BEHAVIORAL, NEUROCHEMICAL, AND INFLAMMATORY ALTERATIONS IN MALE AND FEMALE MICE EXPOSED TO MANGANESE VIA THE DRINKING WATER

by

HELAINA DANIELLE LUDWIG

(Under the Direction of Nikolay M. Filipov)

ABSTRACT

Manganese (Mn) is a ubiquitous essential metal; however, overexposures lead to adverse neurological consequences. The majority of Mn rodent exposure studies utilize males, leaving females under-researched. Mn treatment strategies mainly focus on removal from exposure source, symptomatic treatment, and reduction of body Mn load. This dissertation sought to investigate sex differences in behavior, neurochemical and inflammatory alterations induced by subchronic Mn DW exposure within two rodent strains. The rodent strains utilized herein include 1) CX_3CR1^{GFP} with GFP-tagged monocytes/microglia (WT) and 2) miRNA-155 knockout (KO). After 6-weeks of Mn exposure, WT mice showed both motor and mood alterations. At 8-weeks, Mn-exposed WT males, with and without lipopolysaccharide (LPS) challenge, still exhibited decreased fear/anxiety-like behavior. Mn exposure increased inflammatory cytokines in combination with LPS in males, but not in females. These behavioral alterations were diminished in the KO mice with miRNA-155 absent. Since Mn is known to interfere with neurotransmitter homeostasis and sex-specific behavioral and inflammatory changes were observed in WT mice, major neurotransmitters systems, astrocytes, and microglia were evaluated. The striatal levels of GABA and GLU, ventral hippocampal levels of

GLN and GABA, and prefrontal cortex (PFC) levels of 5-HT, NE, 5-HIAA, GLN, and GABA were all impacted differently by Mn and sex. Females had higher numbers of microglia and astrocytes present in multiple hippocampal areas and Mn exposure increased microglia activation while LPS increased astrocyte activation, more in the females. Males, but not females, exposed to Mn showed a decrease in TH+ neurons in the substantia nigra, fewer microglia in the VTA, and increased microglial activation in the ventromedial striatum. These findings suggest that the motor and mood alterations induced by Mn exposure are male-biased, partly dependent on intact miRNA-155 signaling, and associated with region-specific neurochemical and inflammatory changes; female mice are affected by Mn differentially, mostly at the neurochemical level, and more by LPS than the male.

INDEX WORDS: Manganese, LPS, Sex Differences, Behavioral Deficits, Cytokines, Neurotransmitters, Microglia, miRNA155, Inflammation

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DEDICATION

I dedicate this work to my family and friends who have provided me with their unwavering support and have sacrificed greatly for me as I accomplished my goals and dreams that this long journey has taken me.

To my Dad -

Who always encouraged me to stay in school, to keep learning and find something in life that I enjoy doing so that my future career would never seem like a tedious task.

To John –

None of this would have been possible without your love and support. You are my best friend and always will be. Thank you for all your help and for believing in me.

To Claire, Zack, and Rory with love

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

Chapter 4

LIST OF FIGURES

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1 Manganese

Manganese (Mn) is an essential metal that is naturally occurring and found ubiquitously throughout the environment (ATSDR, 2012; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Filipov et al., 2005; Krishna et al., 2014). Mn has a silver/grey color and is usually contained in carbonates, oxides, and silicates (Chen et al., 2018; WHO, 2021). It is a transition metal, able to exist in various oxidative states (+2 to +7); while in living organisms the most common oxidative states are Mn^{2+} and Mn^{3+} (Chen et al., 2018; IOM, 2001). Mn can be released naturally into the air, water, and soil through volcanic eruptions and soil erosion (Chen et al., 2018; WHO, 2021).

As an essential metal, Mn is necessary for the synthesis of amino acids, lipids, and carbohydrates (IOM, 2001). It is also an important cofactor for multiple biological processes including free radical defense, bone formation, energy metabolism, reproductive hormone function, immune system function, blood clotting, nervous system function, digestion, and metabolism (ATSDR, 2012; Chen et al., 2018; Erikson et al., 2007; IOM, 2001; Roth et al., 2012). Even though Mn is a cofactor that is required for several enzymes, in some cases, it can be substituted by copper or magnesium; however, there are enzymes that are solely dependent on Mn. Mammalian enzymes that are Mn-dependent include hydrolases, oxidoreductases, lyases, isomerases, transferases, arginase, glutamine synthetase, manganese superoxide dismutase, and phosphoenolpyruvate decarboxylase (Avila et al., 2023; Roth et al., 2013). Manganese is not only an essential metal for animals, but for plants as well. For example, Mn is required as a catalytic center for light-dependent water oxidation in water-plastoquinone oxidoreductase (Pittman, 2005).

The Food and Nutrition Board of the Institute of Medicine (IOM) recommends a dietary intake for Mn at 2.3 and 1.8 mg/day for men and women, respectively. Many foods naturally contain Mn and are an important source of Mn for the general population (Chen et al., 2018; IOM, 2001; WHO, 2021). Foods that contain the highest levels of Mn include: rice, hazelnuts, pecans, almonds, wheat germ, bran, oats, and legumes. Other common foods that contain Mn are fruits, leafy vegetables, meat, poultry, fish, eggs, sweeteners, tea, chocolate, clams, mussels, and milk. Mn may also be obtained from consuming multivitamins or other daily supplements.

In addition to being an essential metal found in the environment and in various food sources, Mn is used in various industrial, agriculture, and medical settings (ATSDR, 2012; Blanc, 2018). The mineral form of Mn has artisanal uses including in pigments, glazes and for glassmaking (Blanc, 2018). Elemental Mn and various Mn compounds including manganese dioxide (MnO_2), potassium permanganate ($KMnO_4$), and methylcyclopentadienyl manganese tricarbonyl (MMT), have been used to manufacture iron and steel alloys as well as silico-manganese slag, batteries, glass, fireworks, cleaning/bleaching disinfectants, antiknock gasoline additive (MMT), fertilizers, varnish, fungicides (such as Mancozeb and Maneb), ceramics, cosmetics, and leather (ATSDR, 2012; Blanc, 2018; Chen et al., 2018; Cota et al., 2023; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; IOM, 2001; WHO, 2021). In medicine, Mn has been used as a contrast agent in magnetic resonance imaging (Chen et al., 2018; Krishna et al., 2014).

2 Manganese Toxicokinetics

2.1 Transporters

Zinc transporter proteins (ZIP) 8, ZIP14, divalent metal transporter 1 (DMT1), SLC39A8 (solute carrier family 39 member 8), citrate transporter, choline transporter (found on the brain microvascular endothelial cells as well as on the mitochondria membrane), ceruloplasmin and SPCA1 (secretory pathway Ca^{2+}/ Mn^{2+} ATPase isoform 1) are all capable of Mn influx,

SLC30A10 and ferroportin have the ability for both Mn influx and efflux, and sodium-calcium exchanger is a Mn exporter (Figure 1) (Chen et al., 2018; Roth et al., 2013; Roth, 2006). Divalent metal channels that have the capability of passively transporting Mn^{2+} include voltage regulated channels, the ionotropic glutamate receptor Ca^{2+} channels, and the store operated Ca^{2+} channel (Figure 2) (Roth et al., 2013; Roth, 2006). Table 1 lists the organelle/plasma membrane cellular location of receptors, channels, and transporters involved with manganese homeostasis. Mn is able to cross the blood-brain barrier through various mechanisms including transferrin (Tf)-dependent transports, facilitated diffusion, and DMT1 mediated transport (Figure 3) (Hu et al., 2010). Within the central nervous system, the direct Tf-independent pathway utilizing DMT1 is responsible for the transport of Mn^{2+} into cells (Figure 3). The non-Tf-dependent pathway is responsible for Mn entry into neuronal cells via a retrograde transport along axonal projections. When Mn concentrations are elevated, the choroid plexus is a key entry route for Mn into the brain. The major Mn transport mechanism within the choroid plexus and across the blood-brain barrier has been suggested to be a Tf-dependent pathway (Hu et al., 2010; Roth et al., 2013; Roth, 2006). A Tf-Mn^{3+} complex will form and attach to transferrin receptor (TfR) on the cell surface, which in turn will be internalized by the cell within an endosomal vesicle. This vesicle with endure acidification within the endosome by a hydrogen ion ATPase pump, which will cause Mn to be released from the Tf/TfR complex, causing Mn^{3+} to be reduced to Mn^{2+} (Figure 3) (Roth et al., 2013; Roth, 2006). In order to be transported by DMT1, a reduction of Mn must occur, and Mn^{2+} is the only form found in cells. The zinc transporters ZIP8 and ZIP14 play a role in Mn transport, since the interstitial fluid of the brain has a pH that is around 7.3, which for ZIP transporters is close to optimal – compared to the pH 6.0 that is optimal for DMT1 function (Chen et al., 2018; Roth et al., 2013; Roth, 2006). ZIP14 and ZIP8, on the other hand, have a high affinity for binding with Mn and overexpression of ZIP8 has been shown to stimulate intracellular accumulation of Mn and cadmium (Girijashanker et al., 2008; He et al., 2006).

Mutations and/or polymorphisms within the Mn transporters' genes can result in Mn disorders. Deficiency with SLC39A8 (ZIP8) is associated with glycosylation disorders and Mn deficiency (Chen et al., 2018). SLC30A10 (which encodes for a divalent cation transporter on the cell membrane, present in both brain and liver) gene mutations have resulted in Mn metabolism disorders, hypermanganesemia, abnormal iron levels, chronic liver disease, polycythaemia, and increased blood Mn levels (Chen et al., 2018; Tuschl et al., 2016). Homozygous mutations in ZIP14, in the SLC39A14 gene, has been found to lead to excessive accumulation of Mn and rapid progression of parkinsonism-dystonia in patients with the mutation (Tuschl et al., 2016).

