms reported in ~10 – 25% of ADHD children that are co-morbid for both ADHD and Obsessive Compulsive Disorder (OCD) (87–91).

Collectively, our findings demonstrate a constellation of behavioral/cognitive impairments in developmentally Mn-exposed animals that are also seen in Mn exposed children and those with ADHD more generally; namely, impairments in attention, impulse control, and sensorimotor function, as well as a reduced response to committing an error and a heightened sensitivity to reward/reward omission (3,4,72,73). These latter *new* findings relating to Mn-induced dysfunction in behavioral reactivity/emotion regulation and error monitoring are highly relevant clinically, since the ability to maintain emotional and cognitive functioning in the face of unexpected events, as well as to learn from trial history and performance and adjust behavioral strategy accordingly, provides benefits in a variety of situations (85,92,93). These findings are also clinically relevant in light of evidence showing that emotion dysregulation occurs in ~25-45% of children and ~30-70% of adults

with ADHD, and that emotional problems have a greater negative impact on well-being and quality of life than other ADHD symptoms such as hyperactivity and inattention (3–5). Thus, our rat model of environmental Mn exposure, used here and in prior studies (18–24), exhibits the hallmark areas of dysfunction seen in the ADHD phenotype (2), and this study substantially extends our understanding of the lasting behavioral impacts of developmental Mn exposure.

4.2 MCS offers some protection against the adverse effects of early developmental Mn exposure.

Supplementing the maternal diet with additional choline during pregnancy and lactation offers protection against some of the effects of developmental Mn exposure, although the degree of benefit varied by cognitive domain. Two areas where MCS significantly benefited the Mn animals are in the realms of behavioral reactivity/emotion regulation and attention. In the realm of behavioral reactivity, the pattern of findings indicates that MCS offers protection against the effects of Mn exposure on reward sensitivity and reaction to errors. Specifically, animals born to dams on the choline-supplemented diet and then exposed to Mn in the early postnatal period did not exhibit a drop in performance on trials following a reward omission (similar to the unexposed unsupplemented controls), and very different from the Mn animals born to dams on the unsupplemented diet (Figure 5A). In addition, MCS partially normalized the reaction to committing an error for the Mn animals; this was seen for %Correct in the second focused attention task where the drop in performance

after an error was similar for the MCS control and Mn animals. In addition, in the reward omission task, MCS normalizes correct response latency on trials following an error for the Mn animals; that is, the Mn+MCS animals exhibit the same degree of post-error slowing to that seen in the control+MCS animals (Figure 5B). Finally, MCS reduced perseverative responding of the Mn animals in the reward omission task on trials after a correct (rewarded) response trial to levels below the control+MCS animals (Figure 5C), and this effect of MCS was accompanied by a reduction in food pellet reward collection latency (Figure 5D). Together, these findings support that MCS normalized compulsivity and reward omission sensitivity in the Mn animals, and partially normalized error reactivity.

Maternal choline supplementation also offered protection against the detrimental effects of Mn exposure on attentional function. This normalization of attentional function is seen in the second focused attention task, where MCS lessens the impairment of the Mn animals at the longer and more challenging pre-cue delays (Figure 2C and D). Similarly, in the selective attention task, MCS normalizes the performance of the Mn animals on distraction trials, such that they no longer differ from controls (Figure 3). In contrast to the protection provided by MCS for the adverse effects of Mn in the realms of behavioral reactivity and attention, no protection was provided with respect to the impairments seen in learning and sensorimotor function. In the first focused attention task, the Mn animals learned to wait for the visual cue more slowly than controls, and MCS did not alter this Mn

effect (Figure 2A, B). Further, the grasping and reaching impairments of the Mn animals born to choline-supplemented dams was as pronounced as that seen in the unsupplemented animals (Figure 6).

Collectively, these findings demonstrate that supplementing the maternal diet with additional choline during pregnancy and lactation partially, but not completely, normalizes Mn deficits in the domains of behavioral reactivity/emotion regulation, compulsivity, and attention, but not learning or sensorimotor function. These neuroprotective effects are consistent with other studies that have shown that MCS lessens the adverse cognitive effects of various developmental insults. Specifically, MCS has been shown to lessen deficits in learning, attention, emotion regulation, and spatial cognition in a down syndrome mouse model (31–33,94), spatial learning and memory in a multi-generational Alzheimer's mouse model (95), anxiety-related behaviors in a rat model of maternal stress (30), social behavior and anxiety in a mouse model of autism (96), seizure-induced memory impairment in rats (97,98), recognition memory in a rat model of fetal-neonatal iron deficiency (99,100), deficits in working memory, spontaneous alternation behavior, and reflex development in a fetal alcohol syndrome rat model (28,29). MCS has also been shown to lessen deficits in visual recognition memory, working memory, verbal memory, eyeblink conditioning (EBC), postnatal growth, and cognition in human infants born to alcohol-using mothers (36–38). Of particular interest to the present study, MCS has been shown to lessen the attentional dysfunction, as well as to normalize behavioral

reactivity to committing an error or not receiving an expected reward in a mouse model of Down syndrome (33). Collectively, these studies provide additional support for increasing choline intake during pregnancy, particularly for women at risk of toxicant exposure.

4.3 MCS benefits cognitive function in offspring not exposed to Mn

In addition to the benefits provided to the Mn exposed animals, supplementing the maternal diet with additional choline was also beneficial for the control animals in the realms of behavioral reactivity, attention, and learning. Specifically, for control animals, MCS lessens the disruptive effect of committing an error on the prior trial (Figure 4, 5A; discussed above in section 4.2), suggesting an improved ability to regulate the behavioral disruption/emotional reaction to committing an error. For attentional function, the MCS benefit in control animals is especially evident at the 0 sec pre-cue delay in the second focused attention task (Figure 2C), an effect which may also partly reflect this reduction in error reactivity because trials with a 0 sec pre-cue delay are closest temporally to the prior trial, some of which were error trials. In addition, trending effects for improved attentional performance were seen for the rate of improvement across blocks in the first focused attention task (Figure 2A, B), as well as for performance in the reward omission task on trials following a correct response (Figure 5A).

Altogether, these findings showing cognitive benefits of MCS in control animals are consistent with the MCS benefits reported in prior studies in humans and animal models (39–42,44,47,48,50). For example, a number of studies by Meck, Williams and colleagues demonstrate that MCS improves attention and memory in rodents (42–45). Additionally, studies in humans have demonstrated that increased choline intake during pregnancy produces lasting benefits for offspring attention, memory, and executive functioning (39–41). Notably, in one controlled feeding study that followed a cohort of children until 7 years of age, random assignment of mothers to a higher choline intake (vs the Adequate Intake) during the third trimester produced faster processing speed during infancy (40), as well as superior memory, sustained attention, and executive functioning at 7 years of age (39).

4.4 Putative neurobiological mechanisms

Our rodent model of developmental Mn exposure recapitulates many of the adverse behavioral symptoms reported in ADHD children. While the neurobiological mechanisms underlying ADHD and related symptoms are not well understood, studies suggest that the combination of multiple environmental and biological risk factors lead to hypofunctioning of the catecholaminergic system within the fronto-cortico-striatal loop and other neural circuits underlying executive attention and emotion regulation (101–105). Similarly, studies from our lab and others have demonstrated that developmental Mn exposure causes hypofunctioning of the catecholaminergic system in the prefrontal cortex and striatum (22–24), including

reducing stimulated dopamine and norepinephrine release (24,25), and altered levels of dopaminergic and noradrenergic system proteins (22–24). The catecholaminergic system in the prefrontal cortex/anterior cingulate cortex has been well-established as critical to executive functioning, including attention, emotion regulation, error monitoring, and behavioral inhibition (106–112), and thus Mn-induced alterations to this system may well underlie the ADHD-like behavioral phenotype of the Mn exposed animals. This inference is further supported by studies from our group showing that oral methylphenidate (a catecholamine reuptake inhibitor) ameliorates the Mn behavioral deficits in attention and sensorimotor function (19,21,59). Notably, our group has also demonstrated that developmental Mn exposure causes lasting alterations in DNA methylation and expression of genes related to DNA methylation, neuronal development and catecholaminergic neuronal systems in our rat model (113), and other studies have also shown that Mn exposure can lead to altered DNA methylation (114–117). Related to this, it is known that choline serves as, among other things, a methyl donor in key biochemical reactions, and supplementing the maternal diet with additional choline has been demonstrated to alter DNA methylation in both humans and animal models (35,118–124). Additionally, MCS has been shown to normalize alterations in DNA methylation caused by fetal alcohol exposure (34,35). Therefore, it is plausible that the benefits of MCS to partially normalize Mn-induced behavioral reactivity and attention deficits in the present study may be due in part to normalization of Mn-induced epigenetic alterations, though this suggestion needs investigation.

4.5 Conclusion

The present study provides new evidence that developmental Mn exposure produces lasting dysregulation of behavioral reactivity/emotion regulation, in addition to its detrimental effects on attention, impulse control, and sensorimotor function in male rats. These findings largely recapitulate the constellation of symptoms reported in Mn-exposed children, and separately in ADHD children, thus further supporting concerns over developmental Mn exposure as a significant risk factor for ADHD and related symptoms. In addition, the present study demonstrates that supplementing the maternal diet with additional choline offers some protection to the adverse effects of early developmental Mn exposure, as well as provides lasting cognitive benefits for the unexposed offspring. In light of the markedly increased physiological demand for choline during pregnancy and the low choline intakes of most pregnant women, these results provide additional support for efforts to increase choline intake during pregnancy, via diet and/or adding choline to standard prenatal vitamin regimens.

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Chapter 3

DEVELOPMENTAL MANGANESE EXPOSURE CAUSES BROAD CHANGES IN THE PREFRONTAL CORTEX METHYLOME AND TRANSCRIPTOME THAT ARE ASSOCIATED WITH DEFICITS IN LEARNING AND BEHAVIORAL ADAPTATION IN ADULTHOOD, AND MATERNAL CHOLINE SUPPLEMENTATION PROTECTS AGAINST MANY OF THESE MANGANESE EFFECTS

Abstract

Environmental manganese (Mn) exposure is a risk factor for ADHD, and studies in children have reported associations between elevated Mn exposure and ADHD-related symptoms of inattention, impulsivity/hyperactivity, and psychomotor impairment. Maternal choline supplementation (MCS) during pregnancy/lactation may hold promise as a protective intervention against the neurotoxic effects of elevated Mn because it has been shown to improve cognitive function in typically-developing children, and to lessen cognitive dysfunction caused by numerous insults during neurodevelopment. Our objectives were to determine whether (1) developmental Mn exposure leads to lasting alterations in prefrontal cortex gene expression and DNA methylation that may underlie the behavioral dysfunction from Mn, and (2) MCS protects against these Mn-induced molecular alterations. Pregnant Long-Evans rats were given a standard diet, or a diet supplemented with ~4x additional choline throughout gestation and lactation (GD 3 - PND 21). Male

offspring were exposed orally to 0 or 50 mg Mn/kg/day over PND 1–21. In adulthood, animals underwent behavioral testing before sacrifice at PND 200. Previously reported findings from these same animals showed that developmental Mn exposure causes lasting behavioral dysfunction consistent with ADHD symptomology, including deficits in attention, behavioral reactivity, learning, impulsivity, and sensorimotor function. Here we analyzed prefrontal cortex brain tissue from those animals to show that developmental Mn exposure causes lasting alterations in DNA methylation at 16,641 CpG loci that were accompanied by altered expression of 952 genes in the prefrontal cortex of adult animals, including genes involved in neuronal function, inflammation, and epigenetic function. Maternal choline supplementation protects against some, but not all, of the DNA methylation and gene expression changes caused by Mn. This MCS protection is seen in genes spanning a variety of functions, including neuronal function and inflammation. One particular gene of interest whose expression is protected by MCS is *Chrna7*, which encodes for the α7 Nicotinic acetylcholine receptor that is involved in neurodevelopment and cognition and has been implicated in ADHD. In the absence of Mn exposure, MCS caused lasting alterations in DNA methylation and expression of 821 genes, many of which are involved in epigenetic function and neuronal function. These findings provide evidence that the lasting cognitive deficits caused by Mn exposure may be due in part to Mn-induced changes in DNA methylation and expression of genes involved in proper neurobiological development and function, and that the MCS effect to protect against some Mn-induced cognitive deficits may be

in part due to normalization of some gene expression and DNA methylation changes caused by Mn. This work adds to the growing evidence that maternal choline supplementation is neuroprotective for offspring, and may be beneficial for individuals in communities at high risk of Mn exposure.

1.0 Introduction

ADHD is the most common behavioral disorder in children, affecting 5-11% of children under age 18 years (1–3). Environmental manganese (Mn) exposure is associated with increased risk of ADHD and related symptoms in children and adolescents (4–14). Pre-clinical studies have shown that developmental Mn exposure causes lasting ADHD-like impairments, including deficits in attention, impulse control, behavioral reactivity, and sensorimotor function (15–20).

The mechanism(s) by which Mn exposure causes lasting cognitive impairments is not fully understood, but studies have shown that Mn-induced behavioral deficits are associated with a hypofunctioning catecholaminergic system in the prefrontal cortex and striatum - brain areas that mediate attention, impulse control, emotion regulation, and sensorimotor function (21–23). In particular, developmental Mn exposure alters catecholaminergic protein levels (24–26) and reduces K^+-stimulated release of dopamine and norepinephrine (16,26,27). In addition to these studies of the catecholaminergic system, studies from our lab and others have shown that Mn exposure more broadly alters gene expression in animal model brain tissue (28), *C. elegans* (29), and cell lines (30,31). Finally, Mn has been shown to cause

lasting alterations in DNA methylation (28,32,33). Because DNA methylation plays a role in regulating gene expression (34,35), and many catecholaminergic system genes are regulated in part by epigenetic mechanisms (36–40), Mn-induced alteration in DNA methylation likely plays a role in some changes in gene expression caused by developmental Mn exposure. These Mn-induced changes in gene and protein expression likely underlie some of the behavioral deficits seen in animals and children (4–7,9–14,41). However, there are no established treatments available for children exposed to high levels of Mn during neurodevelopment, and it is unknown whether there is an intervention that may protect against alterations in DNA methylation, gene expression, and protein levels that have been associated with Mn-induced cognitive deficits.

A number of nutritional interventions have been shown to mitigate the effects of a variety of environmental insults, including maternal supplementation with choline, folic acid, genistein, and L-methionine (42,43). Specifically, maternal choline supplementation (MCS) has been shown to lessen cognitive dysfunction caused by a variety of perinatal insults and genetic abnormalities in humans and animal models (44–58), as well as provide benefits to cognitive functioning in typically developing children (59–61) and animal models (62–70). These cognitive benefits may be partly due to maternal choline supplementation altering gene expression, including expression of genes related to neurological function (71–76), and DNA methylation (73,77–80). Importantly, MCS has also been shown to normalize some gene expression and epigenetic molecular alterations caused by

perinatal alcohol exposure (76,81). However, it is unknown whether MCS can protect against Mn-induced neurobiological alterations, including in gene expression and DNA methylation.

The present study used our rodent model of early childhood environmental Mn exposure to test two hypotheses: 1) That developmental Mn exposure causes lasting alterations in the expression of specific catecholaminergic system genes, as well as broader alterations in the DNA methylome and transcriptome associated with neuronal function, inflammation, and epigenetic regulation in the brain prefrontal cortex; and 2) that supplementing the maternal diet with additional choline during pregnancy and lactation will protect against some or all of these adverse effects of developmental Mn exposure. Notably, the animals used in the present study are the same behaviorally-tested animals from a recent prior study (20). In that prior study, we reported that developmental Mn exposure caused lasting dysfunction in attention, reactivity to errors and reward omission, learning, and sensorimotor function, recapitulating the constellation of symptoms seen in ADHD children, and that MCS lessened the Mn-induced attentional dysfunction and partially normalized reactivity to committing an error or not receiving an expected reward, but provided no protection against Mn-induced learning or sensorimotor dysfunction (20) (summarized in Figure 2). Altogether, the goal of the present study is to determine the lasting molecular changes in the prefrontal cortex and their association with a suite of ADHD-like behavioral deficits caused by developmental Mn exposure, and whether MCS protects against these Mn effects. This will provide much-needed insights into

the molecular underpinnings of these deficits and the potential protective effects of MCS as a widely available nutritional supplement.

2.0 Methods

2.1 Subjects

Sixty-four (64) male Long-Evans rats were used for neurobehavioral assessment, the results of which were published in Howard et al. 2024 and shown in chapter 2 of this dissertation. A subset of these animals and their littermates were used to assess gene and protein expression and DNA methylation. Males were used for several reasons. First, the focus on males reflects the evidence that attentional dysfunction is two to three times more prevalent in boys than girls (1,82). Second, human and animal studies have shown that males are more sensitive than females to developmental Mn neurotoxicity (5,24,83,84). Finally, we have previously established in male rats that developmental Mn exposure causes lasting alterations in gene and protein expression (24–26,28); thus, we deemed it important to build upon these findings to investigate the potential benefits of perinatal choline supplementation to protect against those known Mn deficits.

Subjects were born in-house from 16 nulliparous timed-pregnant dams (Charles River; dams were 8-9 weeks old and pregnant at gestational age 3 days). Twelve to 24 hours after parturition (designated postnatal day (PND) 1, birth = PND 0), litters were weighed, sexed, and culled to eight pups per litter such that each litter was composed

of as many males as possible (2-8) and the remainder females. Each treatment group contained n = 16 rats that underwent behavioral testing, a subset of which (n=3-6/treatment group) were used to assess the molecular outcomes presented here, and no more than 1-2 males per litter were assigned to a particular treatment group. At PND 22, all pups were weaned and pair-housed with a rat of the same treatment group and maintained on a reversed 12:12 hour light/dark cycle. Rats were housed in polycarbonate cages at a constant temperature of 21 ± 2°C. All aspects of behavioral testing and feeding were carried out during the active (dark) phase of the rats' diurnal cycle, as described in chapter 2 and Howard et al. (20). All animal care and treatments were approved by the institutional IACUC (protocol Smitd1803) and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals (85).

2.2 Maternal choline supplementation

Pregnant dams were randomly selected to receive one of two concentrations of choline chloride in their diet (Dyets AIN-76A Purified Rodent Diet), both of which were provided ad libitum during gestation and lactation, from gestational day (GD) 3 until offspring weaning on PND 21 (note that pre-weaned offspring also had direct access to the dam's diet as they approached weaning age and were large enough to access the diet directly). The control purified diet (unsupplemented) contained 1.1 g choline/kg, whereas the choline-supplemented purified diet contained 5.0 g choline/kg; both diets are reported by the manufacturer to contain 54 mg Mn/kg.