2.2 Absorption

Mn that is inhaled can bypass the liver and consequently the first pass metabolism. Inhaled Mn is taken up in the nasal cavity through the presynaptic nerve endings of the axonal projections from the olfactory bulb and trigeminal nerves by the zinc transport proteins ZIP8 and ZIP14, as well as by retrograde transport to the respective neurons in the central nervous system (Chen et al., 2018; Roth et al., 2013; Roth, 2006). ZIP8 and ZIP14 are Mn-bicarbonate symporters and members of the solute carrier-39 family and are expressed on endothelial cells of brain capillaries. By utilizing a HCO_3^- gradient, these symporters can allow for Mn uptake across the plasma membrane (Avila et al., 2023). Alternatively, inhaled Mn can be transported across the pulmonary epithelial lining, then pulmonary macrophages will engulf the inspired Mn, and it will become solubilized within the macrophage. Then, Mn will either cause degeneration of the pulmonary macrophage or it will be expelled back into the pulmonary fluid. After release, the solubilized Mn is transported across the lung's epithelial lining via Tf-dependent or Tf-independent pathway (depending on the valence state of the Mn); Mn^{3+} will bind to Tf and Mn^{2+} will bind to albumin (Roth et al., 2013; Roth, 2006).

Approximately 3-5% of dietary Mn is absorbed through the intestinal epithelium, with the remainder undergoing first-pass metabolism in the liver, where it is taken up by hepatocytes and

then excreted into the feces via the bile (Figure 4) (Avila et al., 2023; Chen et al., 2018; Efsa Panel on Nutrition et al., 2023). Ingested Mn is absorbed in the gastrointestinal tract and enters intestinal cells through both passive diffusion and active transport with DMT-1 via either a Tf independent or Tf-dependent pathway (Figure 4A). DMT-1 is a non-transferrin bound membrane transporter that has non-selective affinity for divalent cations, such as Mn^{2+}, and regulates the influx and transporting of additional metals such as iron, copper, zinc, and calcium (Ca) (Chen et al., 2018; Yokel, 2009). In the intestines, the Tf-independent pathway is used for direct absorption of Mn^{2+} on the enterocyte's apical side (Figure 3). After Mn enters the enterocytes, it is transferred to the basolateral surface, by a process that has yet to be determined (Roth et al., 2013; Roth, 2006). Prior to exiting the cell and entering the blood stream for distribution, some Mn^{2+} is oxidized to its trivalent state, this forms a stable complex with Tf (through the Tf-dependent pathway but does not outcompete Fe^{3+} for binding) and the remaining Mn^{2+} binds with α2-macroglobulin (Figure 4B). DMT1 and ferroportin are used to transport metals, including Mn, across the alveolar epithelial cells that line the pulmonary/blood partition to the lymphatic system or directly into the blood. Manganese from food sources is typically absorbed slower than water-born Mn, because it is bound to various compounds, including proteins and amino acids, which need to be broken down to release manganese for absorption. The ionic form of manganese (Mn^{2+}) is what typically exists in water, and it is readily absorbed into the bloodstream through the intestines.

2.3 Distribution

After Mn is absorbed in the gastrointestinal tract, it will enter the blood stream and be distributed to the different tissues of the body. Erythrocytes are responsible for transporting Mn with the help of transporters including DMT1 and TfR on their cell surface (Aschner et al., 2005; Chen et al., 2018; Roth et al., 2013). The majority of blood Mn is delivered to the soft tissues (~60%) while the liver (~30%) > pancreas (~5%) > bone (0.5%) > kidney (~5%) > brain (0.1%)

are the organs that contain the highest Mn levels after Mn is absorbed in the liver and intestines (Chen et al., 2018).

Within liver cells, Mn influx is regulated by various Mn transporters that are expressed on the liver cell membrane including DMT1, Tf, and ZIP14; Mn efflux is regulated by the Mn exporters SLC30A10, ferroportin, and SPCA1 (Figures 1 and 2) (Chen et al., 2018). As blood circulates through the liver, an adequate amount of Mn for physiological functions will remain in the plasma and typically the excess Mn is conjugated to bile and excreted in the feces (Figure 4) (Chen et al., 2018).

Although the brain has Mn levels that are much lower than those found in the liver, kidney, pancreas, etc. it is the organ that is most susceptible to Mn intoxication. Under typical conditions, brain Mn levels are heterogeneous with the highest concentrations, reported in humans, in the palladium and putamen (Avila et al., 2023). With excess Mn exposure, the basal ganglia have the highest Mn levels (ATSDR, 2012; Avila et al., 2023; Chen et al., 2018; IOM, 2001), but Mn levels increase throughout the brain. Magnetic resonance imaging (MRI) has shown that Mn will accumulate in the globus pallidus, putamen, caudate, midbrain, cerebellum, pituitary gland, olfactory bulb, substantia nigra, hippocampus, pons, striatum, hypothalamus, medulla, and cortex (Avila et al., 2023; Chen et al., 2018; Krishna et al., 2014).

2.4 Excretion

Normal liver function is needed to maintain stable manganese concentrations and is the main route of Mn excretion (J. L. Aschner & M. Aschner, 2005; Avila et al., 2023; Chen et al., 2018; IOM, 2001) and ingested Mn has an average turnover of around ten days. Absorbed manganese is excreted from the blood very rapidly by the liver via conjugating with bile, then entering the intestines. After biliary secretion into the intestines, the greater part of Mn will then be excreted through the feces (>90%), additionally low levels of excretion can occur via urine (<1%), sweat (<1%), and milk (<0.1%), or by being reabsorbed via enterohepatic circulation (<1%) (J. L. Aschner & M. Aschner, 2005; ATSDR, 2012; Chen et al., 2018; IOM, 2001). Health

conditions, such as congenital venous anomaly or cirrhosis of the liver may make those individuals more susceptible to Mn intoxication due to impaired function of the liver to eliminate excess Mn through the bile, which can allow Mn to accumulate in the blood and eventually in the brain (ATSDR, 2012; Avila et al., 2023). Genetic mutations in Mn exporters or transporters such as SLC30A10, SLC39A14, or ZIP8 as well as changes in epigenetic mechanisms that regulate gene expression (i.e., microRNAs) can also lead to Mn not being regulated in the body as it should be (Chen et al., 2014; Tarale et al., 2016; Tuschl et al., 2016; Yu & Zhao, 2023).

3 Adverse Effect of Inadequate Manganese Intake (Manganese Deficiency)

Due to manganese being present in many commonly consumed foods, inadequate dietary intake of manganese is not frequently reported (WHO, 2021). Manganese deficiency has been associated with impaired growth, glucose tolerance, and reproductive function (i.e. reduced fertility and birth defects) as well as alterations in lipid, protein, and carbohydrate metabolism (Avila et al., 2023; IOM, 2001). Inadequate intake of Mn, genetic issues involving eliminating Mn, or diseases (asthma, osteoporosis, and dyslipidemia) are amongst the factors associated with Mn deficiency (Freeland-Graves et al., 2016). In women, decreased Mn plasma concentrations correlated with increased rate of osteoporosis (IOM, 2001). Experiments that maintained animals on manganese-deficient diets have resulted in impaired growth, reproductive deficits, defects in lipid and carbohydrate metabolism, ataxia in newborns, and skeletal abnormalities (WHO, 2021).

4 Adverse Effects of Excess Manganese (Manganese Toxicity)

While manganese is an essential metal and important for many biological processes, excessive exposure to it can result in adverse health outcomes. Excess Mn exposure, in occupational settings, including Mn-mining or Mn-welding, and environmental scenarios, such as Mn-contaminated drinking water, are neurotoxic (Chen et al., 2018; Erikson et al., 2007). Occupational Mn toxicity was first reported in 1837 by John Couper involving bleach factory workers who displayed signs of loss of function in the lower limbs, running forward when

attempting to walk, quiet speaking, and vacant expressions (Blanc, 2018). These employees were grinding black oxide of Mn to be used in the production of bleaching powder and their working conditions led to continuous dermal and respiratory Mn exposure (Blanc, 2018). Inhaling Mn as particulate matter is a great concern due to the metal being readily absorbed by the pulmonary tract (Avila et al., 2023; Elsner & Spangler, 2005; Gonzalez-Cuyar et al., 2014; Laohaudomchok et al., 2011; Utembe et al., 2015). The general public can become exposed to excess Mn via contaminated drinking water. Consumption of Mn-contaminated drinking water is becoming increasingly associated with adverse neurological outcomes (Ljung & Vahter, 2007) and has been correlated with memory deficits and lower cognitive performance in children and with increased infant mortality across multiple studies (Bouchard et al., 2011; Brna et al., 2011; Hafeman et al., 2007; Sahni et al., 2007; Wasserman et al., 2006; Woolf et al., 2002). Notably, Bouchard et al. (2011) reported that children exposed to Mn through DW demonstrated significant intellectual impairment, whereas dietary Mn exposure did not show similar effects.

In the brain, Mn will preferentially target the basal ganglia and cause symptoms similar to Parkinson's disease (Racette et al., 2017) At very high Mn exposures (or chronic lower level exposure), this can lead to a condition known as manganism (Laohaudomchok et al., 2011), characterized by a variety of neurological and motor disturbances that has Parkinson's disease like symptoms (Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Filipov et al., 2005; Grogg et al., 2016; Krishna et al., 2014). In manganism, cock-walk gait is a specific clinical symptom (Chen et al., 2018; Laohaudomchok et al., 2011). Idiopathic Parkinson's disease related symptoms that have been associated with excess Mn exposure include rigidity, emotionless/vacant 'mask-like' facial expression, speech impairment, monotonous tone of voice, postural instability, locomotor deficits, such as reduced response speed, tremors at rest, and gait alterations (Bowler et al., 2006; Bowler et al., 2007; Park et al., 2006; Sahni et al., 2007). Additionally, Mn overexposure causes neuropsychiatric disturbances, e.g. irritability, compulsive behaviors, anxiety and/or depression in humans (Bouchard et al., 2007; Bowler et al., 2003;

Laohaudomchok et al., 2011; Sahni et al., 2007), as well as in laboratory animals (Dodd et al., 2005; Krishna et al., 2014; Liu et al., 2019). Furthermore, other Mn related conditions can be attributed to iron status. Conditions associated with excessive iron accumulation, such as hemochromatosis, can reduce the availability of transferrin binding sites for Mn by saturating transferrin with iron, which would limit the amount of Mn absorption (Roth et al., 2013; Roth et al., 2012; Ye et al., 2017). Conversely, iron deficiency can increase the risk of manganese intoxication due to increased Mn absorption under low Fe conditions (Chen et al., 2018).