Upon weaning on PND 21, all pups were provided Irradiated Teklad Global Soy Protein-Free Extruded Rodent Diet 2920, reported by the manufacturer to contain 80 mg Mn/kg and 1.2 g choline/kg, which fall within the range typically provided in rodent chow. Figure 1A depicts the 2x2 study design with the four treatment groups, while Figure 1B shows the timeline of maternal choline supplementation, Mn dosing, and behavioral testing.

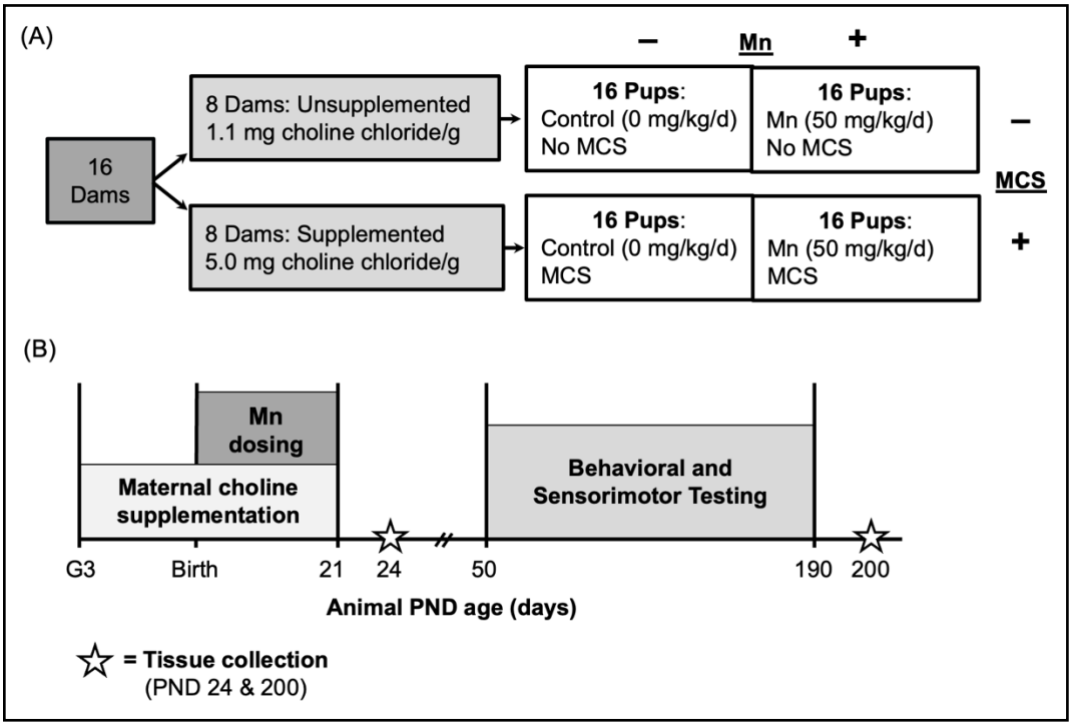

Figure 1. Study timeline with task parameters, and study design. (A) 2x2 study design, ending with 16 pups per treatment group that were used for behavioral testing. A subset of animals from each treatment group were used for molecular analyses presented here. (B) Study timeline, including MCS and Mn dosing, as well as behavioral tasks and tissue collection ages (PND 24 and 200). X-axis is age in days (PND).

2.3 Manganese exposure

Neonate rats were orally exposed to 0 or 50 mg Mn/kg/day in a vehicle solution (5% w/v stevia in Milli-Q water) from PND 1–21, as in our prior studies (15–18,24–26). This Mn exposure level is not overtly toxic and does not measurably affect neonate health or nutrition, based on neonate milk intake from the lactating dam, growth rate, or blood hematocrit levels at weaning (18). Briefly, dosing was delivered once a day directly into the mouth of the pups in a volume of 15-25 μL/dose via a micropipette fitted with a flexible polyethylene gel loading tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received only the deionized water + stevia vehicle. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both, as pre-weaning exposure to 50 mg Mn/kg/day produces relative increases in Mn intake that approximates the relative increases reported in infants and young children exposed to Mn-contaminated water or soy-based formulas (or both) (18,24).

2.4 Behavioral testing summary

See chapter 2, and Howard et al. (20) for detailed information regarding behavioral testing. Briefly, 16 identical automated 5-Choice Serial Reaction Time Task (5-CSRTT) testing chambers fitted with odor delivery systems (#MED-NP5L-OLF, Med Associates, Inc., St. Albans, VT) were used to assess specific cognitive processes, including learning, focused and selective attention, inhibitory control, and behavioral reactivity, as described previously (17,18,20,86). Each rat was assigned to one of the 16 testing chambers, and each chamber was balanced for the four treatment conditions. A daily test session consisted of 150 trials or 50 min, whichever came first. All behavioral testing was conducted by individuals blind to the treatment condition of the subjects.

Behavioral testing began at ~PND 50. 5-CSRTT assessment is conducted via a series of progressively more difficult tasks that challenge the ability of animals to learn the contingencies of the task, including to sustain attention, inhibit premature responses, and ignore olfactory distractors while maintaining focus on the predictive visual cue. Recorded response types for all 5-CSRTT tests included: 1) premature responses (responses made after trial onset but before presentation of the visual cue; assessed in the first focused attention, second focused attention, and selective attention tasks); 2) correct responses (responses made to the correct port following presentation of the visual cue); 3) incorrect responses (responses made to the incorrect port following presentation of the visual cue); and 4) omissions (failure to respond within 15 sec after visual cue presentation). Premature and incorrect responses and omission errors

were not rewarded and were immediately followed by a 5 sec time-out, in which the house light was turned off for 5 sec. The calculated response outcomes were: (1) %Correct, calculated as number of correct responses/(correct + incorrect + premature + omissions) × 100; (2) %Incorrect, calculated as above but with incorrect responses in the numerator; (3) %Premature, calculated as above but with premature responses in the numerator; and (4) %Omissions, calculated as above but with omissions in the numerator. Additionally, we assessed attentional accuracy via a %Accurate outcome, calculated as number of correct responses/(correct + incorrect) × 100.

Following nose-poke training and a 5-choice visual discrimination learning task, several tasks were administered. Two successive focused attention tasks (FA1 and FA2) were administered to assess the ability of rats to detect and respond to a visual cue presented unpredictably in time and location (one of the five response ports). The first focused attention task used variable pre-cue delays of 0, 1, 2, or 3 sec and a fixed visual cue duration of 0.7 sec and was administered for 15 daily sessions. The second focused attention task included variable pre-cue delays of 0, 3, 4, or 5 sec and variable visual cue durations of 0.5 or 0.7 sec and was administered for 15 sessions. One selective attention (SA) task was administered, in which the pre-cue delay varied between 3 and 4 sec, and on one third of the trials in each session an olfactory distractor was presented 1 or 2 sec after trial onset and 1 – 3 sec before visual cue presentation. A reward omission (RO) task included the random omission of a food pellet reward on three out of 10 correct trials. Attentional performance on the reward

omission task was assessed as a function of whether the preceding trial was either (1) correct (and rewarded), (2) incorrect (including omission errors), or (3) correct but food reward omitted.

In addition to the 5-CSRTT operant tasks, the Montoya staircase test of sensorimotor function was conducted using plexiglass devices with a platform and a left-and-right side staircase. The staircases contained 6 descending steps on each side, and each step was baited with three 45 mg food pellets for a total of 36 pellets available at the start of a session. To determine the step of origin and final location of each pellet, pellets were color coded with a unique color per step level. Skilled forelimb performance was evaluated step-by-step for (1) the number of pellets taken (i.e., pellets that were grasped and removed from their step of origin), (2) the number of pellets eaten (pellets that were taken and consumed), (3) the corresponding percent of success (the ratio of the number of pellets eaten/pellets taken x 100), (4) the number of pellets misplaced (all the pellets that were grasped but ended up on a different step level than their step of origin), and (5) the number of pellets lost (pellets that ended up on the floor of the apparatus, out of reach of the rats).

2.5 Sacrifice and tissue collection

For gene expression, protein expression, and DNA methylation analysis, tissue samples were collected from weaned pups on PND 24, and from behaviorally-tested animals on PND 200. Briefly, half of the rats (n=8/treatment group) were euthanized

via CO_2 asphyxiation, upon which the whole brain was immediately removed, gross-sectioned and immediately frozen on dry ice; the section containing the prefrontal cortex was later dissected and tissue punches used to sub-sample prefrontal cortex tissue for RNA expression and DNA methylation analysis, as described below. The remaining animals (n=8/treatment group) were processed for protein immunohistochemical staining analysis; animals were deeply anesthetized with sodium pentobarbital and perfused intracardially with ice-cold 0.9% (wt/vol) saline, followed by perfusion with ice-cold 4% (wt/vol) paraformaldehyde. Whole brains were extracted and cryopreserved as described elsewhere (25). The paraformaldehyde-fixed cryopreserved brains were sectioned (Leica CM3050 S) into 20 μm coronal sections at − 20°C in preparation for protein immunostaining for D1 and D2 receptors, and brain sections were stored in 30% (wt/vol) sucrose in 0.01 M phosphate-buffered saline (PBS) cryoprotectant solution at − 20°C until immunostaining.

2.6 Targeted catecholamine gene expression analysis

Gene expression of key catecholaminergic (Drd1, Drd2, Th, Dat, Net, α2a) genes was determined by RT-qPCR in prefrontal cortex (PFC) tissue from PND 24 littermates and PND 200 behaviorally tested rats from Howard et al. (20) (n=4-5 rats/treatment for PND 24, n=6 rats/treatment for PND 200). Briefly, partially thawed fresh-frozen PFC tissue punches (1.39 mm tissue section thickness, 1.5 mm tissue punch diameter, yielding PFC tissue punches of 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 using the Quick- DNA/RNA™ Miniprep Kit (Zymo: Cat. #d7001) following the manufacturer's instructions. RNA quality and quantity was measured through Nanodrop and RNA band integrity was confirmed through gel electrophoresis prior to DNAse Treatment and Reverse Transcription (RT) using the Invitrogen™ SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme kit (Invitrogen™: Cat. #11766050) following the manufacturer's instructions. RT-qPCR cycle quantity thresholds were measured using the ThermoFisher Scientific TaqMan Advanced Master Mix (Applied Biosystems: Cat. #4444556) and TaqMan primers (Supplemental Table 1) on a Bio-Rad CFX95 instrument following the manufacturer's protocol. Gapdh, ActB, and Ubc expression were used as reference genes. RT-qPCR was performed on duplicate reverse transcription reactions per animal, with triplicate samples per animal included in each of the two reverse transcription reactions. RT-qPCR gene expression data statistical analyses were performed on ΔCt values by mixed-model ANOVA, with choline (2 levels) and Mn treatment (2 levels) as between-subject factors, and rat ID as a random effect; p values ≤ 0.05 were considered significant. Significant main effects or interactions were followed by pair-wise treatment group comparisons using Tukey's post hoc test. Analyses were performed using JMP Pro (version 16.1.0; SAS Institute, Inc.).

2.7 Immunostaining, fluorescence microscopy, and image quantification

Immunohistochemistry was used to assess protein levels in the mPFC and striatum of behaviorally tested animals, as described previously by us (26). For immunohistochemical analyses, behaviorally-tested animals were sacrificed at PND 200 as described above (n = 6/animals treatment group, 24 animals total). From each animal/brain, two sections within the mPFC were selected for staining, ranging from bregma AP + 2.16–1.56 mm (corresponding to plates 15-20 in Paxinos & Watson, 2007). Prior to immunostaining, brain sections underwent antigen retrieval for 15 min in a 10 mM sodium citrate buffer solution composed of sodium citrate dihydrate in Milli-Q water, and heated in a hot water bath at 80°C. Following antigen retrieval, sections were washed three times in 0.01 M PBS for 10 min each. Brain sections were double-stained for D1 and D2 receptor proteins in each section. For staining, brain sections were free-floated in a blocking solution containing 1% (vol/vol) normal donkey serum (Jackson Immunoresearch), 0.3% (vol/vol) Triton X-100 (Sigma Aldrich), and 1% (vol/vol) bovine serum albumin (Sigma Aldrich), in 0.01 M PBS for 1 hour. Primary antibodies were incubated for 40 hours at 4°C in 0.5% (vol/vol) Triton X-100 in 0.01 M PBS, using the following primary antibodies and dilutions: mouse monoclonal anti-D2 receptor, 1:200 (Santa Cruz Biotechnology, Sc-5303); rabbit polyclonal anti-D1 receptor, 1:100 (Alomone Labs, ADR-001). Next, brain sections were washed three times and incubated in a secondary antibody solution composed of 10% (vol/vol) normal donkey serum and corresponding secondary antibodies in 0.5% (vol/vol) Triton X-100 in 0.01 M PBS for 2 hr at 23°C. Secondary antibodies were: donkey anti-mouse Alexa Fluor 594, 1:1000 (Invitrogen Thermo

Fisher. A21203); donkey anti-rabbit Alexa Fluor 488, 1:1000 (Abcam. #ab 150073). Brain sections were then washed three more times in 0.01 M PBS and incubated for 10 min in a 1:1,000 4',6-diamidino-2-phenylindole (DAPI) stain in 0.01 M PBS to label cell nuclei. Finally, sections were washed three times in 0.01 M PBS and mounted on slides and cover-slipped with Fluoromount G mounting media (Southern Biotech) in preparation for fluorescence microscopy. Four sections representing two animals were mounted per slide, balanced by Mn treatment condition.

mPFC and striatum regions were imaged for D1 and D2 receptor levels using a Zeiss AxioImager microscope at 40× magnification. To avoid bias in image acquisition, an oval was drawn in both subregions of interest using the Zeiss ZEN imaging software, and a pseudo-randomization tool determined three non-overlapping fields of view for image collection. This yielded a total of six images per brain region per animal (three images per brain region and section x two sections per animal). All images were captured under identical microscopy imaging settings, including exposure time and gain, for each protein. Each image was collected in a z-stack format at 0.5 μm intervals between each z-focal plane over a total imaging range of 17 μm in the 20 μm brain slice. Following image collection, the number of z-plane images was reduced to 12 (6 μm z-plane distance) to remove out-of-focus z-planes, and then deconvolved using AutoQuant X3 software (version 3.1). Deconvolved images were imported into Imaris (software version 9.8.2) for fluorescence intensity quantification.

Fluorescence quantification was performed using the Imaris 'Surfaces' tool, with unique quantification algorithms applied to each fluorescence channel and protein. Algorithms were customized to each protein, first by applying automated thresholds for absolute fluorescence intensity to determine whether quantified surfaces matched the number of fluorescent objects in the image based on visual inspection. If the automated thresholding appeared incongruous with the staining pattern, the 'Background Subtraction' tool was used to improve the specificity of the algorithm. The primary quantification outcome used for analysis was the sum total fluorescence per three-dimensional object summed across all objects per image. In all cases of image acquisition and quantification, the experimenter was blinded to the treatment condition.

2.8 Transcriptomics of differentially expressed genes and functional pathway analysis

Because the maternal choline supplementation and Mn treatments likely affected DNA methylation and gene expression beyond the key catecholaminergic system genes/proteins investigated in our targeted analyses (described above), we used an unbiased 3' Tag RNA-sequencing approach to determine the lasting effects of these treatments on the PFC transcriptome more broadly. RNA aliquots from the same PND 200 PFC RNA extraction in Methods section 2.5 (n=5-6 rats/treatment) were used 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 2021.1.1). Briefly, read files were cleaned using BBDuk version 38.84 and aligned to the reference genome (mRatBN7.2) using the Geneious Prime mapper. Differential gene expression was calculated using DESeq2, which assessed differences in gene expression in a pairwise fashion, calculating the fold-change and p-values to determine which genes were differentially expressed genes (DEGs). Four different pairwise analyses were conducted to accommodate our treatment group comparisons, including: (1) control vs. Mn ; (2) control vs. Mn+MCS ; (3) control vs. MCS ; and (4) Mn vs Mn+MCS. Principal component analysis (PCA) was conducted using JMP Pro (version 16.1.0; SAS Institute, Inc.) using normalized (TPM) gene counts to visualize the gene expression profile of each animal. A PCA plot generated from counts of all genes were used to identify and remove outliers (three total animals; one from Control, one from MCS, one from Mn+MCS), and a PCA plot using counts from differentially expressed genes was generated to visualize the effect on each treatment group. For each pairwise comparison, genes were ranked and analyzed using Fast Preranked Gene Set Enrichment Analysis (FGSEA) to determine enriched Gene Ontology-associated biological processes, molecular functions, and cellular components. Gene Ontology biological processes groups were further reduced, filtered, and visualized using REVIGO version 1.8.1, which uses an algorithm for semantic similarity of parent and child terms (87). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were conducted on several subsets of differentially expressed genes of interest ($p < 0.05$ unadjusted) using EnrichR (88–

90). KEGG analysis integrates gene function databases and describes differentially expressed genes in the context of disease and pharmaceutical outcomes (91).

2.9 Methylomics of differential DNA methylation

We conducted reduced representation bisulfite sequencing (RRBS) to determine whether developmental Mn exposure caused lasting alterations in DNA methylation, and whether MCS normalized these changes. DNA was extracted from the same PND 200 fresh-frozen PFC samples detailed in Section 2.5, using the Quick-DNA/RNA™ Miniprep Kit (Zymo: Cat. #d7001) following the manufacturer's instructions. 500 ng of DNA was bioanalyzed for DNA integrity, bisulfite converted for RRBS using the Premium RRBS kit V2 (Diagenode: Cat. #C02030036), and sequenced by Illumina NovaSeq PE150 for approximately 40 million reads each sample by the University of California Davis Genome Center (n= 3 rats/treatment). Following sequencing, the data were cleaned using Trim Galore, version 0.6.7, with cutadapt version 4.1. Cleaned sequences were aligned to the reference genome (GRCm39), and CpG methylation was identified and quantified using Bismark (version 0.22.3). Differentially methylated loci and regions were identified using DMRcate (version 3.50.3) as described by Peters et al. (92). The statistical significance is defined by a p-unadjusted value less than 0.01. The minimum number of CpGs required within a window of 1000bp to be considered for differential methylated region analysis was two. Methylation data were aggregated from all samples (rats). The raw number of

methylation loci was 9,281,361, and these were filtered to include loci with read coverage of at least five in all samples, leaving 2,374,207 loci.