5 Manganese Exposure Routes

5.1 Inhalation

Exposure to Mn can occur when air that contains Mn is inhaled and low-level exposures can result in adverse neurological effects. In occupational settings there is a legal limit of 5 mg/m^3 air over an 8-hour work day and Mn air levels can vary depending on the closeness to the source and the amount of clean air circulation (Bowler et al., 2007; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; WHO, 2021). Release of Mn into the air can occur from manmade sources such as power plants, Mn mining operations, welding job sites, automobile exhaust, and alloy production facilities as well as natural sources such as volcanic eruptions. Homeostatic control systems that are in the gastrointestinal tract are lacking in the pulmonary tract, allowing inhaled Mn to be readily absorbed (Roth, 2006) and transported to the brain. Mn absorption via the pulmonary tract can be due to transport into the olfactory nerves that are located in the nasal mucosa, transport across the epithelial lining, and mucociliary involvement in lung clearance (Roth, 2006), bypassing the liver and subsequent first-pass metabolism and excretion (ATSDR, 2012; Bowler et al., 2011; Bowler et al., 2003; Gonzalez-Cuyar et al., 2014; Laohaudomchok et al., 2011; Sen et al., 2011). Factors that can contribute to the amount of inhaled Mn that enters circulation include the site of Mn deposition of the lungs, how efficiently Mn is solubilized by lung macrophages, as well as the size, density, mass, and composition of Mn within the particulate matter (ATSDR, 2012; Roth, 2006).

5.2 Dermal Contact

Mn can enter via cuts in the skin, in small amounts, when in contact with liquids that contain Mn or Mn dust, however, dermal exposure, unless working in a heavily contaminated indoor environment (Blanc, 2018), is not a typical pathway for neurotoxicity of Mn as it does not readily penetrate the skin (ATSDR, 2012).

5.3 Ingestion

The primary source of Mn ingestion is by consuming foods that contain Mn. Mn may also be consumed through drinking water, which may have been contaminated with Mn by natural leaching or though human contamination. The concentration of dietary Mn determines the amount that is absorbed through the gastrointestinal tract along with the amount that is eliminated through the bile (Avila et al., 2023; Chen et al., 2018). Absorption of dietary Mn tends to be lower than Mn absorption from water (ATSDR, 2012) and Bouchard et al. (2011) reported that there was an increase in Mn deposition in children's hair samples that significantly correlated with Mn intake from water, but not from food. Environmental contamination sources of Mn include fertilizers, certain fungicides, dry-cell batteries, methylcyclopentadienyl manganese tricarbonyl (MMT; a fuel additive), mining operations, and manufacturing of alloys (Crittenden & Filipov, 2011; Filipov & Dodd, 2012). Surface and ground water Mn levels in the U.S ranges are, respectively, 400-800 µg/L and 2,900-5,600 µg/L (ATSDR, 2012), with excess levels of Mn DW (≥ 0.2 mg/L) associated with adverse neurological outcomes (ATSDR, 2012). The world health organization has a provisional guideline of 80 µg/L for Mn in drinking water. Mn falls under US EPA's secondary standards, which are non-enforceable, but recommend that Mn US drinking water levels are 0.05 mg/L. In the US, Mn has been detected in 97% of surface water with an average concentration of 16 µg/L and high levels of Mn in drinking water (400-1700 µg/L) have been reported in several countries including India, Myanmar, China, and Bangladesh, as well as in the US (WHO, 2021).

6 Manganese Toxic Mechanisms (Toxicodynamics)

Excess Mn has been previously reported to cause disruptions in energy production and to induce mitochondrial oxidative stress (Fernandes et al., 2023; Gunter et al., 2012; Pajarillo et al., 2021); mitochondria are important organelles in mediating Mn neurotoxicity and Mn preferentially accumulates within them. The mitochondrial fusion-fission is involved in the maintenance of mitochondrial DNA stability, morphology, response to cellular stress and apoptosis (Pajarillo et al., 2021). The mitochondria require Mn as a cofactor for mitochondrial superoxide dismutase 2 (SOD2) (Roth et al., 2013), with Mn^{2+} as the main state in mitochondria and bound to ATP (Tuschl et al., 2013). The mitochondrial membrane expresses the Mn transporters DMT1, TfR, Ca transporter, and citrate transporter. A Ca uniporter imports cytosolic Mn^{2+} into the mitochondrial lumen and excess Mn is exported via Na-independent mechanisms. Mn accumulation within the mitochondria can be due to a slow Mn efflux from the organelle (Gavin et al., 1999). Excess Mn exposure can interfere with oxidative phosphorylation and cause reactive oxygen species production. In the mitochondria and cytoplasm, oxide will be converted to hydrogen peroxide by the Mn and copper/tin superoxide dismutase and further converted to hydroxyl radicals when in the presence of Mn or other metals (Tuschl et al., 2013). Given the high metabolic activity and oxidative environment of the basal ganglia, the accumulation of Mn within this region could exacerbate the detrimental effects of excess Mn by promoting oxidative stress and influencing dopamine.

The basal ganglia are a brain region that has high oxidative activity and the dopaminergic neurons that make up the basal ganglia reside in the substantia nigra pars compacta. Regions that have a high number of oxidative enzymes and high metabolic rate/activity can lead the cells in those zones to generate a large quantity of reactive oxidative species, and the basal ganglia is the region of the brain that Mn preferentially accumulates. In the context of Mn, the high metabolic activity can be utilized to promote Mn oxidation from Mn^{2+}

to Mn^{3+} (Tuschl et al., 2013), which is the Mn species that has a greater prooxidant potential, consequently enhancing the autoxidation of dopamine.

6.1 Neuronal Effects

In astrocytes, excess Mn has resulted in reduced levels of optic atrophy 1 dynamin-like GTPase (OPA1) and mitofusin 2 (MFN2) as well as in increases in dynamin-1-like protein (DRP1) resulting in an imbalance of mitochondrial fusion-fission (Pajarillo et al., 2021). Mitochondrial depolarization, from excess Mn exposure, has resulted in astrocyte dysfunction by increasing DRP1 levels and an Mn-induced decrease in MFN2 levels has been found to increase inflammation in astrocytes and neuronal damage in PC12 cells (Pajarillo et al., 2021). Mitophagy, the natural process of removing damaged mitochondria and its components, may be impaired by excess Mn exposure, this impairment can lead to an accumulation of damaged mitochondria which will produce excess reactive species leading to cellular injury. Mn induced mitophagy dysregulation will also lead to a disruption in mitophagy regulating proteins, also resulting in mitophagy impairment and apoptosis among dopaminergic neurons (Pajarillo et al., 2021). Inducible heme oxygenase is increased in response to oxidative stress and Mn induced hydrogen peroxide release can be decreased by inducible heme oxygenase (Dodd & Filipov, 2011). In addition to causing reactive oxygen species generation, Mn competes with Ca^{2+} binding sites within mitochondria and interfere with Ca^{2+} homeostasis. Disruption of Ca^{2+} homeostasis and oxidative stress will lead to increased permeability of the mitochondrial membrane, leading to increased permeability of the membrane for ions, rapid swelling, and structural changes. This further leads to loss of membrane potential and ATP synthesis, and impaired oxidative phosphorylation thus inducing apoptosis and then neurodegeneration (Tuschl et al., 2013). Nitric oxide (NO) has functions in neurotransmission, vasodilation, and intracellular signaling and is generated by nitric oxide synthases (NOS). Mn can induce cellular injury in the brain by causing nitrosative stress by an overproduction of NO, when NO levels are elevated

this can lead to neurotoxicity by an increase in inducible nitric oxide synthase (iNOS) expression in astrocytes and microglia (Pajarillo et al., 2021).

6.2 Inflammation and Manganese Neurotoxicity

A typical inflammatory response, whether it's instigated in the periphery or in the central nervous system, will result in the production of cytokines including interleukin-1β, tumor necrosis factor-α, and interleukin-6 (IL-1β, TNF-α, and IL-6 respectively). These cytokines, along with various other mediators, act not only locally; they circulate in the blood to communicate with other regions of the body and induce systemic inflammatory response (Ostberg et al., 2000; Perry, 2004). An example of an induced systemic response would be the stimulation of hepatocytes to synthesize acute phase proteins (APP) such as C-reactive protein, haptoglobin, acid glycoprotein, and serum amyloid A. Acute phase proteins consist of both positive and negative APPs. Positive APPs, including C-reactive protein and serum amyloid A, ceruloplasmin, and lipocalin 2 stimulate and contribute to the innate immune system response while negative APPs (i.e. retinol-binding protein, albumin, transthyretin, and transferrin) work to decrease inflammation (Cray et al., 2010; Fernanda et al., 2011; Wait et al., 2005).