3. Results

3.1. Manganese exposure caused lasting behavioral deficits in attentional function, error monitoring, and behavioral reactivity

The animals from the present study underwent extensive behavioral testing, as described in chapter 2 and reported in Howard et al. 2024 (20). A summary of those behavioral outcomes is presented in Figure 2 to provide important context for caused lasting deficits in an array of functional domains including attention and behavioral reactivity - domains that are subserved to a large extent by catecholaminergic system function in the prefrontal cortex and striatum (21–23).

(A)

Functional domain	Mn effect (compared to control)	MCS protection?
Attention (Depicted in panel A below)	Mn Deficit – poorer performance in sustained/focused attention under most challenging trial conditions	Yes – partial protection
Behavioral reactivity (Depicted in panel B below)	Mn Deficit – more reactive to omitted reward and less reactive to error on prior trial	Yes – partial protection
Learning/Impulsivity	Mn Deficit – delay in learning to withhold impulsive responses	No
Sensorimotor function (Depicted in panel C below)	Mn Deficit – impairment in reaching/grasping success	No

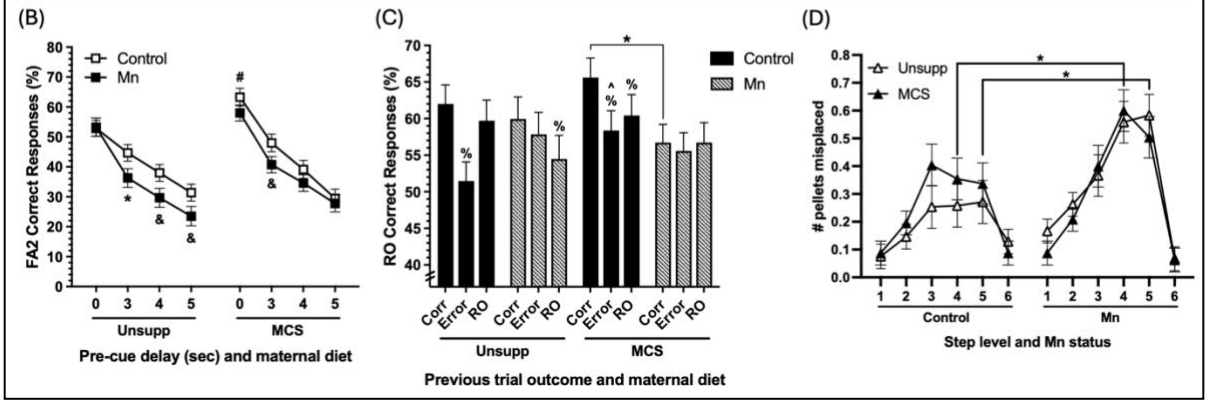

Figure 2. Overview of behavioral outcomes assessed in the animals that provided brain tissue for the molecular outcomes reported in this chapter (Howard et al. 2024), including the effects of Mn (compared to control) and MCS protection. Panel (A) summarizes the behavioral outcomes. (B) depicts performance in the second focused attention task, as a function of the pre-cue delay (0, 3, 4, or 5 sec). (C) depicts performance on the reward omission task, as a function of the prior trial outcome. Prior trial outcomes are Corr (correct response), Error (incorrect or omission error), or RO (correct with reward omitted). (D) depicts performance on the Montoya staircase task of sensorimotor function, as a function of apparatus step. For A-C, % indicates $p \leq 0.05$ versus following a correct response; * and & indicate $p \leq 0.05$ and $p \leq 0.10$

versus controls, respectively; # and ^ indicate $p \leq 0.05$ and $p \leq 0.10$ versus unsupplemented animals, respectively. Data are lsmeans ± SEM (n=14-15/group).

3.2 Manganese exposure increased brain Mn concentrations in PND 24 littermates of behaviorally tested animals, and returned to baseline in PND 200 behaviorally tested animals

Mn exposure over PND 1-21 increased Mn levels in brain tissue in PND 24 weanlings. Specifically, Mn exposure causes an approximately three-fold increase in brain Mn levels in PND 24 offspring, from approximately 3.8 to 9.3 µg/g in control versus Mn-dosed unsupplemented animals, as demonstrated by a significant main effect of Mn [$F(1, 36) = 129.7$, $p < 0.0001$] (Figure 3). There is also a trending main effect of MCS to alter brain Mn levels in offspring [$F(1, 36) = 3.96$, $p = 0.054$], and a trending Mn x MCS interaction [$F(1, 36) = 3.13$, $p = 0.085$]. Tukey's pairwise comparisons revealed that Mn animals who received MCS had significantly higher brain Mn levels than their unsupplemented counterparts. The mean brain Mn levels of MCS animals are 3.8 µg/g in control and 14.3 µg/g in Mn animals.

In the PND 200 behaviorally-tested animals, brain Mn levels return to baseline. Although there remained a trending but biologically unremarkable effect of MCS to reduce brain Mn levels in the Mn-exposed group [$F(1, 20) = 3.19$, $p = 0.089$] (Figure 3), Tukey's pairwise comparisons revealed no significant difference between any groups.

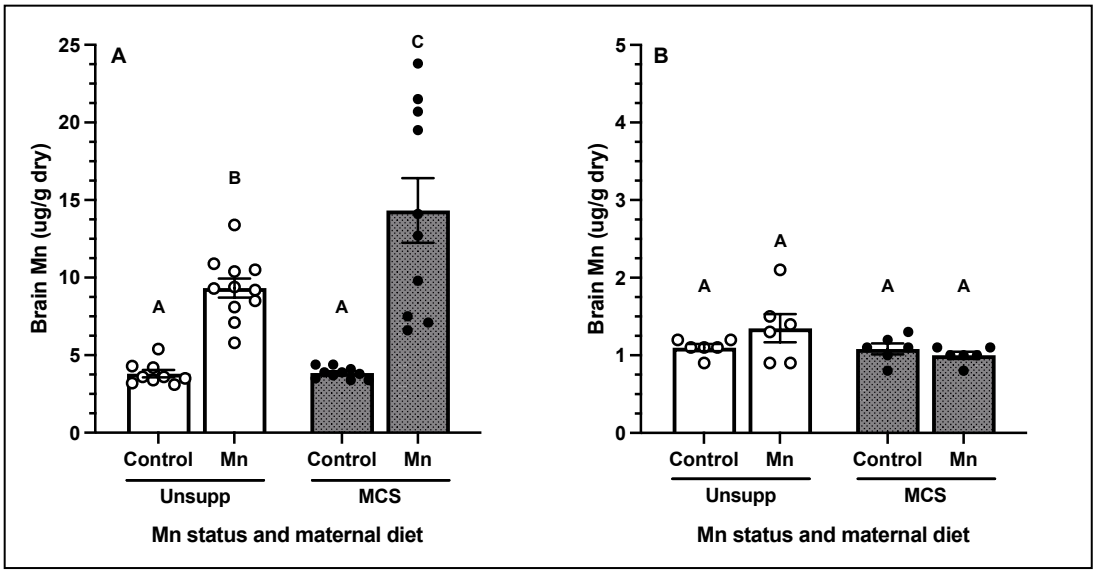

Figure 3: Oral Mn dosing increased brain Mn concentrations in weanling littermates of the behaviorally tested animals (PND 24), and returned to baseline in the behaviorally tested animals at sacrifice (PND 200). PND 24 animal brain (n = 9-11) (A) measurements show that Mn was increased in Mn-exposed animals. PND 200 animal brain (n = 6) (B) measurements show that Mn levels return to baseline. Graphs show arithmetic means with standard error. PND 24 brain statistics were performed on log-transformed data; PND 200 statistics were performed on non-transformed data. Within an age group, treatment groups with different capital letter superscripts are statistically different from one another ($p < 0.05$), based on Tukey's post hoc test.

3.3 Manganese and maternal choline supplementation alter the expression of key catecholaminergic system genes in the prefrontal cortex

We utilized RT-qPCR and immunohistochemistry in a targeted approach to interrogate the effects of Mn and MCS on expression levels of the key

catecholaminergic system genes *Drd1, Drd2, Th, Dat, Net*, and *a2a* and proteins levels for D1R and D2R that have been shown in prior studies to be altered by developmental Mn exposure (24–28). Gene expression analysis was performed in prefrontal cortex of PND 24 littermates to the behaviorally tested animals, and in the PND 200 behaviorally tested animals following completion of testing. The catecholaminergic system in the fronto-cortico-striatal loop is critical for executive functioning and has been shown to underlie some of the behavioral deficits caused by Mn (21–23).

3.31 Mn tends to increase Drd1 and Drd2 expression in PND 24 weanlings, and MCS protects against the Mn effect on Drd2 expression

In PND 24 littermates of behaviorally tested animals, the dopamine 2 receptor gene *Drd2* had a significant Mn x MCS interaction [$F(1, 23.6) = 5.25$, $p = 0.031$], reflecting that *Drd2* expression was higher in Mn animals versus both the controls and the Mn+MCS groups, indicating that MCS normalized the Mn effect to increase *Drd2* expression (Figure 4A; note that a lower ΔCt value equates to higher gene expression). Specifically, contrasts showed that *Drd2* expression in Mn animals trended to be higher than Control animals ($p = 0.065$), and that *Drd2* expression in the Mn group was significantly higher than in the Mn+MCS group ($p = 0.041$) (Figure 4A). For *Drd1*, which encodes for the dopamine 1 receptor, there was a trending main effect of Mn to increase expression [$F(1, 24.7) = 3.18$, $p = 0.087$] in PND 24 weanling prefrontal cortex, but there were no specific contrasts between groups that trended or reached significance (p's ≥ 0.18) (Figure 4A), and there was no main effect

of MCS on expression (p = 0.18). For the tyrosine hydroxylase gene *Th*, there was a trending main effect of MCS to decrease expression [F(1, 24.3) = 3.11, p = 0.090], but specific contrasts showed no significant differences between treatment groups (p's ≥ 0.13) and there was no main effect of Mn on expression (p = 0.20) (Figure 4A). Neither Mn nor MCS altered the expression of the other genes measured (*Dat, Net*, and *a2a*) (p's ≥ 0.23 for the Mn and MCS main effects and their interaction).

3.32 MCS increases Drd1 and Drd2, but not Th, Net, Dat and a2a gene expression, while Mn exposure did not alter expression of any of these genes in PND 200 behaviorally-tested adults.

We assessed whether Mn and/or MCS caused lasting alterations in gene expression levels in PND 200 behaviorally-tested adults for the same catecholaminergic system genes assessed in their PND 24 littermates. For both *Drd1* and *Drd2* there was a significant main effect of MCS to increase expression [F(1, 20.0) = 4.42, p = 0.048, and F(1, 20.0) = 5.37, p = 0.031, respectively], but no main effect of Mn or Mn x MCS interaction for either gene (p's > 0.47; Figure 4B). There also were no specific contrasts between treatment groups that reached significance for either *Drd1* or *Drd2* (p's ≥ 0.09) (Figure 4B).

Due to the main effect of MCS, but not Mn, to alter the expression of *Drd2* and *Drd1* genes, and the fact that prior studies have shown lasting (~PND 100) protein level decreases (D1R) or increases (D2R) in the prefrontal cortex of animals

developmentally exposed to Mn (24–26), immunohistochemistry was conducted to assess levels of these receptor proteins in the prefrontal cortex and dorsal striatum of the PND 200 behaviorally tested animals. These immunohistochemistry findings showed no measurable effects of Mn or MCS on either D1R or D2R protein levels in the prefrontal cortex (p's > 0.23; Figure 5). The lack of a Mn effect is in line with the gene expression results, while the lack of a MCS effect is in contrast with the significant increases in both *Drd1* and *Drd2* gene expression noted above.

The gene and protein expression data presented above addresses the targeted, hypothesis-driven approach to assess Mn and MCS effects on the catecholaminergic system specifically. In summary, in the PND 24 animals, Mn increased the expression of *Drd2*, which was normalized by MCS (Figure 4A). There were also trending effects of Mn to increase *Drd1* expression and for MCS to decrease *Th* expression (Figure 4A). In the PND 200 behaviorally tested animals, MCS increased the gene but not protein expression of both *Drd2* and *Drd1,* but there was no lasting effect of Mn to alter the expression of any catecholaminergic gene or protein assessed (Figure 4B, 5).

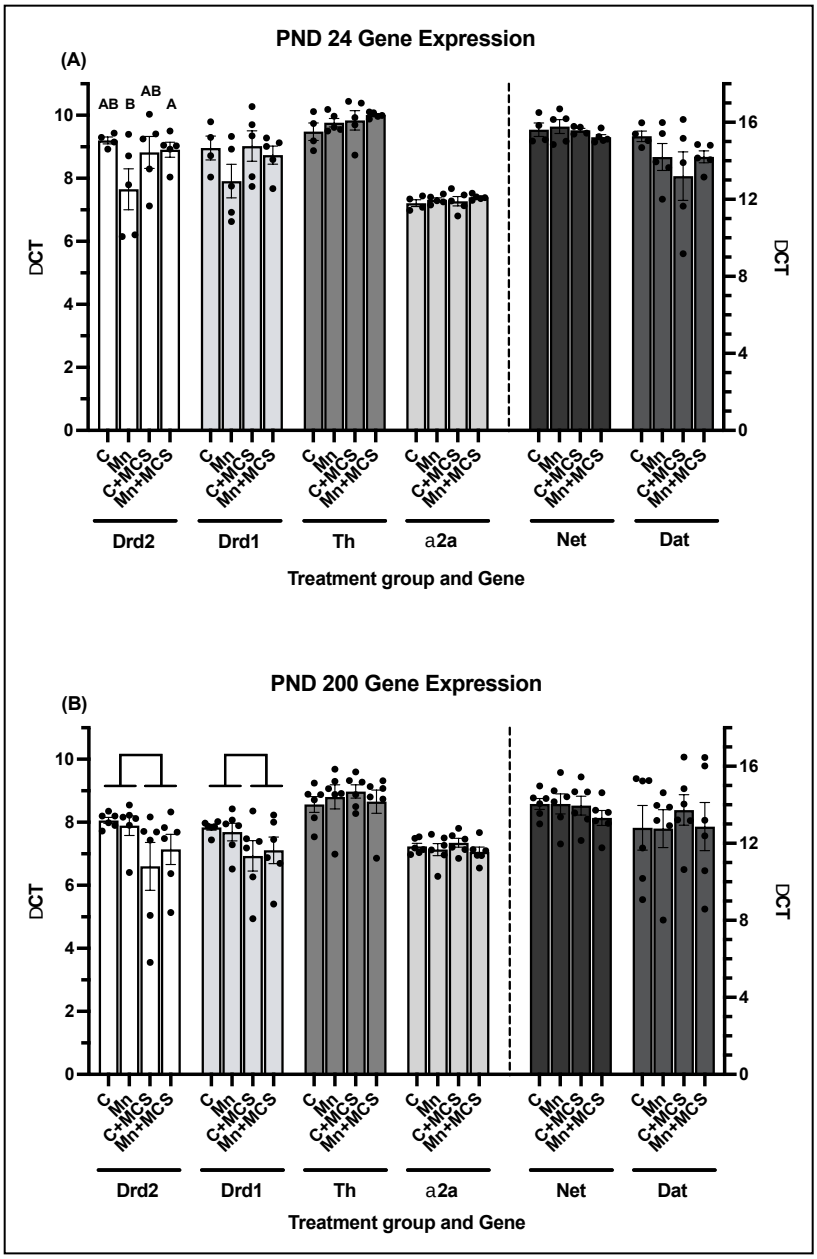

Figure 4. Manganese and MCS alter the expression of catecholaminergic genes in the prefrontal cortex. PND 24 (A) and PND 200 (B) prefrontal cortex RT-qPCR gene expression results; gene expression values are presented as ΔCt values, where a lower value means higher expression. Statistical analyses were performed on ΔCt values. Note that in (A) and (B) *Drd1, Drd2, Th*, and *a2a* expression is reflected on the left Y-

axis, while *Net* and *Dat* expression is shown on the right Y-axis scale. In panel (A), treatment groups with different capital letter superscripts are statistically different from one another ($p < 0.05$), based on Tukey's *post hoc* test. In panel (B), * indicates significant main effect of MCS ($p < 0.05$). Data are arithmetic means ± SEM; n=4-5/trt group for PND 24 (panel (A)), and n=6/trt group for PND 200 (panel (B)).

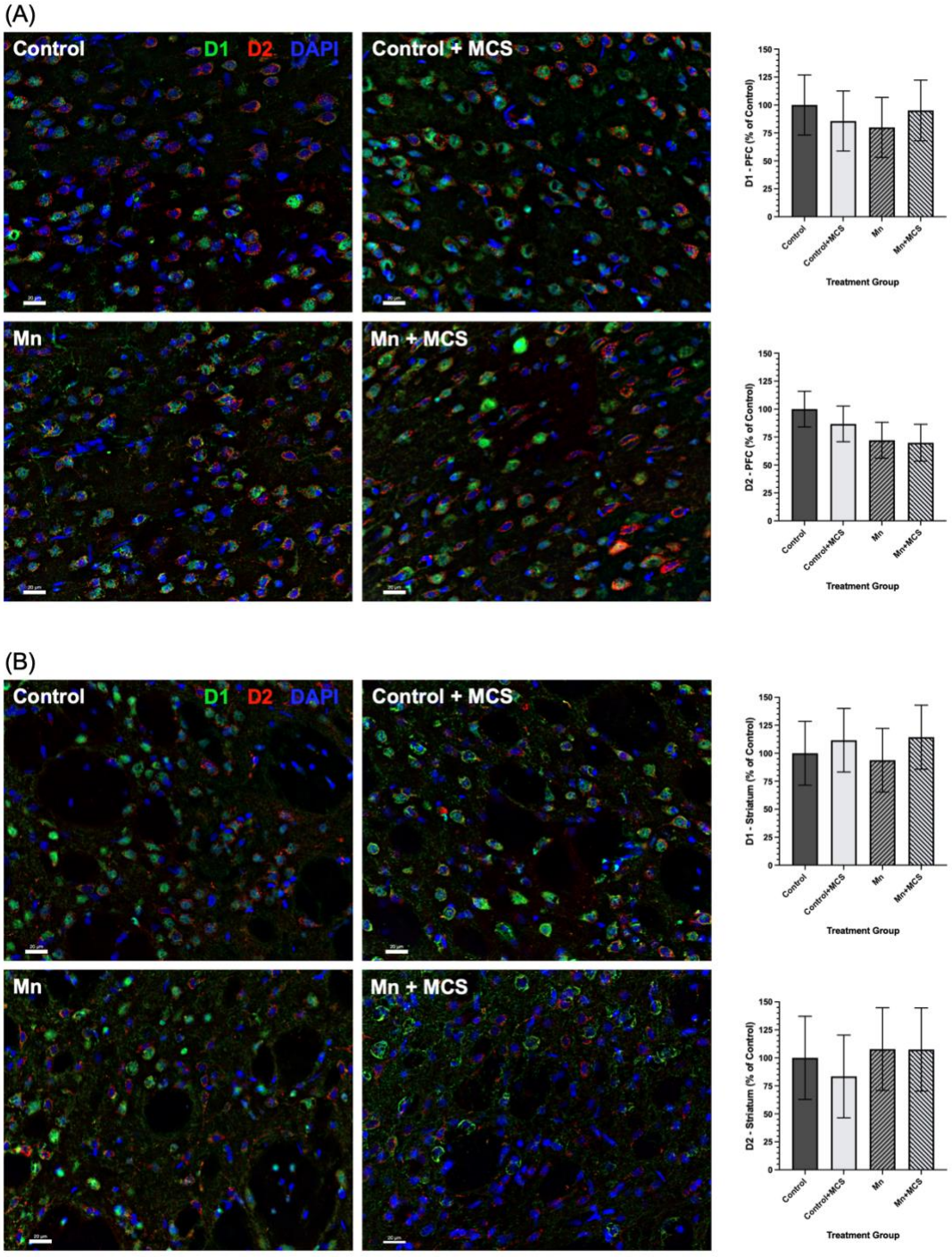

Figure 5. Prefrontal cortex and striatum D1R and D2R protein levels were not altered by early postnatal Mn exposure or MCS. Representative immunohistochemistry

images of (A) mPFC, and (B) striatum brain sections of control, control+MCS, Mn, and Mn+MCS treatment groups. Each image shows the merge of three fluorescence channels; D1R is green, D2R is red, and DAPI is blue. Representative images at 40× magnification (scale bars, 20 μm). Bar charts show quantified fluorescence intensity for D1R and D2R normalized to DAPI cell count for each treatment group. Data are least squares means ± SEM shown as percent of Control group values generated from the statistical mixed model that included all four treatment groups (n = 6 animals/treatment group, and ~6 images/brain region/animal, based on two sections/animal x 2 - 3 images/brain region/section).

3.4 Manganese exposure causes broad lasting changes in gene expression and DNA methylation in the prefrontal cortex

The above analysis was conducted to assess the expression of catecholaminergic genes and proteins, because the catecholaminergic system is critical to executive function and has been shown to be altered by developmental Mn exposure. However, we also assessed the effects of Mn and MCS on the PFC gene transcriptome and methylome more broadly using an unbiased RNA sequencing and reduced representation bisulfite sequencing (RRBS) approach to complement the targeted hypothesis-driven approach described above. This analysis was done on tissue punches collected from the PFC of behaviorally tested animals upon sacrifice at PND 200.

Both Mn and MCS caused broad changes in both gene expression and DNA methylation spanning a wide range of cellular biological functions. Table 1 shows a count of differentially expressed genes in each pairwise treatment group comparison (of 41,882 loci assessed), and shows that each comparison generates hundreds of differentially expressed genes (p-unadj < 0.05; a full list of differentially expressed genes with p-values and fold changes is provided in the Appendix supplemental data document). Table 2 shows a count of differentially methylated regions in each pairwise comparison, and shows that thousands of loci were differentially expressed (p-unadj < 0.01) due to the Mn and MCS treatments.

To visualize the effects of the different treatments on gene expression, volcano plots were generated for each of the pairwise comparisons of interest (Figure 6A). To determine whether the four different treatments (Control, Mn, Control+MCS, Mn+MCS) produced different prefrontal cortex gene expression changes that distinguished the four treatment groups, we performed PCA analyses using normalized gene transcript counts from all genes that were differentially expressed (p-unadjusted < 0.05) in any of the four pairwise treatment group comparisons. The resultant PCA plot (Figure 6B) qualitatively shows that, while there is some overlap between the clusters of animals from each treatment group, there are notable separations between control and Mn animals, and the Mn animals that received choline (Mn+MCS) cluster separately from the Mn animals (see appendix Figure S4

for a PCA plot containing all genes, including those not differentially expressed in any of the group comparison).

Treatment Group Comparison	# Upregulated Genes	# Downregulated Genes	Total DEGs
Control vs Mn	483	469	952
Control vs MCS	430	391	821
Control vs Mn+MCS	504	529	1033
Mn vs Mn+MCS	455	502	957

Table 1. Mn and MCS alter gene expression. Each row represents a different pairwise comparison analyzed with DESeq2, showing the number of differentially expressed (p-unadjusted < 0.05) genes (DEGs), including whether these genes were up or downregulated.

Treatment Group Comparison	**# Hyper-methylated CpG loci**	**# Hypo-methylated CpG loci**	**Total differentially methylated CpG loci** (% of total loci differentially methylated)
Control vs Mn	7,819	8,822	16,641 (0.70%)
Control vs MCS	4,260	14,195	18,455 (0.78%)
Control vs Mn+MCS	7,093	12,747	19,840 (0.84%)
Mn vs Mn+MCS	5,491	10,132	15,623 (0.66%)

Table 2. Mn and MCS alter DNA methylation. Each row represents a different pairwise treatment group comparison analyzed with DMRcate, showing the number of differentially methylated (p-unadjusted < 0.01) CpG loci (of 2,374,207 total loci with read coverage of > 5 in all samples), including whether these regions were differentially hyper or hypomethylated.

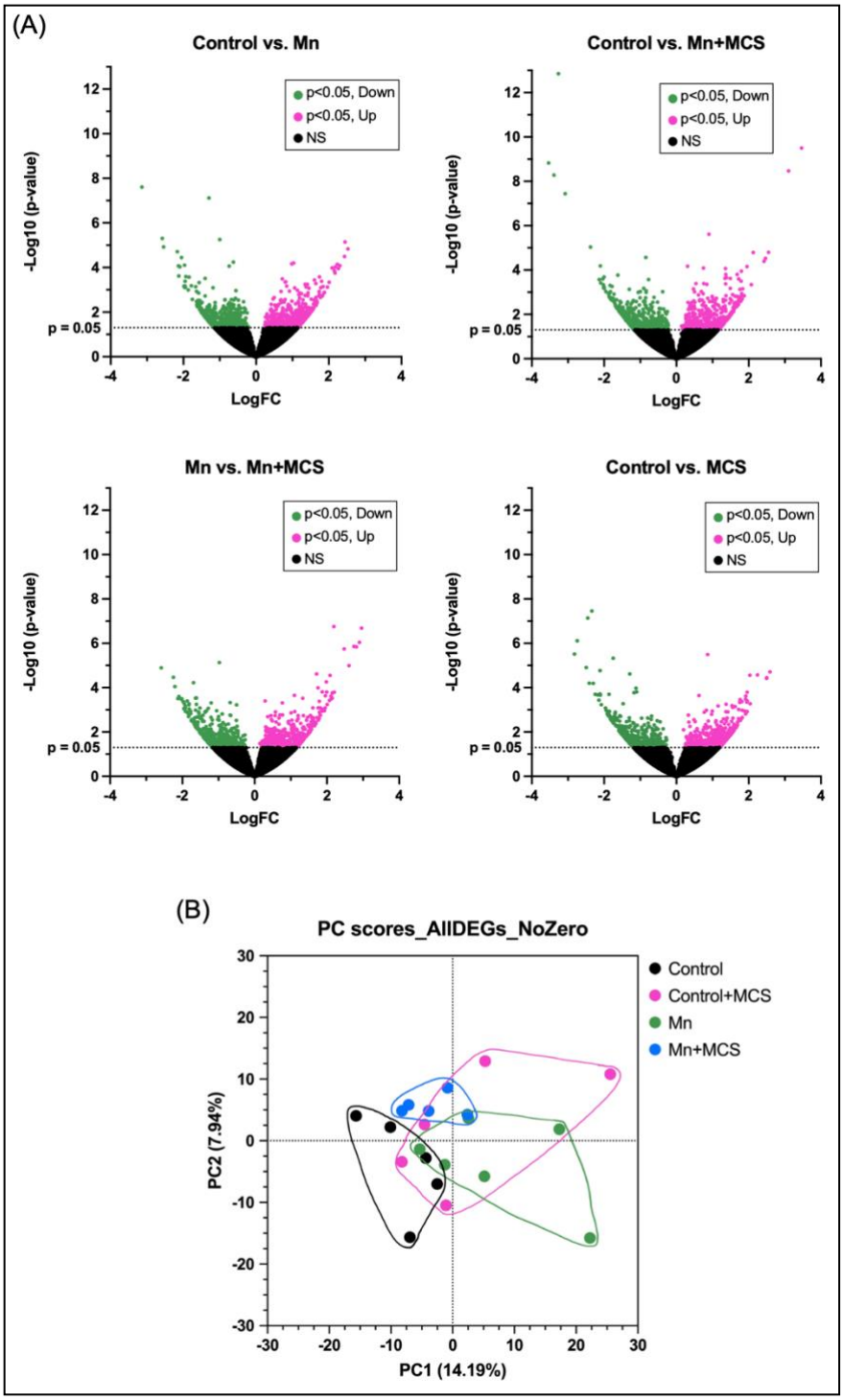

Figure 6. Mn and MCS alter gene expression. (A) Volcano plots show upregulated and downregulated genes based on DESeq2 analysis (each gene is one dot), from

each pairwise comparison. Green dots indicate $p < 0.05$ downregulated, pink dots indicate $p < 0.05$ upregulated, and black dots indicate genes that are not significantly differentially expressed. (B) PCA showing overall gene expression profiles for each rat, calculated using normalized gene counts from all 2823 genes that were differentially expressed (p-unadj < 0.05) in one or more pairwise treatment group comparisons. Lines encircling treatment groups are for qualitative visualization.

3.41 Mn exposure alters the expression of genes in the prefrontal cortex at PND 200, including genes related to neuronal function and inflammation

RNA-seq analysis indicates that compared to control animals, rats that received developmental Mn exposure had lasting changes in the expression of 952 genes (p-unadjusted < 0.05; Table 1 and Figure 6A). Fast Gene Set Enrichment Analysis (FGSEA) was performed to gain additional insight into the function of genes whose expression was altered by Mn exposure. Results identify 154 down- and 346 up-regulated (p-unadjusted < 0.05) Gene Ontology groups (see the Appendix supplemental data document for a list of all GO terms and associated enrichment scores and P-values). These GO term biological process groups were further reduced, filtered, and visualized using REVIGO to generate a tree map (Figure 7). This tree map provides a visual representation of the functions of genes altered by Mn, which are genes involved in a wide variety of functions, including neuronal function and inflammation. Due to the numerous enriched GO groups seen in Figure 7, a list of selected GO terms that are relevant to physiological alterations in the PFC that may

play a role in the lasting behavioral phenotype seen in these animals including Mn-induced dysfunction in attention and behavioral reactivity is shown in Table 3 (Howard et al 2024, see a summary of behavioral findings in Figure 2). This list includes positively enriched GO terms (GO:0032225) *regulation of synaptic transmission, dopaminergic*, (GO:0055093) r*esponse to hyperoxia*, (GO:0032715) *negative regulation of interleukin-6 production*, and (GO:0048663) *neuron fate commitment*, as well as negatively enriched terms (GO:0007269) *neurotransmitter secretion*, and (GO:0023019) *signal transduction involved in regulation of gene expression*.

Detection of tumor cell
Negative regulation of hepatocyte differentiation
Positive regulation of hepatocyte epithelial cell differentiation
Renal sodium ion absorption
Regulation of branching morpho-genesis of a nerve
Regulation of epithelial cell differentiation
Positive regulation of fever generation
Regulation of muscle filament sliding speed
Regulation of T cell differentiation in thymus
Voluntary musculoskeletal movement
Negative regulation of hair follicle development
2
Positive regulation of vaso-constriction
Positive regulation of skeletal muscle fiber development
Regulation of calcium-mediated signaling
Sensory perception of pain
Muscle filament sliding
Intestinal cholesterol absorption
Negative regulation of muscle hyperplasia
Inorganic anion transport
Regulation of embryonic cell shape
Complement activation, alternate pathway
Sulfate transport
Carbo-hydrate transport
Regulation of respiratory gaseous exchange by nervous system processes
1
Neurotransmitter transport
Positive regulation of smoothened signaling pathway
4
Regulation of tumor necrosis factor-mediated signaling pathway
Positive regulation of synaptic vessel exocytosis
Positive regulation of JNK cascade
Positive regulation of transport
Regulation of thyroid stimulating hormone secretion
Positive regulation of corticotropin secretion
Protein folding
Allantoin metabolic process
Chlorophyll biosynthetic process
Gene expression
snRNA pseudouridine synthesis
RNA secondary structure unwinding
Proteolysis
Ubiquitin-dependent protein catabolic process
Response to amphetamine
Response to fructose
3
Response to lithium ion
Cellular response to tumor necrosis factor
Grooming behavior
Behavioral response to pain
DNA duplex unwinding
Septin cytoskeleton organization
Cellular component organization
Actin crosslink formation
Cytoskeletal matrix organization at active zone
Non-motile cilium assembly
Protein hetero-tetramerization
Nucleosome assembly
Spliceosomal complex assembly
Microtubule depolymerization
Female meiosis sister chromatid cohesion
Response to muscle activity
Lipoxygenase pathway
UDP-N-acetylglucosamine biosynthetic process
Succinate metabolic process
Creatine metabolic process
Glucose-1-phosphate metabolic process
Beta-alanine metabolic process
Taurine metabolic process
Uracil metabolic process
dITP catabolic process
Superoxide anion generation
Glycerol-3-phosphate catabolic process
Ether lipid metabolic process
Intracellular potassium ion homeostasis
Intracellular oxygen homeostasis
Retina homeostasis
Neuron cellular homeostasis
Positive regulation of cell projection organization
Positive regulation of dephos-phorylation
Activation of Janus kinase activity
Positive reg. of integrin biosynthetic process
Positive regulation of cell matrix adhesion
Positive regulation of synaptic assembly at neuromus-cular junction
Traversing start control point of mitotic cell cycle
5
Regulation of attachment of spindle microtubules to kinetochore
Positive reg. of mitotic cell cycle spindle assembly checkpoint
Meiotic G2/MI DNA checkpoint signaling
Positive reg. of dopamine metabolic process
6
Positive regulation of synaptic vessel endocytosis
Regulation of NMDA receptor activity
Viral mRNA export from host cell nucleus
Response to hyperoxia
Leukotriene production involved in inflammatory response
Righting reflex
Fructose transmembrane transport
Mitochondrial citrate transmem-brane transport
mRNA export from the nucleus
Apical protein localization
Synaptic vesicle clustering
Establishment of protein localization to chromatin
Polyubiquitinated misfolded protein transport
Somatic diversification of immunoglobulins
Protein import into peroxisome membrane
Receptor localization to synapse
AMPA Glutamate receptor clustering
Eosinophil chemotaxis
Cytoplasmic sequestering of protein
Peroxisome proliferator activated receptor signaling pathway
Synaptic transmission, dopaminergic
Smoothened signaling pathway
7
Epithelial-mesenchymal cell signaling
8
Intestinal epithelial cell differentiation
Placenta development
Determination of left/right symmetry in lateral mesoderm
Heart valve development
Atrial septum secundum morphogenesis
9
Stem cell proliferation
Regulation of hair follicle cell proliferation
Positive regulation of fat cell proliferation
Response to mineralo-corticoid
11
Response to growth factor
Response to leptin
Nerve development
10
Lung lobe formation
Embryonic hemopoiesis
Oocyte development
Branching morphogenesis of a nerve
Bipolar neuron differentiation
Habenula development
Central nervous system vasculogenesis
Tooth mineralization
Embryonic heart tube anterior/posterior pattern specification
Renal inner medulla development
Pancreas morphogenesis
Lobar bronchus development
Epithelial cell fate commitment
Renal outer medulla development
Distal tubule morphogenesis
Inner medullary collecting duct development
Seminiferous tubule development
Thymocyte aggregation
Negative regulation of dendritic cell apoptotic process
12
Regulation of DNA-templated DNA replication initiation
Negative reg. of mitotic recombination
tRNA 3'-end processing
Primary miRNA processing
Negative regulation of cell division
Negative reg. of circadian sleep/wake cycle, REM sleep
Negative regulation of protein deubiquitination
Negative reg. of chemokine (C-X-C motif) ligand 2 production
13
Regulation of interleukin-1 beta production
Negative reg. of type B pancreatic cell apoptotic process
14
Regulation of neuron migration
1. Negative regulation of humoral immune response mediated by circulating immunoglobulin
2. Negative regulation of phosphatidylinositol 3/protein kinase B signal transduction
3. Negative regulation of appetite by leptin-mediated signaling pathway
4. Regulation of synaptic transmission, GABAergic
5. Positive regulation of protein homooligomerization
6. Negative reg. of collateral sprouting of intact axon in response to injury
7. G-protein coupled purinergic nucleotide receptor signaling pathway
8. Cell-cell signaling involved in mammary gland development
9. Myotube differentiation involved in skeletal muscle regeneration
10. Establishment or maintenance of polarity of embryonic epithelium
11. Phospholipase C-activating dopamine receptor signaling pathway
12. Positive regulation of hemoglobin biosynthetic process
13. Negative regulation of apoptotic process involved in metanephric nephron tubule development
14. Regulation of cilium beat frequency involved in ciliary motility

Figure 7. Mn alters the expression of genes involved in a broad array of biological functions, including neuronal function and inflammation. Gene Ontology tree map of enriched GO groups in the Biological Function category, produced by REVIGO. The Tree Map shows a two-level hierarchy of GO terms - representatives from related GO terms are depicted by high-level groups.