The brain requires barriers to protect its environment from drastic changes in the blood. However, the brain still has to be able to respond rapidly to changes that occur in the rest of the body and one method to do this is by utilizing cytokines. Some circulating cytokines can diffuse into the brain (depending on size or location of entry to the brain) (Perry, 2004; Yarlagadda et al., 2009). Others are transported across via a saturable transport mechanism or bind to the blood-brain barrier (Fernanda et al., 2011), where they will interact with perivascular macrophages and adjacent microglia; this would lead to the synthesis of inflammatory mediators and induce intracellular mechanisms that lead to the formation of prostaglandins and nitric oxide that signal to neurons to initiate sickness behaviors including reduced physical activity, fever, and reduced appetite (Fernanda et al., 2011; Konsman et al., 2002; Perry, 2004). In the brain, microglia are the main cell that generate an inflammatory response to external stimuli, such as

Mn and LPS. Filipov et al. (2005) found that when N9 microglia cells were exposed to Mn that proinflammatory cytokine production of IL-6 and TNF-α increased. When the cells were exposed to LPS and to different concentrations of Mn, there was a dose-dependent increase of cytokine production. They ascertained that the involvement of nuclear factor kappa B (NF-κB) activation had an essential role in the rise of cytokine production. In Crittenden and Filipov (2011), they found that Mn caused over-activation of the mitogen-activated protein kinase (MAPK) pathway and an increase in proinflammatory cytokine production. Mn has been found to increase hydrogen peroxide (H_2O_2) release in microglia and potentiate lipopolysaccharide induced cytokines (TNF-α and IL-6) and nitric oxide (NO) (Dodd & Filipov, 2011). Cytokines that are produced by microglia can also activate adjacent cells including astrocytes and neurons, which can lead to an increase of inflammatory signals resulting from Mn-activated microglia (Pajarillo et al., 2021). If microglia are already primed from a previous stimulus, such as exposure to excess Mn, then interaction with cytokines or APPs from the periphery could cause the microglia to respond excessively leading to an overproduction of proinflammatory cytokines potentially damaging nearby neurons (Crittenden & Filipov, 2011; Filipov et al., 2005).

6.2.1 microRNA-155

microRNAs (miRNAs) are short, single-stranded, 18-25 nucleotides in length, non-coding RNAs that can silence or stimulate target gene expression at the post-transcriptional level, by binding to complementary RNA sequences (Gaudet et al., 2018; Grogg et al., 2016; Mashima, 2015; Yin et al., 2017). miRNA-155 (miR-155) has a regulatory signaling role of various cells including macrophages, B cells, T cells and dendritic cells (Mashima, 2015). miR-155 expression, within monocytes and macrophages, is stimulated by poly(I:C) (a toll like receptor [TLR] 3 ligand), LPS (TLR4 ligand), as well as inflammatory cytokines including interferon gamma (IFN- γ) and interferon beta (IFN-β) (Thounaojam et al., 2013). Grogg et al. (2016) reported that miR-155 underwent a decrease in expression that was dose dependent upon insoluble Mn nanoparticle (NP) exposure and that the expression of proinflammatory

cytokines TNF-α and lL-6 increased with Mn exposure. When Grogg et al. (2016) added a miR-155 mimic into the neuronal and microglia co-culture, miR-155 expression was restored and this resulted in a reduction in the secretion of TNF-α and lL-6. In the study by Yin et al. (2017), BV2 microglia cells showed an increase in miR-155 up-regulation after the cells were exposed to lipopolysaccharide (LPS). When the LPS-exposed cells were also treated with a miR-155 inhibitor, cell viability increased, and apoptosis decreased. Additionally, Yin et al. (2017) found that the down-regulation of miR-155 also deactivated the mitogen activated protein kinase (MAPK)/nuclear factor kappa-B (NF-κB) pathway and mammalian target of rapamycin (mTOR) pathway. Given that soluble Mn increases MAPK and NF-κB alone and especially when combined with LPS (Filipov et al., 2005), effects of soluble Mn found in drinking water might be like the effects of LPS or synergistic to it when it comes to miR-155.

miR-155 is a proinflammatory miRNA that has been found to be upregulated in inflammatory and neurological disorders (Gaudet et al., 2018) and that if there is upregulation of miR-155 then there is an increase in proinflammatory cytokine output. When the TLR4 ligand pathway is stimulated, such as with LPS, miR-155 is found to be upregulated the greatest when compared to other miRNAs such as miR-146a (Gaudet et al., 2018). Therefore, it is probable that when microglia are pathologically activated with a bacterial stimulus, such as LPS, as well as exposure to elevated levels of soluble manganese, that miR-155 is upregulated and it is an important regulator for increase in the proinflammatory cytokine output (Zingale et al., 2021).

6.2.2 Effects of Manganese on Microglia

Microglia are the resident macrophage immune cells of the brain and are important for neuronal cell maintenance and innate immunity (Filipov & Dodd, 2012; Thounaojam et al., 2013). Resting microglia have smaller cell body with many elaborated thin processes and with multiple branches (Hristovska & Pascual, 2016). These processes are actively and constantly surveying their microenvironment as well as monitoring the nearby synapses' functional status. Microglia can be typically found in a quiescent and vigilant state, characterized by a CD45

phenotype with lowered expression of antigen presentation molecules (Thounaojam et al., 2013). Extrinsic signaling will activate microglia; after being activated, microglia will become swollen with a larger cell body and shorter thicker processes – a more ameboid shape – and will upregulate major histocompatibility complex class II, as well as increasing the release of inflammatory signals/cytokines into surrounding areas (Fernández-Arjona et al., 2017). Aged microglia and/or microglia that have been chronically stimulated, are in a primed state; they display features that relate to phagosomes, such as increased phagocytic activity, are hypersensitive to their microenvironment, express increased levels of immune receptors, and will respond quicker to immune stimuli (Holtman et al., 2015). As a major source of proinflammatory cytokine (e.g., IL-6 and TNFα) (Crittenden & Filipov, 2008) production within the brain, excessive activation can lead to an overproduction of cytokines, which may induce cytotoxicity of the neighboring neurons when the inflammatory mediator output is sustained for extended periods of time (Crittenden & Filipov, 2011; Dodd & Filipov, 2011; Filipov et al., 2005) by inducing either nitric oxide dependent or independent cytotoxicity to the neurons.

Microglia can remain in an state of activation by overexposure to Mn, which in turn will lead to an overproduction of proinflammatory cytokines as well as other proinflammatory mediators and increased prostaglandin and nitric oxide production (Crittenden & Filipov, 2008; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Filipov et al., 2005; Zhao et al., 2009). When an inflammatory reaction is already present, the addition of inflammagen to the system, such as lipopolysaccharide (LPS), can intensify the neurotoxic effects of Mn (Filipov et al., 2005). When LPS has been administered peripherally, in rodent models, proinflammatory cytokines in both circulation and in the brain were increased (Perry, 2004). Furthermore, LPS causes sickness behaviors characterized with decreased exploratory activity (Krishna et al., 2016). Microglia can also secrete exosomes, as a form of cell-to-cell communication; many of the inflammatory mediators that are produced by Mn-exposed activated microglia, that are toxic to neurons, may be part of the exosome cargo. In addition to inflammatory mediators, exosome cargo can also

include proteins, such as transcription factors, mRNA and microRNA, including miRNA155. As indicated, increased levels of miRNA155 are associated with neuroinflammation and glia activation (Zingale et al., 2021).

7 Effects of Manganese Overexposure on Major Neurotransmitters

Neurotransmitters are chemical signaling molecules that are released by neurons to stimulate other cells as a form of communication. There are many different types of neurotransmitters and each can have different functions within the nervous system. Common monoamine neurotransmitters include dopamine (DA), serotonin (5-HT), and norepinephrine (NE). Monoamine neurotransmitters, including DA, 5-HT, and NE, are neurotransmitters that have one amino group that is connected, via a carbon-carbon chain, to an aromatic ring (Yousuf & Kerr, 2016). They are involved in regulating mood, cognitive function, motivation, movement, emotional response, as well as fight-or-flight response (Bylund, 2016). Classical amino acid neurotransmitters, including gamma-aminobutyric acid (GABA) which is inhibitory and glutamate (GLU) which is excitatory, are neurotransmitters that are derived from amino acids and are involved in various aspects including mood regulation, motor control, cognitive processes, and sensory processing (Yousuf & Kerr, 2016). Proper balance and function of the various neurotransmitters are critical for maintaining neurological health as well as overall brain function. Imbalances or disruptions in the neurotransmitter systems can lead to a range of psychological and/or physiological issues including epilepsy (imbalance between GLU and GABA), Parkinson's disease (decreased dopamine in nigrostriatal and mesolimbic pathways), depression (dysfunction in GABA and GLU systems, and reduced concentrations of 5-HT, NE, and DA), anxiety (decreased GABA and elevated NE) (Mittal et al., 2017; Teleanu et al., 2022).

While Mn is an important cofactor for several biological processes throughout the body, including the brain, neurological damage in conjunction with Mn accumulation has been reported (Balachandran et al., 2020; Ennok et al., 2020; Stepens et al., 2014). Excessive accumulation of manganese in the brain has been associated with abnormalities in the

dopaminergic, serotonergic, GABAergic, and glutamatergic systems, leading to parkinsonism like conditions such as motor disfunction, ataxia, speech difficulties, postural instability, tremors at rest, rigidity, gait abnormalities (Bowler et al., 2007; Lin et al., 2020; Park et al., 2006; Racette et al., 2017; Sahni et al., 2007). Additionally, Mn has been shown to inhibit the synthesis of dihydroxyphenylalanine (DOPA), a precursor to DA, while also reducing 5-HT levels and enhancing 5-HT utilization (Balachandran et al., 2020). Overexposure to Mn impacts both the serotonergic and dopaminergic neurons, particularly in the striatum with changes in these systems being implicated in the developments of mood and motor impairments. Tyrosine hydroxylation, a key step in DA biosynthesis, can be inhibited by excess Mn causing a decrease in DA levels (Lu et al., 2023; Pittman, 2005). There are also direct Mn-DA interactions leading to the oxidation of DA and DOPAC (Sistrunk et al., 2007). Mn exposure has been shown to affect GLU and GABA within the basal ganglia which can disrupt striatal DA metabolism, due to these neurotransmitters being connected to DA regulation (Fitsanakis et al., 2006). Disruptions in the balance of excitatory and inhibitory neurotransmitters in the brain can lead to deviations in motor and emotional behaviors.