Category of function	GO Group	P-value (unadj.)	NES	Leading Edge Genes
Neuronal Function	Neuron Fate Commitment (GO:0048663)	0.011	1.54	OTX2, PAX3, GATA2, SMO, NRG1, SMAD4, NOTCH3, OLIG2, PAX7
	Cerebral Cortex Cell Migration (GO:0021795)	0.0018	1.53	EGFR, PEX13, PSEN1
	Regulation of Synaptic Transmission Dopaminergic (GO:0032225)	0.016	1.32	CHRNA7, CHRNB2
	Positive Regulation of Gamma-Aminobutyric Acid Secretion (GO:0014054)	0.048	1.39	HTR2C, TRH, P2RX7
	Neurotransmitter Secretion (GO:0007269)	0.049	-1.46	AP3M2, LIN7B, AP2B1
Inflammation / Neuronal Function	Arachidonic Acid Metabolic Process (GO:0019369)	0.0018	1.66	ALOX5, MAPK3
Inflammation	Response to Hyperoxia (GO:0055093)	0.0018	1.65	ALOX5, IL18, NOS3, TP53, CDKN1A
	Negative Regulation of Interleukin-6 Production (GO:0032715)	0.0054	1.56	CHRNA7, NCKAP1L
	Defense Response (GO:0006952)	0.014	1.53	TAP1, RELA, NAIP5, TAPBP, CD74
	Leukotriene Biosynthetic Process (GO:0019370)	0.015	1.53	ALOX5, FCER1A
Epigenetics	Histone Demethylation (GO:0016577)	0.026	1.31	KDM6B, KDM4A
Cell Development & Metabolism	Cell Fate Specification (GO:0001708)	0.0053	1.58	OTX2, ITGB1, SMO
	Mitochondrial Respiratory Chain Complex I Assembly (GO:0032981)	0.018	-1.51	NDUFAF5, NDUFAF7
	Signal Transduction Involved in Regulation of Gene Expression (GO:0023019)	0.02	-1.51	GATA4

Table 3. Mn exposure alters the expression of genes with a variety of functions, including GO groups related to neuronal function, inflammation, epigenetics, and cell development/metabolism. Shown here are selected GO groups from the Biological Function category, that align with the hypothesis that gene expression changes may play a role in Mn-induced behavioral alterations. Included is a "category of function" column that indicates which of four functional categories (inflammation, epigenetics, cell development, and neuronal function), the GO group best fits within. NES = normalized enrichment score.

3.42 Mn exposure alters DNA methylation in the prefrontal cortex at PND 200

Reduced representation bisulfite sequencing analysis indicates that compared to control animals, rats that received developmental Mn exposure had lasting changes in the methylation of 16,641 CpG loci (p-unadjusted < 0.01) of 2,374,207 loci assessed, or 0.7%. Of these differentially methylated CpG loci, 7,819 (47%) were hypermethylated and 8,822 (53%) were hypomethylated (Figure 8 and Table 2; a full list of differentially methylated loci with p-values and fold changes is provided in the Appendix supplemental data document). There are 7,983 unique genes with one or more differentially methylated loci within 1000 BP.

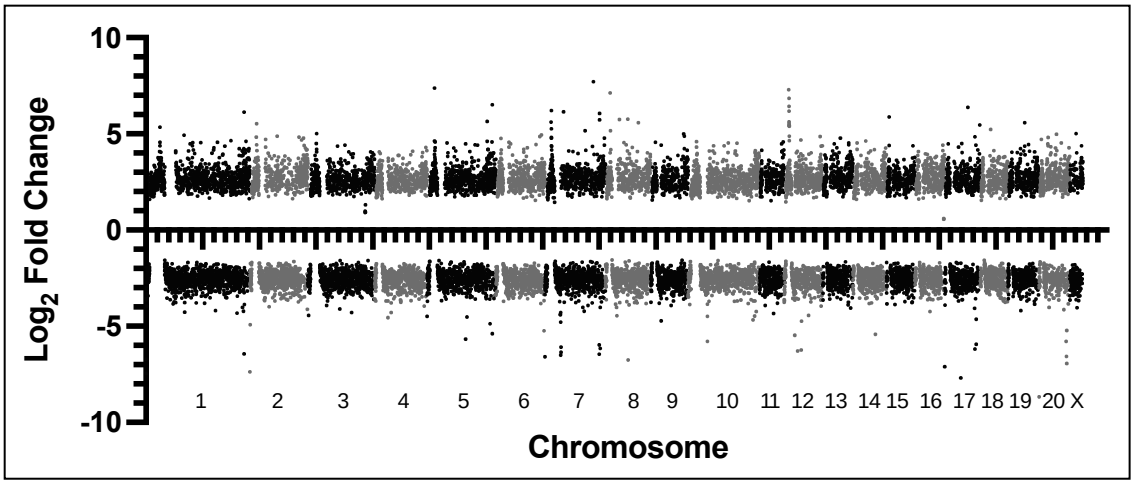

Figure 8. Mn alters DNA methylation. RRBS analysis of PND 200 prefrontal cortex brain tissue; each point is a significantly (p-unadj.<0.01) differentially methylated CpG loci in Mn-exposed animals compared to controls. For visualization, CpG loci from odd-numbered chromosomes are in black; even-numbered chromosomes in grey.

3.5 Maternal choline supplementation protects against some Mn-induced changes in gene expression and DNA methylation

3.51 MCS protects against changes in the expression of a subset of Mn-altered genes, including those related to neuronal function and inflammation

Several lines of evidence were used to determine the effect of MCS to protect against Mn-induced changes in prefrontal cortex gene expression. To determine which Mn-altered genes were protected by MCS, we first assessed which genes were differentially expressed in the Mn animals (i.e., Control vs Mn DEGs) that were no

longer differentially expressed in Mn animals that received MCS (i.e., Control vs Mn+MCS DEGs). Of the 952 genes that were differentially expressed due to developmental Mn exposure, 750 were no longer differentially expressed in the Mn+MCS group (vs Controls), indicating that MCS protected against ~80% of Mn-induced alteration in expression (Figure 9A). Of these 750 genes, 386 were upregulated and 364 were downregulated in the Control vs Mn comparison, and again, no longer differentially expressed in the Control vs Mn+MCS comparison.

In light of the apparent broad benefit of MCS to protect against the majority of differentially expressed genes caused by developmental Mn exposure, we hypothesized that the benefits of MCS to protect against (i.e., normalize) those Mn-caused DEGs would be also evident, but opposite in direction of differential expression, when comparing the Mn versus Mn+MCS treatment groups. In other words, we hypothesized that genes that were differentially expressed by Mn exposure (i.e., Control vs Mn DEGs) and no longer differentially expressed in the Mn animals treated with MCS (i.e., Control vs Mn+MCS DEGs) would now be differentially expressed, but in the opposite direction, in the Mn vs Mn+MCS comparison. The results show that, in fact, of the 750 genes that were differentially expressed due to developmental Mn exposure and that were no longer differentially expressed in the Mn+MCS group (i.e., protected by MCS), 218 (~30%) are also differentially expressed, but in the opposite direction, in the Mn vs Mn+MCS comparison (Figure 9B). This indicates that MCS fully normalized expression of these 218 genes in the

Mn animals. One notable gene whose expression was protected by MCS is *Chrna7*, which encodes for the α7 Nicotinic acetylcholine receptor, which is critical for cognitive function and implicated in ADHD (93–95). Figure 9C shows a heat map of fold change in expression for all the genes differentially expressed in the Control vs Mn comparison (952 total), for each of the pairwise comparisons of interest. Genes that were not differentially expressed (p unadj. < 0.05) in a pairwise comparison are in white. The magnitude of the effect of Mn to alter expression (up or downregulation) is inversely correlated to the effect of MCS to alter expression in the Mn vs Mn+MCS comparison, as depicted in a regression plot in Figure 9D.

For the 193 genes that were not rescued by MCS, showing that the magnitude of change in expression caused by Mn correlates to the magnitude of expression change in the Mn+MCS animals (compared to control; Figure 9E). This indicates that MCS treatment did not alter the Mn effect on these genes. Therefore, follow-up analyses were completed separately on the protected and non-protected genes.

KEGG analysis was conducted on the 218 genes whose Mn-induced alterations in expression were protected by MCS as determined by Figure 9B, to interrogate the biological processes that these genes are involved in (see the Appendix supplemental data document for a list of enriched terms from two KEGG analyses of the 750 protected genes in Figure 9A, and the 193 non-protected genes in Figure 9B). Table 4

shows the GO Biological Process terms that were enriched in this gene set, including those involved in oxidative stress/inflammation, and neuronal function.

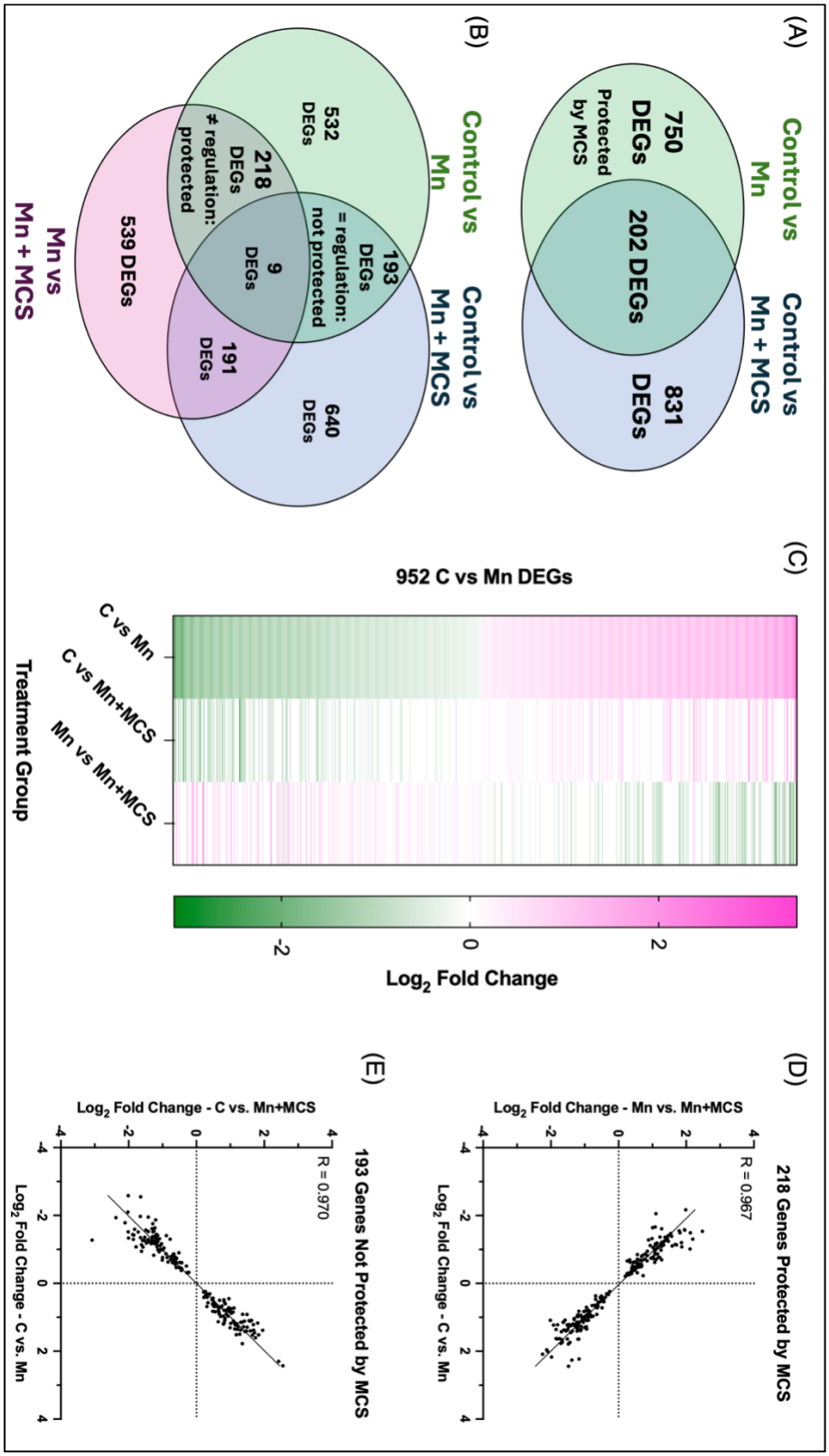
(A)
Control vs Mn
Control vs Mn + MCS
750 DEGs Protected by MCS
202 DEGs
831 DEGs
(B)
Control vs Mn
Control vs Mn + MCS
Mn vs Mn + MCS
532 DEGs
218 DEGs ≠ regulation: protected
193 DEGs = regulation: not protected
9 DEGs
640 DEGs
539 DEGs
191 DEGs
(C)
952 C vs Mn DEGs
C vs Mn
C vs Mn+MCS
Mn vs Mn+MCS
Treatment Group
Log2 Fold Change
-2
0
2
(D)
218 Genes Protected by MCS
Log2 Fold Change - Mn vs. Mn+MCS
Log2 Fold Change - C vs. Mn
R = 0.967
(E)
193 Genes Not Protected by MCS
Log2 Fold Change - C vs. Mn+MCS
Log2 Fold Change - C vs. Mn
R = 0.970

Figure 9. MCS protects against Mn-induced changes in expression of some, but not all, genes. (A) Venn diagram including DEGs (p-unadj < 0.05) from two comparisons; Control vs Mn in green (left side) and Control vs Mn+MCS in blue (right side). (B) Venn diagram including DEGs (p-unadj < 0.05) from three comparisons; Control vs Mn in green (left side) and Control vs Mn+MCS in blue (right side), and Mn vs Mn+MCS in pink (bottom). (C) Heat map includes Log_2 Fold Change for all the 952 differentially expressed genes from the Control vs Mn comparison (p-unadj < 0.05). It also includes Log_2 Fold Change for the other two pairwise comparisons (Control vs Mn+MCS and Mn vs n+MCS), but this value is white for those genes that did not reach significant differential expression in the given pairwise comparison. (D) Regression plot comparing the fold change of the 218 genes that were protected by MCS in two pairwise comparisons: on the x-axis is fold change in the Control vs Mn comparison, and on the y–axis is fold change in the Mn vs Mn+MCS comparison. (E) Regression plot comparing the fold change of the 193 genes that were not protected by MCS in two pairwise comparisons: on the x-axis is fold change in the Control vs Mn comparison, and on the y–axis is fold change in the Control vs Mn+MCS comparison.

Category of function	Term	P-value	Genes
Neuronal Function	Postsynapse Organization (GO:0099173)	0.0024	CHRNA7;ARHGAP39
	Dendritic Spine Organization (GO:0097061)	0.0324	CHRNA7;DIP2A
Inflammation	Positive Regulation Of Natural Killer Cell Mediated Cytotoxicity (GO:0045954)	0.0217	IL21;NECTIN2
	Cellular Response To Interleukin-6 (GO:0071354)	0.0257	ST3GAL6;ST18
	Positive Regulation Of Natural Killer Cell Mediated Immunity (GO:0002717)	0.0279	IL21;NECTIN2
	Regulation Of Natural Killer Cell Mediated Cytotoxicity (GO:0042269)	0.0474	IL21;NECTIN2
Epigenetics	Heterochromatin Formation (GO:0031507)	0.0474	ZNFX1;SMARCA5
Cell Development/ Epigenetics	Regulation Of DNA Replication (GO:0006275)	0.0487	INO80C;SMARCA5;WAPL
Cell Development & Metabolism	Peptidyl-Arginine Modification (GO:0018195)	0.0017	PRMT7;NDUFAF5
	Regulation Of Mesenchymal Stem Cell Differentiation (GO:2000739)	0.0024	LTBP2;SOX5
	Substrate-Dependent Cell Migration (GO:0006929)	0.0085	ITGA2;CD2AP
	Regulation Of Transforming Growth Factor Beta Production (GO:0071634)	0.0128	ITGB6;CD46
	Integrin-Mediated Signaling Pathway (GO:0007229)	0.014	ITGA2;ITGB6;CDH17;DOCK1
	Positive Regulation Of Peptidyl-Lysine Acetylation (GO:2000758)	0.0144	SMARCA5;DIP2A
	Positive Regulation Of Stem Cell Differentiation (GO:2000738)	0.0144	LTBP2;SOX5
	Regulation Of Mitochondrial Membrane Permeability (GO:0046902)	0.0161	MUL1;SPG7
	Regulation Of Tissue Remodeling (GO:0034103)	0.0179	IL21;RUNX1
	Mesoderm Formation (GO:0001707)	0.0197	ITGA2;TBX19
	Regulation Of Organelle Organization (GO:0033043)	0.0239	USP6NL;INO80C;CLIC4;MUL1

	Monoatomic Anion Transport (GO:0006820)	0.0243	CLIC4;PACC1;ANO6
	Glycosaminoglycan Biosynthetic Process (GO:0006024)	0.0254	HS3ST3A1;SLC10A7;ST3GAL6
	Inorganic Anion Transport (GO:0015698)	0.0254	CLIC4;PACC1;ANO6
	Regulation Of Lysosomal Lumen pH (GO:0035751)	0.0301	VPS33A;ATP6V0A1
	Regulation Of Small GTPase Mediated Signal Transduction (GO:0051056)	0.0403	ARHGAP8;FAM13A;ARHGAP39;CD2AP
	Regulation Of Notch Signaling Pathway (GO:0008593)	0.041	TM2D3;CD46;SREBF2
	Intracellular pH Reduction (GO:0051452)	0.0474	ATP6V0A1;RAB7A
	Mitochondrial Membrane Organization (GO:0007006)	0.0474	SAMM50;SPG7

Table 4. KEGG analysis of genes protected by MCS indicates that MCS protects against Mn-induced expression changes in genes involved in oxidative stress/inflammation and neuronal function. KEGG analysis of the 218 genes that were differentially expressed in the Control vs Mn comparison, but were oppositely expressed in the Mn vs Mn+MCS comparison. Shown in the table are the significantly enriched GO Biological Process terms derived from the genes that were protected by MCS. Included is a "category of function" column that indicates which of four functional categories (inflammation, epigenetics, cell development, and neuronal function), the GO group best fits within.

3.52 MCS protects against changes in DNA methylation caused by Mn

To determine the effect of MCS to protect against Mn-induced changes in DNA methylation of specific CpG loci, we used the same logical framework as for the differentially expressed genes (reported above). We first assessed which CpG loci

were differentially methylated in Mn animals (i.e., Control vs Mn differentially methylated loci) that were no longer differentially methylated in the Mn+MCS animals. With this, 16,641 loci were differentially methylated due to developmental Mn exposure, and of these 13,374 were no longer differentially methylated in the Mn+MCS group (vs Controls), indicating that MCS protected against ~80% of Mn-induced alteration in methylation of these loci. (Figure 10A), which is similar to the 79% of Mn-induced differentially expressed genes that are no longer DEGs when supplemented with MCS. Further, of these 13,374 differentially methylated CpG loci, 6,764 (51%) were hypermethylated and 6,610 (49%) were hypomethylated in the Control vs Mn comparison.

We further hypothesized that the benefit of MCS to protect against (i.e., normalize) those Mn-induced alterations in DNA methylation would also be evident, but opposite in direction of differential methylation, when comparing the Mn vs Mn+MCS treatment groups. In other words, CpG loci that were differentially methylated by Mn exposure and no longer differentially methylated in Mn animals treated with MCS would now be differentially methylated, but in the opposite direction, when assessing the Mn vs Mn+MCS comparison. Results show that, of the 13,374 loci that were differentially methylated due to developmental Mn exposure and that were no longer differentially methylated in the Mn+MCS group (i.e., protected by MCS), 2,506 (~19%) are also differentially methylated, but in the opposite direction, in the Mn vs Mn+MCS comparison (Figure 10B), indicating that MCS normalized methylation of

these loci in the Mn animals. This is comparable to the 30% of genes that were altered by Mn and fully protected by MCS.

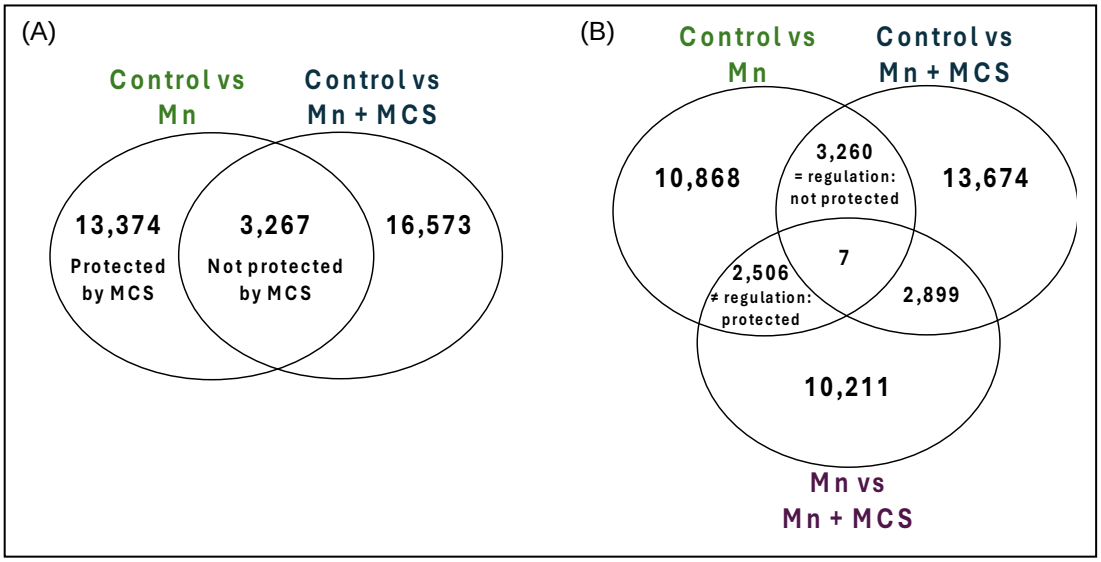

Figure 10. MCS protects against Mn-induced changes in methylation at some, but not all, loci. (A) Venn diagram including differentially methylated loci (p-unadj < 0.01) from two comparisons; Control vs Mn in green (left side) and Control vs Mn+MCS in blue (right side). (B) Venn diagram including differentially methylated loci (p-unadj < 0.01) from three comparisons; Control vs Mn in green (left side) and Control vs Mn+MCS in blue (right side), and Mn vs Mn+MCS in pink (bottom).

3.6 In the absence of Mn exposure, MCS alters DNA methylation and gene expression, including genes related to neuronal function and epigenetics

3.61 MCS alters the expression of genes in the prefrontal cortex at PND 200, including genes related to neuronal function, inflammation, and epigenetics

Animals that received MCS, in the absence of developmental Mn exposure, had lasting changes in the expression of 821 genes (p-unadjusted < 0.05; of 41,882 loci assessed) compared to control animals (See Table 1 and the volcano plot in Figure 6; a full list of differentially expressed genes with p-values and fold changes is provided in the supplemental data document). Fast Gene Set Enrichment Analysis (FGSEA) was performed to gain additional insight into the function of genes whose expression was altered by MCS. Results identify 206 down- and 190 up-regulated (p-unadjusted < 0.05) Gene Ontology groups (see Appendix supplemental data document for a list of all GO terms and associated enrichment scores and p-values). These GO term biological process groups were further reduced, filtered, and visualized using REVIGO to generate a tree map, which provides a visual representation of the functions of genes altered by MCS, and recapitulates the effect of MCS to alter expression of genes involved in neuronal function and inflammation (Figure 11). Of these enriched GO terms, Table 5 contains a list of selected terms that are relevant to physiological alterations in the PFC that may play a role in the lasting cognitive benefit provided by MCS (20). These include positively enriched GO terms (GO:0043966) *Histone H3 acetylation*, (GO:2000463) *positive regulation of excitatory postsynaptic membrane potential*, and (GO:0001963) *synaptic*

transmission, dopaminergic, as well as negatively enriched terms (GO:0006635) *fatty acid beta-oxidation*, and (GO:0046330) *positive regulation of JNK cascade*.

1. Negative regulation of humoral immune response mediated by circulating immunoglobulin
2. Negative regulation of phosphatidylinositol 3/protein kinase B signal transduction
3. Negative regulation of appetite by leptin-mediated signaling pathway
4. Regulation of synaptic transmission, GABAergic
5. Positive regulation of protein homooligomerization
6. Negative reg. of collateral sprouting of intact axon in response to injury
7. G-protein coupled purinergic nucleotide receptor signaling pathway
8. Cell-cell signaling involved in mammary gland development
9. Myotube differentiation involved in skeletal muscle regeneration
10. Establishment or maintenance of polarity of embryonic epithelium
11. Phospholipase C-activating dopamine receptor signaling pathway
12. Positive regulation of hemoglobin biosynthetic process
13. Negative regulation of apoptotic process involved in metanephric nephron tubule development
14. Regulation of cilium beat frequency involved in ciliary motility

Figure 11. MCS alters expression of genes involved in a number of pathways, including neuronal function and epigenetics. Gene Ontology tree map of enriched GO groups in the Biological Function category, produced by REVIGO. The Tree Map shows a two-level hierarchy of GO terms - representatives from related GO terms are depicted by high-level groups.

Category of Function	GO Group	P-value (unadj.)	NES	Leading Edge Genes
Neuronal Function	Positive Regulation of Excitatory Postsynaptic Membrane Potential (GO:2000463)	0.011	1.46	NLGN1,PTEN
	Nerve Development (GO:0021675)	0.0055	1.46	PRX,NTF3
	Neurotransmitter Transport (GO:0006836)	0.046	1.46	SLC6A5,SLC6A20,RIMS2,SV2C,SLC6A19
	Synaptic Transmission Dopaminergic (GO:0001963)	0.033	1.42	DRD2,ADORA2A,RGD1560648,DRD3,SNCA
	Long-Term Synaptic Potentiation (GO:0060291)	0.049	-1.43	MUSK,TNR
	Negative Regulation of Dendritic Cell Apoptotic Process (GO:2000669)	0.0086	-1.35	AXL
Inflammation	Positive Regulation of JNK Cascade (GO:0046330)	0.0414	-1.49	HIPK2,MAP3K11,NOD1,IL1A,MYD88,WNT5A,TLR4
	Lipoxygenase Pathway (GO:0019372)	0.0018	1.37	ALOX5,ALOX5AP
Epigenetics/ Cell Development	mRNA Export From Nucleus (GO:0006406)	0.0043	-1.61	DDX39B,DDX39A
Epigenetics	Nucleosome Assembly (GO:0006334)	0.0018	1.79	ABTB2,KAT6A,SOS2
	Histone H3 Acetylation (GO:0043966)	0.0019	1.67	KAT6A,MBIP,TADA1,BRCA2
	Gene Expression (GO:0010467)	0.039	1.38	FAS,NR4A2,FMN1

Cell Development & Metabolism	Regulation of Mitochondrial Membrane Potential (GO:0051881)	0.0126	1.47	PID1,ADORA2A, BCL2L1
	Fatty Acid Metabolic Process (GO:0006631)	0.037	-1.46	ECI1,ECH1,ACSM 5,PPARA
	Fatty Acid Beta-Oxidation (GO:0006635)	0.0021	-1.72	ECI1

Table 5. MCS alters the expression of genes with a variety of functions, as seen in enriched GO groups. Shown here are selected GO groups from the Biological Function category, that align with the hypothesis that gene expression changes may play a role in MCS-induced behavioral alterations. Included is a "category of function" column that indicates which of four functional categories (inflammation, epigenetics, cell development & metabolism, and neuronal function), the GO group best fits within. NES = normalized enrichment score.

3.62 MCS alters DNA methylation in the prefrontal cortex at PND 200

Reduced representation bisulfite sequencing analysis indicates that compared to control animals, rats that received MCS had lasting changes in the methylation of 18,455 CpG loci (p-unadjusted < 0.01; of 2,374,207 loci assessed). Of these 18,455 differentially methylated loci, 4,260 (23%) were hypermethylated and 14,195 (77%) were hypomethylated (Table 2; a full list of differentially methylated loci with p-values and fold changes is provided in the Appendix supplemental data document).

4.0 Discussion

Studies in children and adolescents have revealed associations between developmental Mn exposure and ADHD symptoms, including inattention, impulsivity/hyperactivity, emotion dysregulation, and sensorimotor dysfunction (4–14). In animal models, Mn exposure has also been shown to cause molecular changes that lead to hypofunctioning of neuronal systems, including the catecholaminergic system in the prefrontal cortex (PFC) (16,24–28). These molecular changes caused by Mn are believed to contribute to, if not largely underlie the lasting functional ADHD-like deficits that are caused by developmental Mn exposure. However, there are currently no recognized interventions, other than exposure prevention, to mitigate the neurotoxic effects of elevated developmental Mn exposure. Maternal choline supplementation has been shown to lessen cognitive dysfunction caused by a variety of perinatal insults and genetic abnormalities in humans and animals (44–58). Importantly, our prior findings from the same behaviorally-tested animals utilized in the present study show developmental Mn exposure causes lasting dysfunction in attention, behavioral reactivity, learning, and sensorimotor function in male rats, and MCS during pregnancy and lactation was effective in protecting against some of these Mn-induced impairments, particularly in attentional function and behavioral reactivity (Chapter 2, (20)).

Here, we took two complementary approaches to gain mechanistic insights into the molecular changes that may underlie the lasting cognitive deficits caused by developmental Mn exposure, namely 1) a targeted approach in which we

hypothesized that specific catecholaminergic system genes and proteins would be dysregulated, and 2) an unbiased approach, in which we broadly evaluated the transcriptome and methylome in the PFC of animals that received MCS and Mn. The present study shows that (1) developmental Mn exposure causes lasting alterations in the expression of numerous genes in the prefrontal cortex, including genes related ot neuronal function and inflammation, as well as alterations in DNA methylation. Importantly, we found that (2) supplementing the maternal diet with additional choline during pregnancy and lactation offers protection against the adverse effects of early postnatal Mn exposure on gene expression and DNA methylation. This MCS protection is seen in a variety of genes, including those involved in neuronal function. One protected gene of interest is *Chrna7*, which encodes for the α7 Nicotinic acetylcholine receptor that is involved in neurodevelopment and cognition, and has been implicated in ADHD (94,96). Collectively, these findings add to our understanding of the numerous lasting molecular effects produced by developmental Mn exposure, and they also demonstrate that MCS exerts lifelong benefits to offspring, including providing some protection against the neurotoxic impacts of elevated Mn exposure. Given evidence that ~ 90% of pregnant women consume less than the recommended Adequate Intake of choline (61,97–99), these data provide additional support for efforts to increase choline intake during pregnancy and lactation, particularly for women at risk of environmental exposure to Mn and other environmental toxicants. These data and interpretations are discussed below.

4.1 Developmental Mn exposure causes lasting alterations in gene expression and DNA methylation

Our findings demonstrate that developmental Mn exposure over PND 1 - 21 causes lasting expression changes in 952 genes (483 upregulated and 469 down-regulated) in the prefrontal cortex of adult PND 200 animals (Table 1). Further, fast Gene Set Enrichment Analysis (FGSEA) indicates that Mn exposure alters the expression of genes involved in a variety of pathways, most notably genes involved in neuronal function, inflammation, epigenetics/regulation of gene expression, and cell development/metabolism (see Figure 7 and Table 3). Examples of positively enriched gene ontology (GO) terms include (GO:0032225) *regulation of synaptic transmission, dopaminergic*, (GO:0048663) *neuron fate commitment*, (GO:0055093) r*esponse to hyperoxia*, and (GO:0032715) *negative regulation of interleukin-6 production*, and negatively enriched GO terms include (GO:0007269) *neurotransmitter secretion*, and (GO:0023019) *signal transduction involved in regulation of gene expression*. It is noteworthy that gene expression changes measured here were seen in the prefrontal cortex of behaviorally tested animals, which exhibited lasting dysfunction in attention, impulse control, sensorimotor function, and behavioral reactivity (summarized in Figure 2) (20).

Additional research has shown that Mn produces lasting hypofunctioning of the catecholaminergic system, including reduced D1R, TH, DAT, NET, and increased D2R protein levels (24–26). However, in the older and behaviorally tested PND 200 adults assessed here, we did not see evidence of catecholaminergic system disruption,

either by targeted RT-qPCR and immunohistochemistry, or unbiased transcriptomics. At PND 24, we do see a trending effect of Mn to increase the expression of *Drd1* and *Drd2* dopamine receptor genes, so this effect may not persist into adulthood (PND 200) in the animals assessed here. However, we did see evidence that Mn altered the inflammatory state of the PFC, given that the expression of a number of inflammation-related genes was altered. This aligns with prior findings demonstrating that Mn causes a lasting proinflammatory environment (26,28), including an increased prevalence of proinflammatory A1 astrocytes (26). Given that the prefrontal cortex/anterior cingulate cortex is well-established as critical to executive functioning, including attention, emotion regulation, error monitoring, and behavioral inhibition (100–106), these findings suggest that at least some of the Mn-induced gene expression changes in the prefrontal cortex may contribute to the ADHD-like behavioral phenotype of the Mn exposed animals.

Mn exposure also causes lasting changes in the methylation of numerous CpG loci, including 7,819 hypermethylated loci and 8,822 hypomethylated loci (see Table 2 and Figure 8). Our group has also demonstrated that developmental Mn exposure causes lasting alterations in DNA methylation in young adult PND 66 rats (28), and other studies have also shown that Mn exposure can lead to altered DNA methylation in human placenta (107), mouse brain (32), and cells (33,108). Specifically, Wang and colleagues demonstrated that providing excess Mn to pregnant/lactating dams via chow from G10 to PND 21 leads to lasting changes in offspring DNA methylation in the hippocampus, including in promoter regions of genes involved in neurogenesis

and neuronal function, at PND 21 and 77 (32). Because DNA methylation is known to impact levels of gene expression (34,35), Mn-induced alterations in DNA methylation may underlie some Mn-induced changes in gene expression.

4.2 MCS offers some protection against the molecular alterations caused by early developmental Mn exposure

MCS protects against Mn-induced alterations in gene expression and DNA methylation, and this protective effect may play a role in the effect of MCS to lessen Mn behavioral deficits in attention and behavioral reactivity, as seen in Howard et al. 2024 ((20), summarized in Figure 2). The PCA plot shown in Figure 5 shows that, when assessing the gene expression profile of each animal based on differentially expressed genes, there is moderate separation between the treatment groups. Notably, the cluster of points (i.e., individual animals) representing the Mn group appears to be the most separated from the cluster representing the control animals, and MCS reduced this separation in the Mn+MCS animals. Compared to control animals, Mn-exposed animals exhibited differential expression of 952 genes. However, in animals that received MCS and Mn, 750 of these genes are no longer differentially expressed (see Figure 9A), including genes involved in neuronal function, inflammation, regulation of gene expression, and mitochondrial-related genes, based on KEGG analyses. In addition, of the 750 genes that were differentially expressed due to developmental Mn exposure and that were no longer differentially expressed in the Mn+MCS group (i.e., protected by MCS), 218 (~30%) are also differentially

expressed, but in the opposite direction, in the Mn vs Mn+MCS comparison (Figure 9B). These genes are considered to be most protected by MCS against Mn-induced alterations in gene expression.

KEGG analysis of the 218 MCS-protected genes indicates that numerous genes are involved in oxidative stress/inflammation and neuronal function (Table 4). These include (GO:0071354) *Cellular Response To Interleukin-6*, (GO:0045954) *Positive Regulation Of Natural Killer Cell Mediated Cytotoxicity*, and (GO:0097061) *Dendritic Spine Organization*. Numerous studies have shown choline supplementation reduces inflammatory markers such as expression of proinflammatory cytokines in animal models (73,75,109–112). Given that Mn produces a pro-inflammatory environment in the PFC (26,28), reducing levels of Mn-induced inflammation may be one mechanism by which MCS exerts protective effects.

Given that alterations in neuronal function in the prefrontal cortex likely contribute to behavioral deficits caused by developmental Mn exposure, as well as the effect of MCS to benefit Mn and control animals, it is unsurprising that we see numerous neuronal genes here that are protected by MCS. One notable gene that is protected by MCS is *Chrna7*, which encodes for the α7 nicotinic acetylcholine receptor. Here, we found that *Chrna7* expression is upregulated in Mn animals, but fully protected by MCS. As mentioned above, this gene is critical to brain development and cognition, and overexpression/triplication of this gene has been linked to ADHD (94,96) and underexpression had been associated with schizophrenia

and autism (94,96,113). Importantly, *Chrna7* has also been implicated in the cognitive benefits of MCS. Several studies have demonstrated that abolished or diminished expression of the α7 nicotinic receptor negated benefits of MCS to improve cognitive function (114,115), and another study found that the cognitive benefit of MCS seen in children was modulated by a *Chrna7* variant (rs3087454) (116). Activation of the α7 nicotinic receptor has also been shown to reduce inflammatory and microglial activity (117), including protecting against the loss of tyrosine hydroxylase in the substantia nigra and suppression of the overactivation of GFAP+ cells and expression of related inflammatory cytokines in a model with 6-hydroxydopamine-induced lesions, which is relevant to the Mn model which has been shown to decrease tyrosine hydroxylase expression and increase GFAP+ astrocytes in the mPFC, striatum, and nucleus accumbens (25,26). The α7 nicotinic acetylcholine receptor has also been shown to be regulated in part by promoter methylation (118), and thus MCS may also modulate expression via an epigenetic mechanism, and can be agonized by choline itself (119) in addition to acetylcholine. These studies provide several lines of evidence indicating that *Chrna7* is in part responsible for the protective benefit of MCS in our model and others.