GABA, the primary inhibitory neurotransmitter, can be influenced by Mn, though its reported effects on the GABAergic system are equivocal. Studies have reported inconsistent findings, with some indicating that Mn exposure increases GABA release (Dydak et al., 2011), others showing a decrease (Fordahl & Erikson, 2014), and some suggesting release inhibition (Anderson et al., 2008). These differences might be due to variations in experimental conditions, concentration of Mn utilized, duration of exposure, and which brain region that was studied. Glutamate, the most abundant excitatory neurotransmitter in the brain, is highly responsive to changes in energy supply. Mn-induced mitochondrial dysfunction and the resultant generation of reactive oxygen species may directly interfere with glutamate uptake, leading to an accumulation of glutamate in the extracellular space (Gunter et al., 2012; Pajarillo et al., 2021; Soares et al., 2020). Mn exposure has been shown to affect glutamate and GABA within the

basal ganglia which can disrupt striatal DA metabolism, due to these neurotransmitters being connected to DA regulation. Disruptions in the balance of the excitatory and inhibitory neurotransmitters in the brain can lead to deviations in motor and emotional behaviors.

8 Sex-Specific Effects of Manganese Overexposure

From a non-behavioral perspective, manganese has been reported to have sex dependent toxicity (Chi et al., 2017; Dodd et al., 2005; Madison et al., 2011; Riojas-Rodríguez et al., 2010; Zhang et al., 2003). Females tend to have higher blood Mn levels than males, likely due to a higher rate of absorption, and individuals with impaired liver function may accumulate excessive Mn (Chen et al., 2018). Children that were exposed to excess Mn, have been reported to display lower intelligence scores than non-exposed children; girls were more affected than boys (Riojas-Rodríguez et al., 2010). Furthermore, visuospatial learning has been found to have sex-specific vulnerability to neuroactive metals (Rechtman et al., 2020); girls exposed to Mn and copper developed visuospatial learning slower than boys, and this was driven predominantly by exposure to Mn (Rechtman et al., 2020). Another example of a non-behavioral aspect is immune differences between sexes, i.e., cytokine production, which could play a role in the response to Mn and inflammatory stimuli. Among humans, adult females reportedly produce more anti-inflammatory cytokines than males (Barrientos et al., 2019), but also mount a more robust immune/inflammatory response to the same inflammatory stimuli than males (Bouman et al., 2005). Middle-aged female mice challenged with LPS have been reported to exhibit decreased horizontal and vertical locomotor activity (characteristic sickness behavior among rodents) than males, and also had increased levels of peripheral and central cytokines in response to LPS (Dockman et al., 2022). Mn exposure paradigms that have been utilized to investigate behavioral sex differences include injection (Liu et al., 2019), oral gavage (Moreno et al., 2009), or subcutaneous osmotic pump (Sepúlveda et al., 2012). Behavioral effects associated with Mn exposure that were reported by these studies ranged from no sex differences (Liu et al., 2019; Sepúlveda et al., 2012) to male-specific hyperactivity (Moreno et

al., 2009). Non-neurotoxicity focused laboratory studies have reported that Mn exposure through drinking has led to sex specific gut microbiome alterations (Chi et al., 2017), as well as decreased fertility in males, but not in females. However there was a significant increase in ovarian and uterine weights (Elbetieha et al., 2001). Neurologically, Mn drinking water exposure has led to greater changes in striatal neuromorphology in females compared to males (Madison et al., 2011), i.e. morphology changes in females included shorter total dendritic length, decreased total spine number, decreased number of nodes, and increased number of dendritic spines.

9 Treatment for Manganese Intoxication

The current treatment strategy for Mn toxicity is a combination of removing the individual from the source of exposure, symptomatic treatment, chelation therapy to reduce the body's Mn load, along with iron supplementation to reduce the amount of Mn binding to the proteins/transporters that interact with both Mn and iron (Hu et al., 2010; Tuschl et al., 2013). The use of ethylenediaminetetraacetic acid (EDTA) for Mn chelation therapy has resulted in decreased blood Mn levels along with increased Mn urine levels, though neurologic symptoms may fail to improve due to EDTA's poor penetration of the blood-brain barrier and possible irreversible Mn-induced neuronal damage (Hu et al., 2010). When chelation therapy is combined with levodopa there is an initial improvement of extrapyramidal symptoms, however this can be short term with response to treatment reducing after two to three years. Consequently, chelation therapy with EDTA is not likely to succeed in patients with chronic toxicity/advanced manganism in completely reversing the neurologic effects. On the other hand, chelation with para-aminosalicylic acid (PAS) and its metabolites concentrates Mn in the choroid plexus and cerebral spinal fluid from where it is eventually eliminated (Evans, 2022), making it a candidate for chronic toxicity/advanced manganism treatment. PAS also has the ability to chelate both Mn^{2+} and Mn^{3+} and the salicylic acid component has anti-inflammatory properties (Evans, 2022). Sodium para-aminosalicylic acid (PAS-Na) has also been shown to reduce Mn-

induced neuroinflammation in the thalamus of rats (Deng et al., 2023), which was done by inhibiting the expression of proinflammatory cytokines.

10 Direction of Dissertation

The behavioral effects of subchronic low levels of Mn when consumed through drinking water have mainly been studied in males, with limited female data. Especially in conjunction with an inflammagen, which has been shown to potentiate Mn-induced proinflammatory cytokine production. Chapter two focuses on evaluating behavioral (locomotor and mood) alterations of male and female mice that were exposed to Mn in DW with the addition of a lipopolysaccharide (LPS) challenge towards the end of an exposure. The aim was to determine if the Mn effects were sex-specific and to assess if sex plays a role in the inflammatory response.

Mn-induced neurobehavioral impairments have been linked with alterations in neurotransmitter systems (Fordahl & Erikson, 2014; Gwiazda et al., 2002; Krishna et al., 2014; Lin et al., 2020; Tamar et al., 2021). However, there have been different reports regarding which systems were affected, and the majority of studies utilized males, leaving females understudied in this area as well. Chapter three concentrates on assessing Mn-induced changes in both monoamine and amino acid neurotransmitters in males and females exposed to Mn via the drinking water. Additionally, the effects of drinking Mn water when interfaced with an LPS challenge, on astrocyte and microglia activation, will be investigated in the context of sex.

miR-155 is known to regulate proinflammatory cytokine production and secretion in response to inflammatory stimuli, which includes excess Mn. If miR-155 is missing then this regulatory pathway is disrupted, with the potential to alter how immune cells, such as microglia, respond to the inflammatory challenge. Chapter four begins the investigation of how the absence of miR-155 affects male and female mouse behaviors when exposed to subchronic low levels of Mn via drinking water with the addition of an LPS challenge. Additional context on how the absence of miR-155 affects behavior can help to provide insights into behavioral dysfunction that is associated with Mn toxicity and potentially help identifying therapeutic targets to mitigate

the neurotoxic effects of this metal. It is important to determine if same/similar Mn-induced behavioral and inflammatory alterations are present when miR-155 is absent or if the effects are more/less severe compared to when it is present in both males and females. Altogether, these research chapters will increase our understanding of how sex influences the behavioral and inflammatory responses after Mn exposure. By comparing the impacts on both males and females, this work aims to provide a more comprehensive understanding of the neurobehavioral and immunological consequences of subchronic Mn drinking water exposure, as well as the role of miR-155 in modulating these effects.