A prior study from our group showed that the same developmental Mn exposure regimen as that used here similarly produced lasting alterations in gene expression in the prefrontal cortex of younger (PND 66) animals that did not undergo behavioral testing, including genes involved in inflammation, epigenetic regulation, cell development, and neuronal system function, and it identified dysregulation of

mTOR and Wnt signaling pathways as particularly important targets of developmental Mn exposure (28); importantly, these latter signaling pathways are upstream regulators of a number of neurodevelopmental processes (120,121). Interestingly, there were only five genes that were differentially expressed in the same direction (upregulated/downregulated) due to developmental Mn exposure in both the Santiago et al. study and the present study: *EEF1AKMT1, DOK6, CDH13, ISLR2,* and *CALCOCO1*. Of these, *CALCOCO1* is involved in transcriptional activation of target genes in the Wnt/CTNNB1 pathway. Three of these genes, *DOK6, CDH13, and ISLR2,* are involved in neuron growth/differentiation (122–124). *CDH13* is also a risk gene for ADHD and plays a role in learning and memory (123,125,126). With regards to the protection of MCS, of these five genes, *EEF1AKMT1* expression is fully protected by MCS (i.e., this gene is present in the 218 protected genes described in Figure 9B), and *Islr2* and *CALCOCO1* are no longer differentially expressed in the Control vs Mn+MCS comparison (i.e., they are within the group of 750 MCS-protected genes described in Figure 9A, but are not in found in the set of 218 fully protected genes). Finally, *Dok6* and *Cdh13* still differentially expressed in the Control vs Mn+MCS comparison, and so are not protected by MCS. The protection of some, but not all, of these Mn-altered genes aligns with the behavioral phenotype observed in our prior study (20) in which MCS provides partial protection against Mn-induced functional deficits.

Similarly to Mn-induced alterations in gene expression, MCS also protects against changed in DNA methylation caused by Mn exposure. Compared to control

animals, 16,641 loci were differentially methylated due to developmental Mn exposure. Of these loci, 13,374 (~80%) were no longer differentially methylated in the Mn+MCS group (vs Controls) (Figure 10A). In addition, of these 13,374 protected loci, 2,506 (~19%) are also differentially methylated, but in the opposite direction, in the Mn vs Mn+MCS comparison (Figure 10B). These loci are considered to be fully protected by MCS against Mn-induced alterations in methylation. Similar to the protective effect of MCS seen here, studies have also shown that choline supplementation (maternal or direct) can protect against DNA methylation changes caused by fetal alcohol exposure (76,81,127). In one study of MCS in rats, prenatal alcohol exposure suppressed protein and mRNA expression of histone activation marks, but increased the repressive marks, and elevated methylation of the stress regulatory gene *POMC* in adult animals. Supplemented gestational choline normalized the alcohol-altered protein and the mRNA levels of numerous epigenetic genes and proteins, and normalizes the changes in *POMC* gene methylation and gene expression (76). A study in children using postnatal choline supplementation found prenatal alcohol exposure caused increased methylation of *POMC* and *PER2*, but 9 months of postnatal choline supplementation reduced DNA hypermethylation and increased expression of *POMC* and *PER2* in these children (127). Finally, another study of direct choline supplementation in mice found that postnatal alcohol exposure caused altered methylation in the hippocampus and PFC, with an overall phenotype of hypermethylation, which was significantly reduced after choline supplementation (81).

Overall, we have demonstrated that MCS functions to protect against gene expression and DNA methylation changes caused by developmental Mn exposure, and this may underlie the ability of MCS to protect against cognitive deficits caused by Mn exposure (20). These findings recapitulate other studies in which MCS protects against gene expression changes caused by a number of genetic aberrations or insults, such as in a mouse model of Alzheimer's or placental insufficiency (71,74,75,111), and align with numerous studies that have shown MCS protects against cognitive deficits caused by a variety of developmental insults in animal models (46–50,52–58,128) and human infants (44,45,51). However, as mentioned above, a number of differentially expressed genes and differentially methylated loci are not protected by MCS. In the behavioral outcomes measured in these animals (20), MCS protects against Mn-induced deficits in attention and behavioral reactivity, but not learning/impulsivity and sensorimotor function, but more work should be done to investigate whether the differentially expressed genes and methylated loci that were not protected here may be associated with the specific cognitive outcomes that were not protected by MCS.

4.3 MCS in the absence of Mn exposure causes lasting alterations in gene expression and DNA methylation

In the absence of Mn exposure, supplementing the maternal diet with additional choline during pregnancy and lactation causes lasting changes in gene expression and DNA methylation in the prefrontal cortex. MCS increases expression of *Drd1* and *Drd2* (Figure 4B), as well as genes involved in epigenetic function,

neuronal function, and inflammation (see Figure 12 and Table 5). Given that choline is critical to a number of physiological functions, including functioning as a precursor neurotransmitter acetylcholine and membrane phospholipids, and serving as a methyl donor for one-carbon reactions (129), it follows that maternal supplementation of choline may produce changes in a variety of biochemical pathways. However, alterations in expression of catecholaminergic genes and other genes involved in neuronal function may also directly contribute to MCS-induced cognitive benefits in control animals. Several specific genes of interest that are altered by MCS include upregulation of *KAT6A* and downregulation of *KAT8* (both histone acetyltransferases), downregulation of *mTOR* which forms two complexes that are known to regulate neurodevelopmental processes (121), and upregulation of *CEP112*, which helps to regulate GABA receptor surface expression.

MCS also causes lasting changes in the methylation of 18,455 CpG loci (see Table 2). Interestingly, MCS caused an overall phenotype of hypomethylation, where only 4,260 (33%) CpG loci were found to be hypermethylated but 14,195 (77%) were hypomethylated. This is a surprising finding, given that choline is a methyl donor, and a number of studies have shown that MCS does cause global hypermethylation (77–79,81,130). However, hypomethylation is typically correlated with an increase in gene expression (34,35), and thus this may underlie some alterations in gene expression caused by MCS. Further studies are needed to elucidate the reason for MCS-induced hypomethylation, and possibly investigate broader implications of MCS for chromatin remodeling.

Other studies have demonstrated that MCS alters expression of genes with similar functions to those seen here (71,73,75). Alldred et al. found that, in mouse models of Alzheimer's Disease and Down syndrome, MCS produced significant changes in offspring gene expression levels, including genes involved in neurotransmission, the endosomal-lysosomal pathway and autophagy, and transcription factors (71). Another study using the APP/PS1 rodent model of Alzheimer's revealed that MCS significantly changed the hippocampal tissue expression of 27 genes, a number of which were involved in inflammation, histone modifications, and neuronal death regulation functions (111). MCS has also been shown to decrease the expression of pro-inflammatory genes such as TNF-a, IL-1β, and NfκB in mouse placental tissue (73,75). Alterations in neuronal function and inflammation, seen in our studies and others, may underlie some MCS-induced cognitive benefits.

MCS alterations in DNA methylation and expression of genes related to neuronal function, inflammation, epigenetic regulation, and other functions may contribute to the cognitive benefits seen in these same animals in the absence of Mn exposure, including improvements in behavioral reactivity, attention, and learning (20) – benefits of MCS similar to those reported in a number of other humans and animal model studies (59–62,64,67,68,70,131).

4.4 Conclusions

The present study provides new evidence that developmental Mn exposure produces lasting dysregulation of DNA methylation and gene expression in the

prefrontal cortex of male rats, including altering expression of genes involved in neuronal function, inflammation, epigenetics, and cell development/metabolism. These alterations may contribute in part to deficits in behavioral reactivity/emotion regulation, attention, impulse control, and sensorimotor function seen in the same animals, as reported previously (Chapter 2, (20). In addition, the present study demonstrates that supplementing the maternal diet with additional choline over pregnancy and lactation offers some protection to the adverse effects of early developmental Mn exposure. This includes normalization of some, but not all, alterations in DNA methylation and gene expression, including genes related to neuronal function that may play a role in MCS cognitive benefits. This research provides greater insight into the mechanisms by which developmental Mn exposure causes ADHD-like symptoms, and provides additional evidence that MCS is neuroprotective for Mn-exposed offspring. Given these benefits of MCS, and the low choline intake of most pregnant women, these results add to the growing body of evidence supporting efforts to increase choline intake during pregnancy.

Chapter 3 References

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Chapter 4

CONCLUSIONS

Exposure to high levels of environmental manganese (Mn) poses a risk to public health. Studies in children have reported associations between elevated manganese (Mn) exposure and symptoms of inattention, impulsivity/hyperactivity, and psychomotor impairment (1,2,11,3–10). Notably, many of these symptoms of developmental Mn exposure are also hallmarks of attention deficit hyperactivity disorder (ADHD) (12). Rodent model studies have been useful in establishing a causal link between early-life Mn exposure and ADHD-like impairments, recapitulating symptoms seen in Mn-exposed children (13–17), and they have been critical to understanding the underlying neurobiological mechanisms of the Mn-induced deficits. In particular, these animal model studies have demonstrated that elevated Mn exposure causes lasting hypofunctioning of the catecholaminergic system in the prefrontal cortex and striatum (13,14,17–19), which are key brain regions involved in mediating attention, impulse control, emotion regulation, and sensorimotor function (20–22). However, the mechanisms by which Mn causes these lasting alterations, and therapeutic approaches for protecting against these Mn effects, are not well understood.

Therapies such as methylphenidate (Ritalin) have proven to be effective for some children diagnosed with ADHD, but there are no established treatments available for children exposed to high levels of Mn during development. Additionally, primary prevention (i.e., removal of the toxicant from the environment or the body) is

the only established method to prevent neurotoxicity and associated symptoms from occurring (primary prevention of lead exposure and lead neurotoxicity is a prime example (23)). However, this method is not feasible for many individuals and/or populations at risk of elevated Mn exposure because of high cost, not knowing the toxicant is present, ubiquity of toxicants in the environment, etc. Therefore, an overarching goal of my research was to investigate the efficacy of a nutritional intervention to protect against neurobehavioral deficits from occurring upon exposure to elevated Mn.

We hypothesized that supplementing the maternal diet with additional choline during pregnancy and lactation would prove efficacious as an intervention to protect against Mn-induced neurological deficits. Maternal choline supplementation (MCS) has been shown to lessen cognitive and molecular dysfunction caused by various environmental and genetic insults (24,25,34–39,26–33), as well as provide cognitive benefits to typically developing children and animal models (40–48). Given this, the research presented here focuses on determining whether MCS is an effective intervention to protect against Mn-induced deficits, as well as further elucidating the lasting effects of Mn on cognition and neuronal function.

The first chapter of this thesis provides a broad review of the literature related to both Mn and MCS, to provide a framework for understanding the behavioral and neurobiological effects of Mn, and MCS protection reported in chapters 2 and 3. There is a wealth of research that has been done on both Mn and MCS, and this

informed my hypotheses for the effects of Mn and MCS on behavior and underlying neurobiology presented in chapters 2 and 3.

In chapter 2, my first research chapter, I focused on the effects of Mn exposure on behavior, and whether MCS was effective at protecting against Mn-induced deficits. I used a Mn-exposure model and testing paradigm that has been well-validated in our lab, to recapitulate the behavioral phenotype seen in our prior studies (14–16). Consistent with these studies, I demonstrated that early-life Mn exposure produces lasting deficits in attention, learning, and sensorimotor function. In addition, I reported new findings showing that developmental Mn exposure also leads to lasting dysregulation in behavioral reactivity, including decreased sensitivity to errors but a heightened reaction to the omission of an expected reward. Behavioral reactivity in these animals is an essential functional domain comparable to emotion regulation in children (49,50), and similar patterns of emotion regulation dysfunction have been reported in children with ADHD (49–54). Taken together, these findings demonstrate that developmental Mn exposure produces a constellation of deficits consistent with ADHD symptomology, and further validates our rodent model as a model of environmentally-induced ADHD. Importantly, the data presented in chapter 2 also demonstrates that MCS is partly efficacious at protecting against the Mn-induced behavioral deficits. We found that MCS lessened Mn-induced attentional dysfunction and partially normalized behavioral reactivity, but provided no protection against Mn-induced deficits in learning or sensorimotor function. In addition, consistent with literature showing that MCS provides cognitive benefits in typically

developing children and animal models (40–42,44–47,55), we found that in the absence of Mn, MCS was beneficial to control animals in the realms of behavioral reactivity, attention, and learning.

My second research chapter (chapter 3) focused on the molecular underpinnings of this behavioral phenotype. I utilized brain tissues from the behaviorally tested animals reported in chapter 2, to more effectively link neurobiological alterations to cognitive functioning. My main focus was investigating the effects of Mn and MCS on the prefrontal cortex (PFC), because this brain region is critical to executive functioning, including mediating attention, impulse control, emotion regulation, and sensorimotor function (20–22). Mn exposure has been shown to cause molecular changes indicative of a hypofunctioning catecholaminergic system in the PFC and striatum (13,14,17–19,56), so the first section of chapter 3 presents a targeted, hypothesis-driven approach in which I assess levels of catecholaminergic genes and proteins in the PFC. Interestingly, we did not find a lasting effect of Mn exposure on expression of these genes and proteins as seen previously, although this may be due to a number of factors, including using older (PND 200) animals than prior studies (PND 24, 66, or 100 animals), and the extensive behavioral testing these animals had undergone. Therefore, the second section of this chapter focuses on a more exploratory unbiased transcriptomic and methylomic approach, in which I demonstrate that Mn exposure caused changes in expression of a variety of genes, including those related to neuronal function, inflammation, epigenetics/gene expression, and cell development/metabolism. In addition, Mn alters DNA

methylation, which may underlie some of the gene expression changes observed. These Mn-induced changes, including in expression of genes that are critical for neurodevelopment and neuronal functioning, may play a role the ADHD-like behavioral phenotype described in chapter 2. I also demonstrate that MCS is effective in protecting against some, but not all, of these Mn-induced alterations in gene expression and DNA methylation, particularly in genes that mediate neuronal function and inflammation. This normalization of Mn-altered gene expression and DNA methylation may be in part responsible for the effect of MCS to protect against some of the Mn-induced cognitive impairments.

Overall, the findings presented in this dissertation provide further evidence that developmental Mn exposure is a risk factor for ADHD, and add to the wealth of knowledge demonstrating that maternal choline supplementation is neuroprotective for offspring and improves offspring cognitive functioning. They also provide information that may inform future studies that further investigate the neurobiological effects of Mn exposure and the protective effects of MCS or other interventions, as well as the effectiveness of MCS in human populations at risk of Mn exposure. Altogether, these data provide additional support for efforts to increase choline intake during pregnancy and lactation, especially given the evidence that around 90% of pregnant women consume less than the recommended Adequate Intake (42,57–59). For women at risk of environmental exposure to Mn and other environmental toxicants, which disproportionately impact communities of color and low socioeconomic status, providing supplemental choline to pregnant and lactating

mothers is a relatively affordable and tractable intervention that may provide cognitive benefits and lessen the deleterious effects of toxicant exposure in their children.

Chapter 4 References

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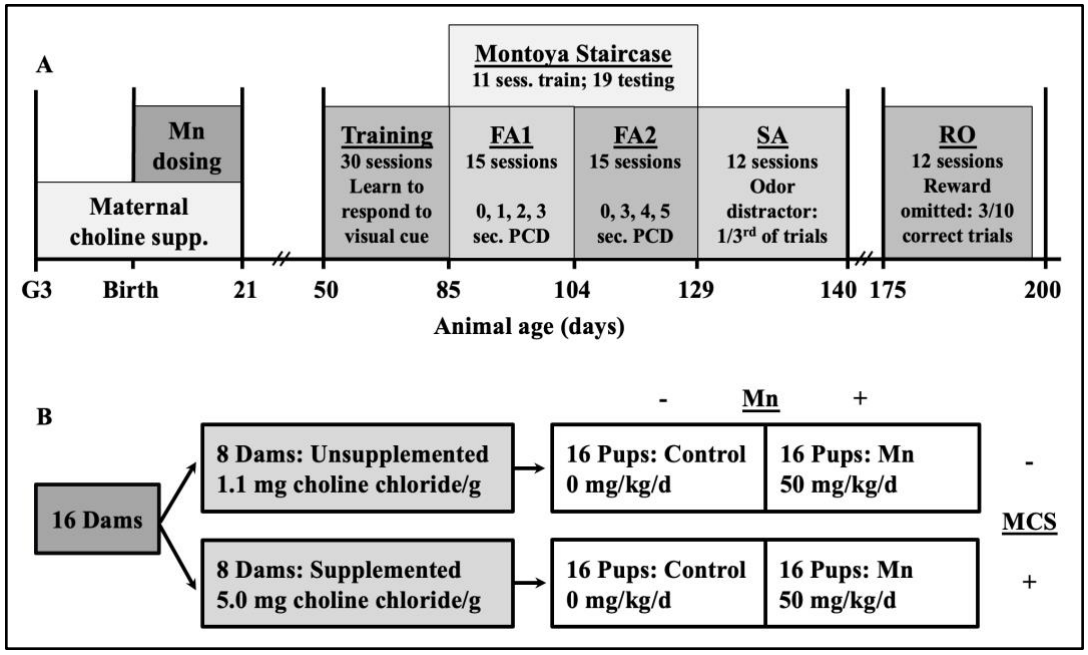
Appendix

Chapter 2 Supplemental Material



Supplementary Figure S1. Study timeline with task parameters, and study design. (A) Study timeline, including MCS and Mn dosing, as well as tasks with parameters. X-axis is days of age (PND). (B) 2x2 study design, ending with 16 pups per treatment group that were used for behavioral testing.

Supplementary section 1. Manganese exposure regimen and rationale.

Neonate rats were orally exposed to 0 or 50 mg Mn/kg/day in a vehicle solution (5% w/v stevia in Milli-Q water) from PND 1–21, as in our prior studies (1–6). This Mn exposure level is not overtly toxic and does not measurably affect neonate health or nutrition, based on neonate milk intake from the lactating dam, growth rate, or blood hematocrit levels at weaning (1,5). Briefly, dosing was delivered once a day directly into the mouth of the pups in a volume of 15-25 µL/dose via a micropipette fitted with a flexible polyethylene gel loading tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received only the deionized water + stevia vehicle.

The environmental relevance of this oral Mn dosing regimen is based on the following: The 50 mg Mn/kg/d exposure level over the pre-weaning period produces relative increases in Mn intake that are ~700-fold over levels consumed from lactation alone, which approximates the relative ~500-fold increase in Mn exposure experienced by infants and young children exposed to Mn-contaminated water or soy-based formulas (or both), compared to Mn ingestion from human breast milk (1,5). For example, human breast milk contains ~6 μg Mn/L, yielding normal infant intake rates of ~0.6 μg Mn/kg/d, based on infant daily milk consumption rates of ~0.8 L/day for an 8 kg 6-9 month old infant. Infants consuming contaminated water (e.g., directly or indirectly to rehydrate powdered formulas) containing 1.5 mg Mn/L, i.e., a level several fold higher than the U.S. EPA health advisory level of 0.3 mg/L and comparable to median well-water levels associated with cognitive deficits and other effects in children, would experience Mn exposure of ~200 μg/kg/d, which is ~300-fold higher than the level of Mn intake from breast milk based on median fluid intake rates of ~1 L/d for infants <1 year of age . Further, infants consuming Mn-contaminated well-water containing 1.5 mg Mn/L mixed with a high Mn soy formula containing up to 1.0 mg Mn/L (total = 2.5 mg Mn/L; see Frisbie et al. 2019 (7)) would ingest ~300 μg Mn/kg/d, which is ~500-times than the Mn intake from breast milk, assuming the above median fluid intake rates for infants <1 year of age. By comparison, rat milk Mn levels are ~200-300 μg Mn/L, and pre-weaning rats consume an average of 260 mL/kg/d over PND 1–21. Thus, pre-weaning control rats consume ~70 μg Mn/kg/d, which is ~100-times higher than normal human infant Mn intake from breast milk. Since normal daily dietary requirements for Mn are not known for either infant humans or rats (8,9), we chose here an exposure regimen that models the relative increase in Mn intake experienced by human infants exposed to contaminated well-water or soy formulas, compared to human breast milk.

Supplementary Table 1: MCS produces measurable changes in choline and some of its metabolites in dam plasma and liver. MCS increases plasma DMG, betaine, and TMAO, and trends toward increasing choline levels, while MCS increases liver choline, betaine, TMAO, LPC, and decreases liver methionine. MCS also trends toward increasing GPC in liver. * indicates $p \leq 0.05$ versus unsupplemented diet; # indicates $p \leq 0.10$ versus unsupplemented diet. Statistical analyses used standard least squares models. Data are mean ± SEM (n=8/group).

Metabolites measured include:

Dam plasma: methionine, choline, betaine, dimethylglycine (DMG), Trimethylamine N-oxide (TMAO). **Dam liver**: methionine, choline, betaine, DMG, TMAO, glycerophosphocholine (GPC), phosphocholine, phosphatidylcholine (PtdCho), sphingomyelin (SM), lysophosphatidylcholine (LPC)

Dam Tissue	Metabolite	Unsupp. diet	MCS diet	MCS effect
Plasma nmol/mL	Dimethylglycine (DMG)	1.57 ± 0.18	3.36 ± 0.40 *	p = 0.0020
	Betaine	78.6 ± 4.6	174 ± 17 *	p = 0.0002
	Trimethylamine N-oxide (TMAO)	1.40 ± 0.18	34.0 ± 11 *	p = 0.0178
	Choline	21.3 ± 4.0	41.4 ± 8.6 #	p = 0.0638
Liver nmol/g	Choline	98.2 ± 22	371 ± 100 *	p = 0.0185
	Methionine	125 ± 7.7	87.6 ± 11 *	p = 0.0145
	Betaine	199 ± 18	662 ± 130 *	p = 0.0034
	TMAO	2.54 ± 0.44	70.0 ± 28 *	p = 0.0322
	Lysophosphatidyl choline (LPC)	445 ± 14	524 ± 13 *	p = 0.0011
	DMG	15.6 ± 1.3	31.9 ± 6.8 *	p = 0.0333
	Glycerophosphoc holine (GPC)	536 ± 46	807 ± 140 #	p = 0.0909

Supplementary Table 2: MCS and Mn produce measurable changes in choline and some of its metabolites in PND 24 pup liver and brain. In offspring, MCS increases GPC in brain, trends toward increasing LPC in brain, increases LPC in liver, and decreases betaine and SM in liver. Oral Mn exposure also alters levels of some metabolites in PND 24 pups, including trending toward decreasing sphingomyelin in brain, and decreasing SM and PtdCho in liver. Data are means ± SE. Statistical analyses used standard least squares models. * indicates a main treatment effect of $p \leq 0.05$; # indicates a main treatment effect of $p \leq 0.10$.

Metabolites measured include:

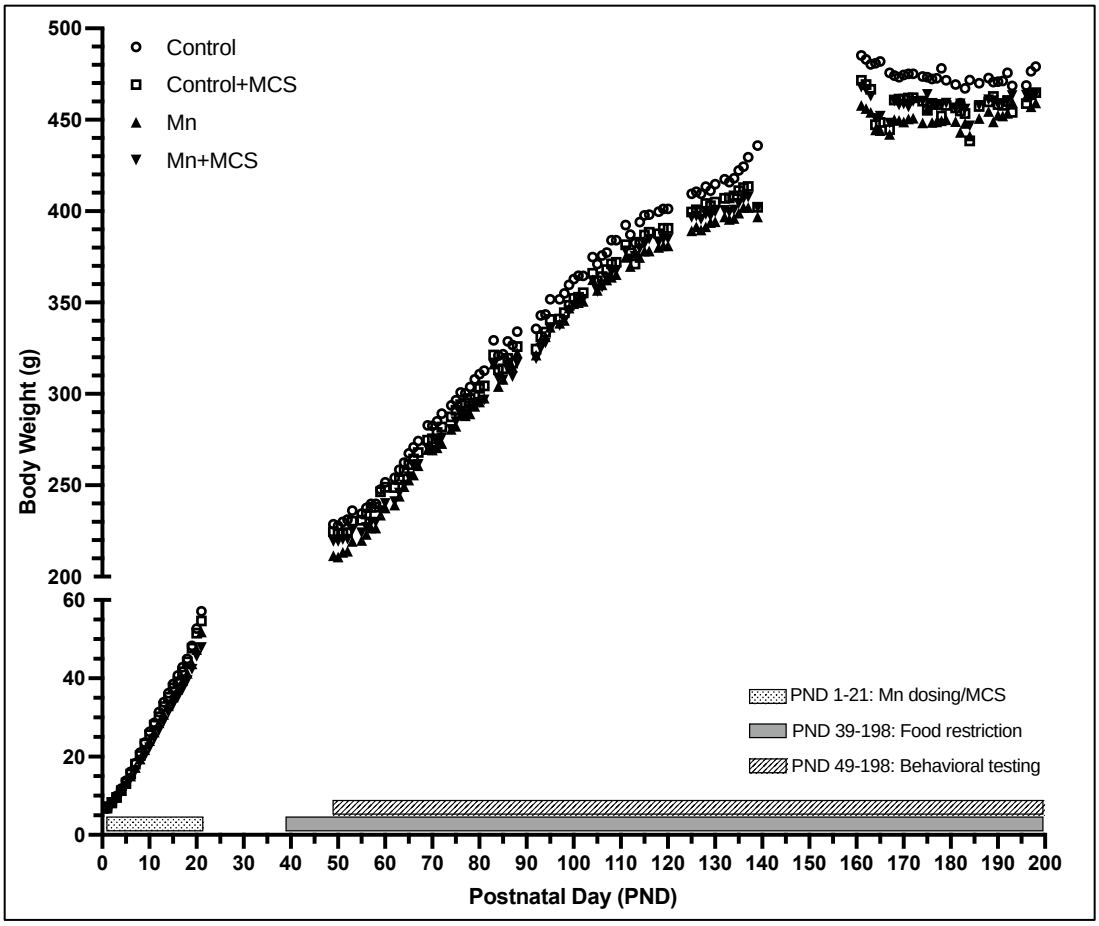
Pup liver: methionine, choline, betaine, GPC, phosphocholine, PtdCho, SM, LPC

Pup brain: methionine, choline, betaine, GPC, phosphocholine, PtdCho, SM, LPC, and acetylcholine

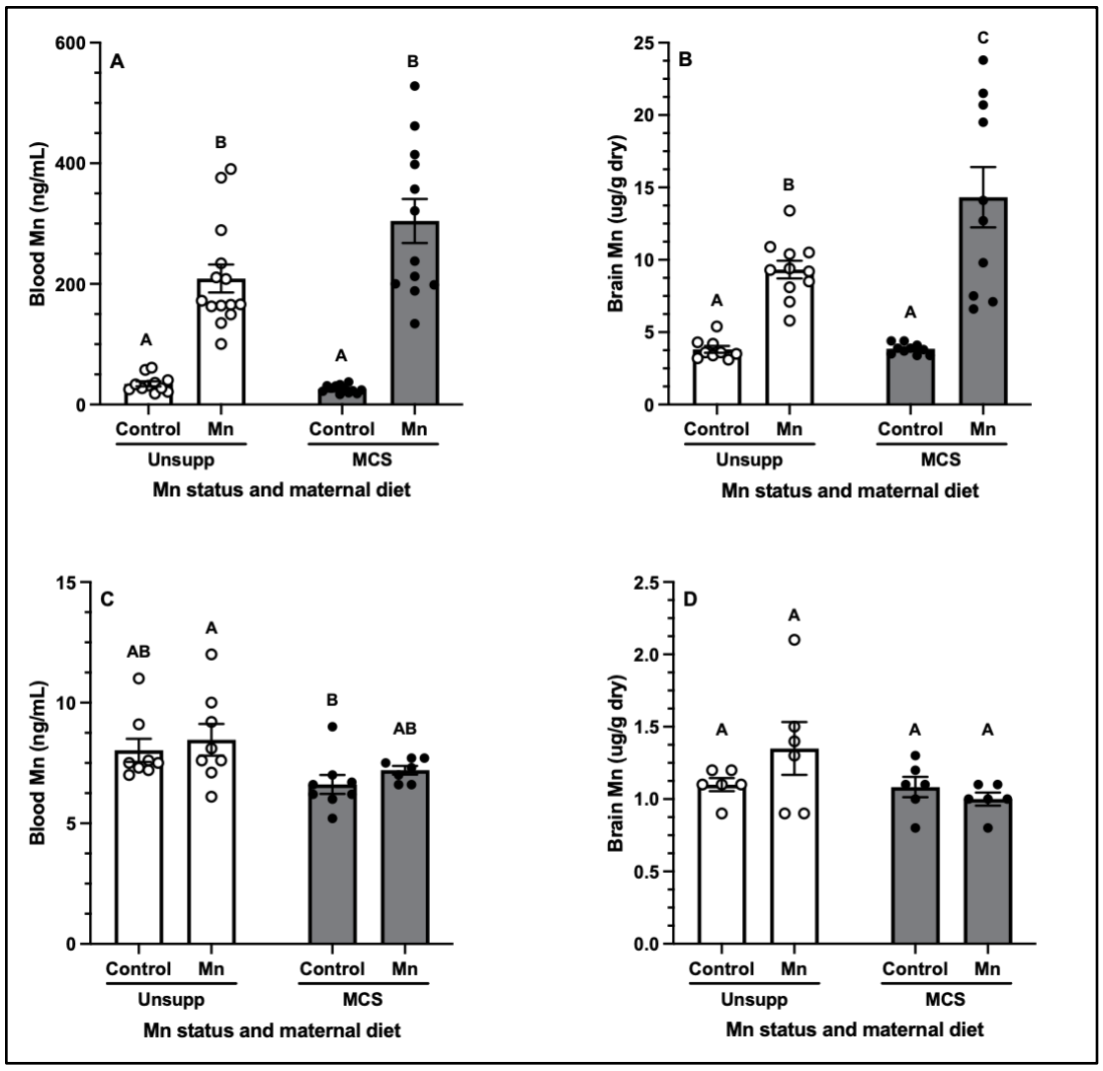
Pup Tissue	Metabolite	Control Unsupp. diet	Control MCS	Mn Unsupp diet	Mn MCS	Mn main effect	MCS main effect	MCS*Mn interaction
Brain nmol/g	GPC	315 ± 15	488 ± 80	349 ± 18	400 ± 36	p = 0.513	***p = 0.0159**	p = 0.158
	LPC	166 ± 7.7	177 ± 7.1	156 ± 11	185 ± 12	p = 0.903	**# p = 0.0570**	p = 0.386
	Sphingomyelin (SM)	5950 ± 290	5330 ± 150	5180 ± 280	4990 ± 280	**# p = 0.0557**	p = 0.147	p = 0.437
Liver nmol/g	Lysophosphatidylcholine	352 ± 13	364 ± 5.1	330 ± 5.9	361 ± 10	p = 0.205	***p = 0.0384**	p = 0.324
	Betaine	3750 ± 170	2610 ± 230	3870 ± 480	3450 ± 330	p = 0.176	***p = 0.0361**	p = 0.307
	SM	1610 ± 29	1450 ± 34	1450 ± 20.5	1190 ± 33	***p < 0.0001**	***p < 0.0001**	p = 0.158
	Phosphatidylcholine	20100 ± 630	21200 ± 490	19400 ± 390	19800 ± 120	***p = 0.0345**	p = 0.130	p = 0.384

Supplementary Table 3: Body weights of animals at the start and end of Mn dosing (PND 1 and 21, respectively) and at the start and end of testing (PND 49 and 198, respectively). Body weight data are in grams (mean ± standard error). A, B, etc. superscripts: within a PND age, treatment groups with different capital letter superscripts are statistically different from one another ($p < 0.05$), based on Tukey's post hoc test. PND 1-21 includes only male animals, n=20-22/trt group. PND 49-128 includes behaviorally tested male animals, n=16/trt group.

Postnatal day (PND)	Control	Control+MCS	Mn	Mn+MCS
1	6.7 ± 0.17^{A}	7.1 ± 0.21^{A}	6.9 ± 0.17^{A}	7.2 ± 0.26^{A}
21	57.1 ± 1.1^{A}	54.7 ± 1.2^{AB}	51.9 ± 1.1^{BC}	47.9 ± 1.3^{C}
49 (Testing start)	229 ± 3.9^{A}	225 ± 6.8^{A}	212 ± 5.4^{A}	219 ± 4.2^{A}
198 (Testing end)	479 ± 15.5^{A}	465 ± 13.0^{A}	459 ± 9.7^{A}	464 ± 10.0^{A}



Supplementary Figure S2: Body weights of animals over the course of the study, annotated with study events (dosing, food restriction, behavioral testing). Data shown are mean body weights; error bars are omitted for clarity. PND 1-21 includes only male animals, n=20-22/trt group. PND 49-128 includes male animals, n=16/trt group.



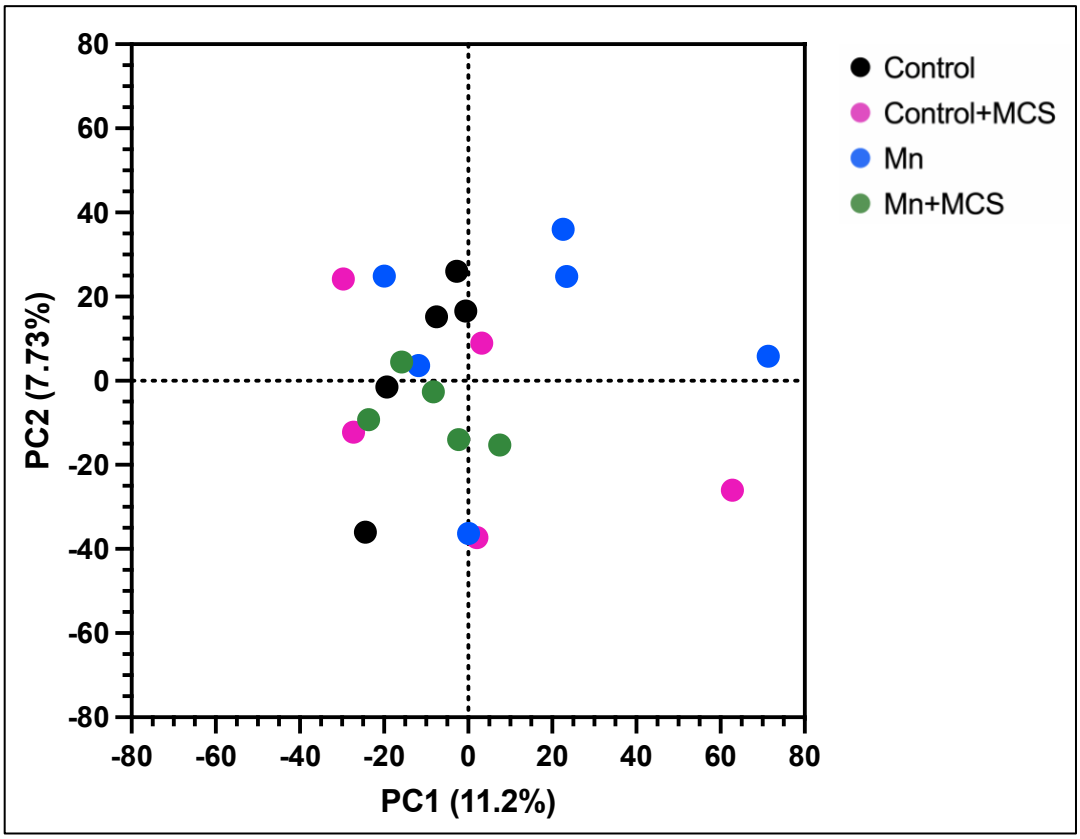
Supplementary Figure S3: Oral Mn dosing increased blood and brain Mn concentrations in weanling littermates of the behaviorally tested animals (PND 24), and returned to baseline in the behaviorally tested animals at sacrifice (PND 200). PND 24 animal blood (n = 11-14) (A) and brain (n = 9-11) (B) measurements show that Mn was increased in Mn-exposed animals. PND 200 animal blood (n = 7-8) (C) and brain (n = 6) (D) measurements show that Mn levels return to baseline. Graphs show arithmetic means with standard error. PND 24 blood and brain statistics were performed on log-transformed data; PND 200 statistics were performed on non-transformed data. Within an age group and tissue, treatment groups with different capital letter superscripts are statistically different from one another ($p < 0.05$), based on Tukey's post hoc test.

Chapter 3 Supplemental Material

Supplemental Data Document

Supplementary Figure S4.

1. A supplemental data document including the raw data from RRBS and RNA-seq analyses, including all differentially expressed genes from each pairwise comparison, FGSEA analysis results including enriched Gene Ontology terms, KEGG analysis results, Venn diagrams and lists of DEGS shared between treatment group comparisons, and differentially methylated loci from each pairwise comparison are available in an excel sheet available upon request.



Supplementary Figure S4: PCA showing overall gene expression profiles for each rat, calculated using normalized gene counts from all genes that contained gene counts for each sample.

Appendix References

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