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Figures and Tables

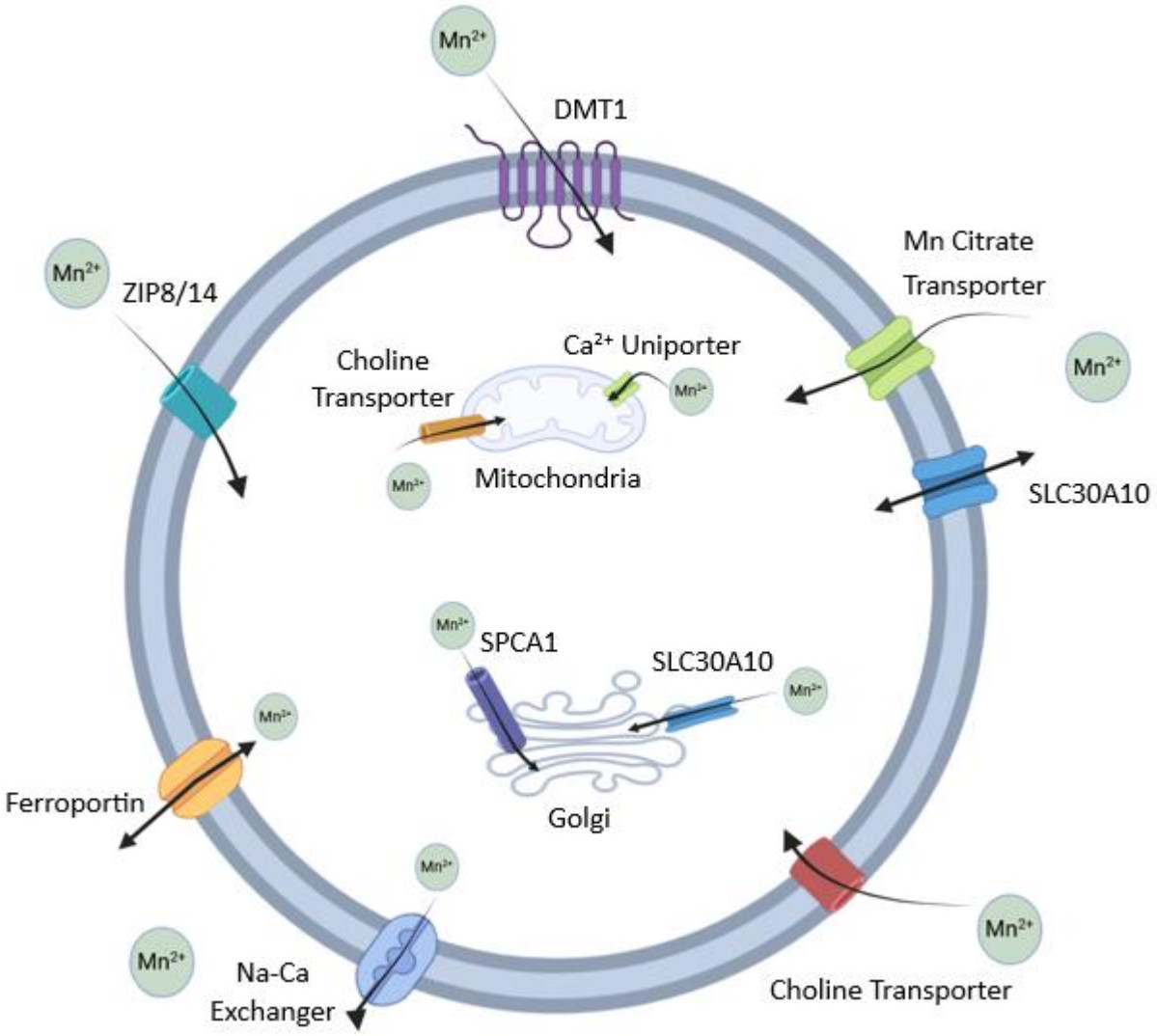

Figure 1.1. Receptors, Channels, and Transporters involved in Mn homeostasis. ZIP8, ZIP14, DMT1, Mn citrate transporter, Ca^{2+} uniporter, SLC30A10, Choline transporter, ferroportin facilitate Mn influx, whereas SLC30A10, Na-Ca exchanger, and ferroportin facilitate Mn efflux (Chen et al., 2018; Harischandra et al., 2019; Roth et al., 2013; Roth, 2006). DMT1: divalent metal transporter 1, ZIP: Zinc transporter protein, Na-Ca exchanger: sodium calcium exchanger, SLC30A10: solute carrier family 30 member 10, SPCA1: secretory pathway Ca^{2+}/ Mn^{2+} ATPase isoform 1. Image created in BioRender.

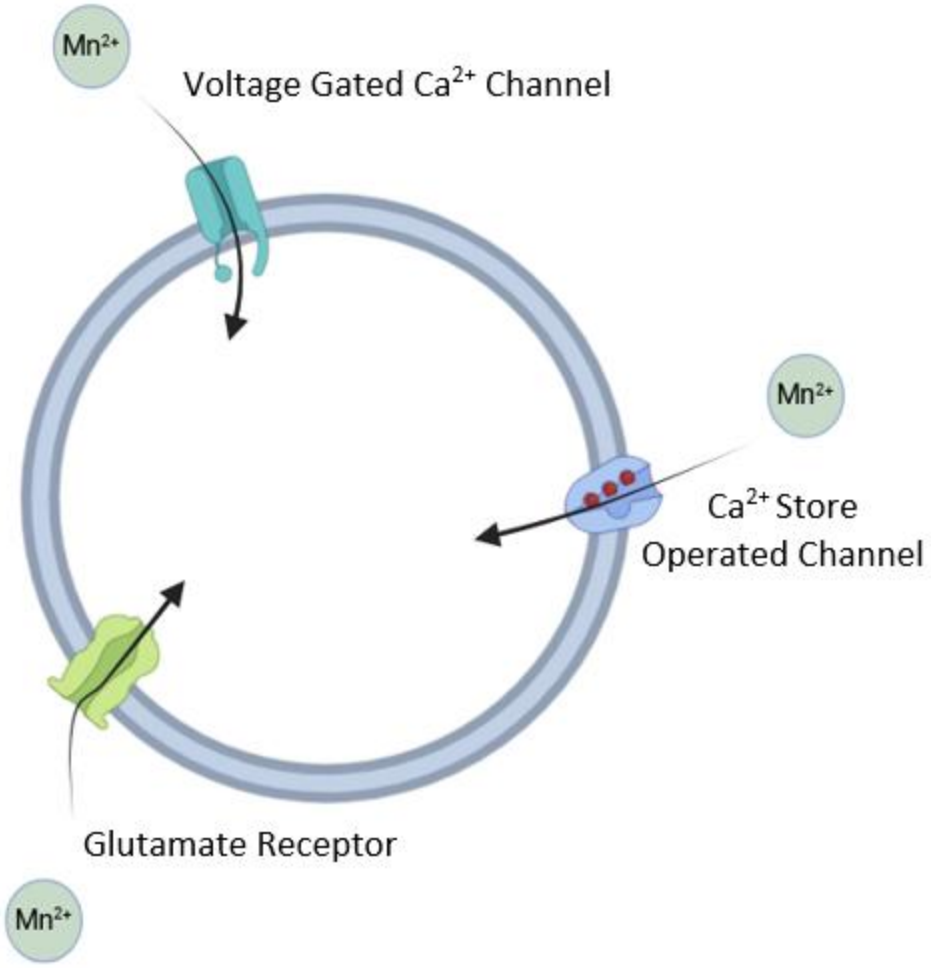

Figure 1.2. Divalent metal channels involved in Mn homeostasis. Voltage gated Ca^{2+} channel located on muscle cells, neurons, and glial cells; Ca^{2+} store operated channel located on hepatocytes, endothelial cells, and neurons; glutamate receptor located on neurons, glia, astrocytes, T cells (Chen et al., 2018; Harischandra et al., 2019; Roth et al., 2013; Roth, 2006). Image created in BioRender.

Table 1.1. Organelle/plasma membrane location of receptors, channels, and transporters involved with manganese homeostasis.

Receptor/Channel/Transporter	Location	Reference
DMT1	Enterocytes, Ependymal Cells, Endothelial Cells, Astrocytes, Microglia, Schwann Cells, Oligodendrocytes, Hepatocytes, Macrophages	(Cheli et al., 2018; Coates, 2014; Harischandra et al., 2019; Roth et al., 2013; Skjørringe et al., 2015)
Ca^{2+} Uniporter	Mitochondria	(Harischandra et al., 2019; Wu et al., 2021)
SLC30A10	Golgi, Hepatocytes, Basal Ganglia Neurons, Enterocytes	(Harischandra et al., 2019; Taylor et al., 2019)
Mn Citrate transporter	Hepatocytes, Endothelial Cells	(Gunter et al., 2012; Harischandra et al., 2019; Nyarko-Danquah et al., 2020)
Choline Transporter	Mitochondria, Cholinergic Neurons, Endothelial Cells	(Koehl et al., 2023; Misawa et al., 2001; Patil et al., 2025)
Na-Ca Exchanger	Cardiac, Smooth Muscle, Skeletal, And Neuronal Cells	(Iwamoto, 2006; Roth et al., 2013; Roth, 2006)
Ferroportin	Enterocytes, Hepatocytes, Macrophages, Astrocytes, Endothelial Cells	(Harischandra et al., 2019; Kasvosve, 2013; Siah et al., 2005)
ZIP8	Epithelial Cells, T Cells, Choroid Plexus Papilloma Cells, Urothelial Cells, Endothelial Cells	(Ajjimaporn et al., 2012; Aydemir et al., 2009; Harischandra et al., 2019; Morgan et al., 2020; Steimle et al., 2019; V. Zhang et al., 2023)
ZIP14	Choroid Plexus Papilloma Cells, Endothelial Cells, Hepatocytes, Astrocytes	(Harischandra et al., 2019; Maxel et al., 2019; Morgan et al., 2020; Routhe et al., 2020; Steimle et al., 2019)
SPCA1	Golgi, Endothelial Cells, Smooth Muscle Cells	(Harischandra et al., 2019; Steimle et al., 2022)
Voltage Gated Ca^{2+} Channel	Muscle Cell, Neurons, Glial Cells	(Buraei & Yang, 2013; Dziedzic et al., 2021)
Ca^{2+} Store Operated Channel	Hepatocytes, Endothelial Cells, Neurons	(Prakriya & Lewis, 2015; Zhang & Hu, 2020)
Glutamate Receptor	Neurons, Glia, Astrocytes, T Cells	(Harischandra et al., 2019; Vizcarra et al., 2023)

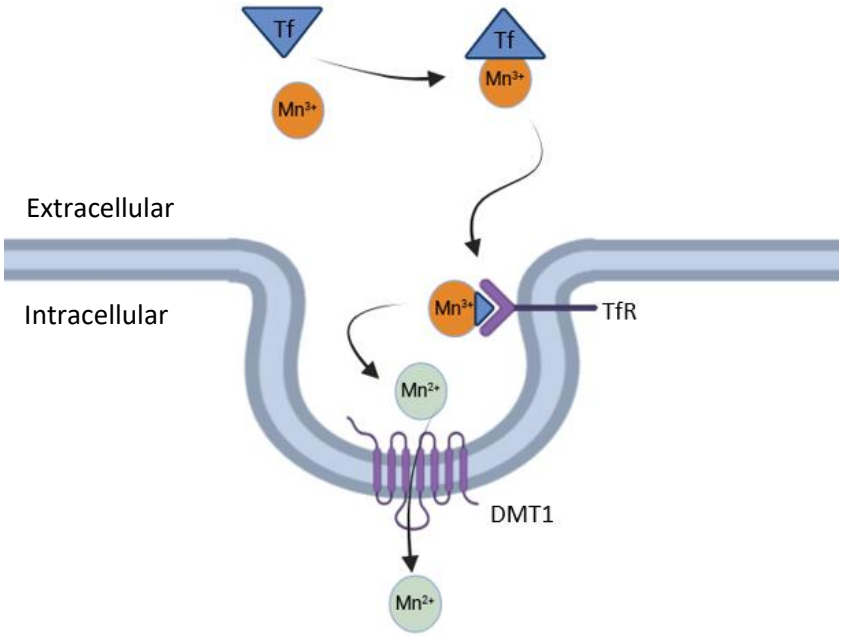

Figure 1.3. Mn^{3+} entry into cell via transferrin (Tf)/transferrin receptor (TfR) complex. The Tf/TfR complex can reduce Mn^{3+} to Mn^{2+} (Chen et al., 2018; Harischandra et al., 2019; Roth et al., 2013; Roth, 2006). Image created in BioRender.

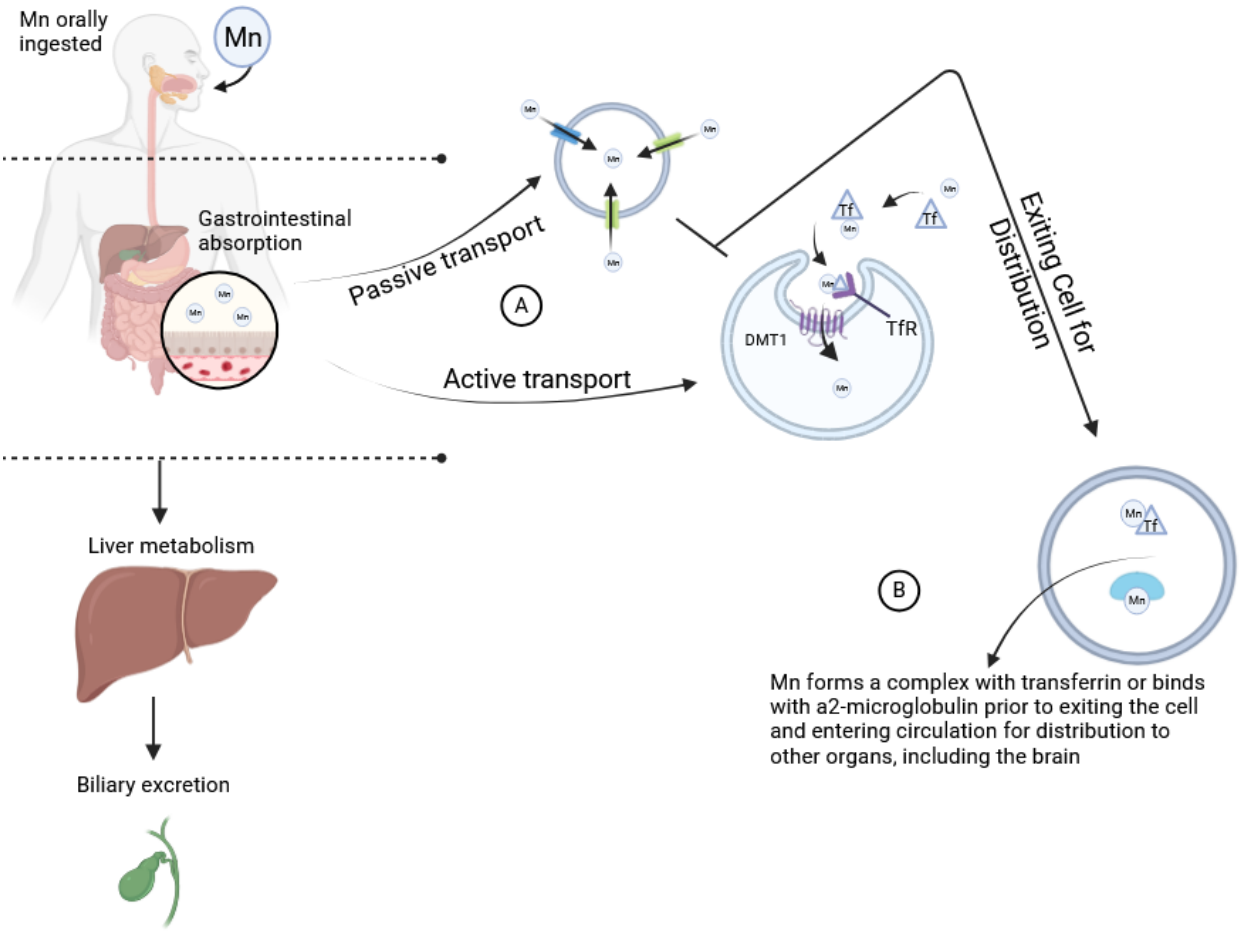

Figure 1.4. Typical Ingested Mn absorption route. Mn is absorbed through intestinal epithelium and the majority is excreted through the bile. A) After Mn is in gastrointestinal tract, Mn can enter cells both through passive diffusion utilizing various divalent metal channels and/or by active transport utilizing DMT-1 and transferrin. B) Before exiting the cell, into the plasma for distribution, Mn can form a complex with transferrin or bind with a2-macroglobulin (Chen et al., 2018; Harischandra et al., 2019; Peres et al., 2016; Roth et al., 2013; Roth, 2006). Image created in BioRender.

CHAPTER 2

BEHAVIORAL ALTERATIONS IN MICE EXPOSED TO MANGANESE VIA THE DRINKING WATER: EFFECTS OF SEX AND A LIPOPOLYSACCHARIDE CHALLENGE

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Abstract

Manganese (Mn) is an essential and important metal; however, overexposures lead to adverse neurological outcomes. Nonoccupational Mn overexposure occurs primarily through consumption of Mn-contaminated drinking water (DW). Sex differences in terms of nervous and immune systems' responsiveness to excessive Mn in the DW are understudied. Thus, this study investigated behavioral and sex differences in response to Mn DW treatment (0.4 g Mn/L for up to 8 weeks) and a lipopolysaccharide (LPS) challenge of adult C57BL/6 mice with GFP-tagged monocytes/microglia. After 6 weeks, in motor function tests, Mn exposure resulted in decreased activity and gait deficits. In two different mood tests (open field test [OFT]/elevated zero maze), Mn-exposed mice exhibited decreased fear/anxiety-like behavior. Two weeks after behavioral assessment, when mice were challenged with LPS, circulating inflammatory cytokines, and acute phase proteins increased in both sexes. After 8 weeks of Mn exposure, liver and brain Mn levels were increased, but Mn alone did not affect circulating cytokines in either sex. Notably, Mn-exposed/LPS-challenged males had potentiated plasma cytokine output, whereas the reverse was seen in females. Males, but not females, continued to exhibit increased fearlessness (i.e., increased OFT center time), even when challenged with LPS. Overall, our results show that Mn DW exposure increases brain Mn levels and it leads to behavioral alterations in both sexes. However, males might be more susceptible to the effect of Mn on mood, and this effect is recalcitrant to an inflammagen challenge. Mn augmented post-LPS cytokine production only in males, further indicating that important Mn effects are sex-biased.

Keywords: acute phase proteins | behavioral deficits | cytokines | LPS | manganese | sex differences

1 Introduction

Manganese (Mn) is an essential metal, necessary for synthesis of amino acids, lipids, and carbohydrates (IOM, 2001). It is also an important cofactor for multiple biological processes including free radical defense, bone formation, and metabolism (Chen et al., 2018; Erikson et al., 2007; IOM, 2001). Mn can be transported across the blood–brain, as well as the blood–CSF barriers, by multiple transporters including, but not limited to, transferrin, SLC30A10, and divalent metal transporter-1 (Aschner & Aschner, 1990; Aschner et al., 2007; Liu et al., 2021). Although an essential metal, overexposure to Mn is neurotoxic (Chen et al., 2018; Erikson et al., 2007). In the brain, Mn preferentially targets the basal ganglia and causes parkinsonism (Racette et al., 2017), which, at very high exposures, can result in manganism (Laohaudomchok et al., 2011). In manganism, cock-walk gait is a specific clinical symptom (Chen et al., 2018; Laohaudomchok et al., 2011), whereas parkinsonism-related symptoms associated with excess Mn exposure include rigidity, speech impairment, postural instability, and locomotor deficits, such as reduced response speed, tremors at rest, and gait alterations (Bowler et al., 2006; Bowler et al., 2007; Park et al., 2006; Sahni et al., 2007). Additionally, Mn overexposure causes mood alterations, for example, irritability, compulsive behaviors, anxiety, and/or depression in both humans (Bouchard et al., 2007; Bowler et al., 2003; Bowler et al., 2006; Laohaudomchok et al., 2011; Sahni et al., 2007) and laboratory animals (Dodd et al., 2005; Krishna et al., 2014; Liu et al., 2019).

Exposure level, route, and duration all influence the severity of Mn-induced neurological dysfunction (Chen et al., 2018; Lucchini & Tieu, 2023; Lucchini et al., 2009). In occupational settings, such as Mn mining operations or welding, inhaled Mn is the greatest concern (Elsner & Spangler, 2005; Gonzalez-Cuyar et al., 2014; Laohaudomchok et al., 2011; Utembe et al., 2015). Although occupational exposure to Mn remains a major concern, excess exposure for the general public via contaminated drinking water (DW) is increasingly associated with adverse neurological outcomes (Ljung & Vahter, 2007). For instance, Bouchard et al. (2011) reported

intellectual impairment in children exposed to Mn through DW, but not diet. Consumption of Mn-contaminated well water correlated with memory deficits, lower intelligence scores, and increased infant mortality across multiple studies (Bouchard et al., 2011; Brna et al., 2011; Hafeman et al., 2007; Sahni et al., 2007; Wasserman et al., 2006; Woolf et al., 2002). Surface and groundwater Mn levels in the US range, respectively, 400–800 μg/L and 2900–5600 μg/L (ATSDR, 2012). Excess levels of Mn DW (≥ 0.2 mg/L) are associated with adverse neurological outcomes in chronic exposure scenarios (ATSDR, 2012) and at least 2.6 million Americans potentially drink water with elevated Mn (McMahon et al., 2019).

In rodents, peripheral lipopolysaccharide (LPS) administration increases proinflammatory cytokines in both circulation and the brain (Perry, 2004). Additionally, it causes sickness behaviors characterized, among others, with decreased exploratory and rearing activity, as well as increased thigmotaxis and corner preference (Godbout et al., 2005; Krishna et al., 2016). Ongoing inflammation, or the presence of an inflammagen, such as LPS, can exacerbate the neurotoxic effects of Mn (Filipov et al., 2005). That Mn treatment causes microglial overproduction of cytokines and other proinflammatory mediators is well established (Crittenden & Filipov, 2008; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Filipov et al., 2005; Zhao et al., 2009). However, the effects of Mn on peripheral cytokines or other inflammatory markers are not.

Behavioral studies centered on the consequences of Mn exposure via the increasingly relevant DW route (Avila et al., 2010; Krishna et al., 2014) are sparse. Mood and motor alterations are two behavioral domains affected throughout multiple studies. A common element that these behavior-focused studies (Avila et al., 2010; Krishna et al., 2014; Sepúlveda et al., 2012) have is that they were male-biased. At nonbehavioral level, Mn has been reported to have sex-dependent toxicity (Chi et al., 2017; Dodd et al., 2005; Madison et al., 2011; Riojas-Rodríguez et al., 2010; Zhang et al., 2003). Females typically have higher blood Mn levels, likely due to higher absorption rates (Chen et al., 2018). Children exposed to excess Mn had lower

intelligence scores than nonexposed children; girls were affected more than boys (Riojas-Rodríguez et al., 2010). In school-aged children, visuospatial learning also has sex-specific vulnerability to neuroactive metals, predominantly Mn (Rechtman et al., 2020). Sex differences in Mn effects when exposure is in adulthood are understudied. Limited Mn exposure studies, in rodents, have been utilized to investigate behavioral sex differences and effects associated with Mn exposure (Liu et al., 2019; Moreno et al., 2009; Sepúlveda et al., 2012), with results ranging from no sex differences (Liu et al., 2019) to male-specific hyperactivity (Moreno et al., 2009).

Immune differences between sexes, that is, cytokine production, could play a role in the responses to Mn and inflammatory stimuli. In this regard, adult females reportedly produce more anti-inflammatory cytokines than males (Barrientos et al., 2019), but mount a more robust inflammatory response to the same inflammatory stimuli too (Bouman et al., 2005). Middle-aged female mice challenged with LPS exhibited a greater decrease in locomotor activity than males and had higher cytokine levels in response to LPS (Dockman et al., 2022). Non-neurotoxicity studies reported that DW Mn exposure causes decreased fertility in males at 8000 mg/L, but not in females (Elbetieha et al., 2001), and results in sex-specific gut microbiome alterations (20 mg/kg/day) (Chi et al., 2017). Neurologically, Mn led to greater changes in mouse striatal morphology in females compared with males injected with Mn subcutaneously (50 mg/kg) (Madison et al., 2011).

Whether Mn neurotoxicity is sex-dependent in the context of DW exposure or inflammagen challenge is unknown. Hence, the main objectives of this study were to (i) evaluate the neurotoxic effects of Mn DW exposure on selected behavioral parameters, (ii) determine if the Mn effects are sex-specific, and (iii) assess whether sex plays a role in the response of Mn-exposed mice to an inflammagen.

2 Materials and Methods

2.1 Reagents

All chemicals, unless stated otherwise, including manganese ($MnCl_2 \cdot 4H_2O$) and LPS (*Escherichia coli* serotype 0111: B4), were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Animals

The University of Georgia Institutional Animal Care and Use Committee (IACUC) approved all procedures that involved animal handling in advance, and they were in accordance with the latest National Institutes of Health and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Homozygous male and female *CX_3CR1^{GFP}* mice, on a C57BL/6 background (Jackson Labs, stock 005582), were used for all experiments. This strain of mice is behaviorally and immunologically indistinguishable from wild-type C57BL/6 (Dockman et al., 2022). Same-sex mice were group-housed (2–5 per cage) with PicoLab Rodent Diet 20 (LabDiet, 5053) available ad libitum in an environmentally controlled room (22°C–24°C) with a relative humidity of 50%–70% and maintained on a 12 h light/dark cycle in an AAALAC accredited facility throughout the study. Mice were acclimated to using water bottles for 3 weeks. Then, they were randomly assigned to DW treatment groups and were 2 months old at treatment onset.

2.3 Animal Treatment and Tissue Collection

In total, 53 mice (males: 25 and females: 28) were exposed to vehicle control (NaCl; 0.4 g Na/L) or $MnCl_2$ (0.4 g Mn/L) (n = 12–15/group) in deionized water for 8 weeks. Water bottles were changed weekly with freshly prepared control or Mn solutions. Body weight (BW), water intake, and estrous cycle stages (females only) were recorded weekly. The Mn DW concentration used in the current study was selected based on previous work that resulted in significant, human-relevant Mn deposition in the brain (Avila et al., 2010; Krishna et al., 2014) and in behavioral alterations in C57BL/6 male mice (Avila et al., 2010; Krishna et al., 2014). Behavioral tests were carried out after 6 weeks of DW treatment (n = 12/group), while

maintaining the mice on their respective DW treatments, and are described in detail below. At the end of the 8-week exposure period, a subset within each sex and treatment group was randomly selected to receive an intraperitoneal (IP) injection of LPS (*E. coli* serotype 0111: B4 1.5×10^{12} EU or 0.3 mg/kg BW) or saline vehicle ($n = 6$/group). Sickness behavior was assessed in an open field test (OFT) 4 h after LPS/vehicle treatment, and the mice were sacrificed 2 h later (6 h post-LPS/vehicle treatment). Brains were extracted and processed similarly to Coban and Filipov (2007) and Carpenter et al. (2021). Briefly, brains were weighed, washed in ice-cold HEPES-buffered Hank's saline solution (pH 7.4), and separated longitudinally into two hemispheres; one half of the brain was fixed in 4% paraformaldehyde, cryoprotected, and stored at −80°C, whereas the other half was frozen on dry ice and stored at −80°C for future analyses. In addition, organs (liver, spleen, thymus, kidneys, and lymph nodes) and fat (subcutaneous, retroperitoneal, epididymal/ovarian, and brown adipose tissue) were collected, weighed, and stored at −80°C; plasma was collected from these mice and analyzed for tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 10 (IL-10), C-reactive protein (CRP), and serum amyloid A (SAA) using enzyme-linked immunosorbent assays (ELISA) as described later.

2.4 Behavioral Tests

The tests used in this study, in the order presented below, evaluated locomotion and mood behavior in rodents that are known to be affected by Mn (Conrad et al., 2011; Krishna et al., 2014; Sepúlveda et al., 2012), and are both altered in Mn-exposed humans (Bouchard et al., 2007). Male and female mice behavioral testing was carried out separately with all equipment receiving a thorough cleaning between testing the different sexes; all mice were subjected to tests in the same order. All animals were naïve to behavioral apparatuses prior to the start of testing. Tests were performed by a treatment-blind experimenter in a room designated for behavioral testing, located in the same facility and near the room where the animals were housed.

2.4.1 OFT

An open field arena (25 cm × 25 cm × 40 cm, divided into a 16 square grid; Coulborn Instruments, Whitehall, PA) was used to conduct the OFT for a 30-min period as in (Dockman et al., 2022; Krishna et al., 2016; Krishna et al., 2014). Limelight tracking software (Actimetrics, Wilmette, IL) was used to monitor and record videos of each test. ANY-maze software (Stoelting Co., Wood Dale, IL) was used to assess and score mouse locomotor activity by an experimenter blinded to the treatments, per 5-min interval and the total 30-min test. Parameters of interest included distance traveled and the number of grid crossings for the first 5-min total time (horizontal locomotor activity), time spent in the center versus the periphery (measures of anxiety-like or fearlessness behavior), and the number of rearings (vertical activity). The same OFT, but ran for 15 min, was used to score and assess sickness behavior post-LPS (detailed in Section 2.4.4).

2.4.2 Gait Test (GT)

Motor function was evaluated by utilizing the GT as described in Carpenter et al. (2021). Briefly, an 82 cm × 5.5 cm × 8 cm (l × w × h) runway was lined with a white paper strip; an empty cage with home cage bedding was placed at the end of the runway. Two trials, 5 min apart, were conducted—a training pre-trial and a test trial that was used for subsequent statistical analyses. Before each trial, front and hind paws were painted with nontoxic red and black ink, respectively (Office Depot, item #839994 and #839967), and then, the mouse was allowed to traverse the runway. Between each trial, the runway was cleaned with 70% ethanol and lined with a new paper strip. Gait parameters (see Figure S1) that were measured included stride length, base width, interstep/intrastep distance, stride variability, total number of steps, and cadence (Carpenter et al., 2021; Mulherkar et al., 2013; Wang et al., 2017).

2.4.3 Elevated Zero Maze (EZM)

An EZM apparatus (50-cm diameter; Stoelting Co., Wood Dale, IL) was used to assess anxiety-like behaviors as in Carpenter et al. (2021), with locomotion also monitored. At the start

of the test, mice were placed at the center of an open quadrant facing inward and allowed to explore the maze freely for 5 min. Parameters of interest included times spent in open and closed arms, latencies to enter or exit a closed area at the start of the test, and the number of head dips and stretch attend posture (SAP) attempts, as in (Grewal et al., 1997). Mice were considered to be in a zone when 70% of the body was in an area. EZM parameters were tracked and scored using ANY-Maze software (Stoelting) in a treatment blind manner.

2.4.4 Post-LPS Challenge (Post-LPS OFT)

At the end of the 8-week exposure period, a subset of mice within each sex and treatment group were randomly selected to receive, via an IP injection, either saline vehicle or 0.3-mg/kg BW LPS, which has been previously found to induce transient peripheral and central inflammatory response and accompanying behavioral changes in adult/middle-aged mice on C57BL/6 background (Dockman et al., 2022; Krishna et al., 2016). Four hours post-LPS/vehicle treatment, mice were subjected to a 15-min OFT. Parameters of interest included locomotor activity, such as distance traveled and the number of grid crossings; rearing activity, that is, standing on hind legs to explore and assess the environment; and times spent in the center versus the periphery of the arena.

2.5 ELISA

Plasma concentrations of IL-6, IL-10, TNFα, CRP, and SAA were analyzed 6 h post-LPS administration using mouse-specific ELISA kits (Bio-Techne, Minneapolis, MN) following the manufacturer's protocol. Samples and standards, namely, IL-6 (1000–15.625 pg/mL), TNFα (2000–31.25 pg/mL), IL-10 (2000–31.25 pg/mL), CRP (1500–23.4 pg/mL), and SAA (16,000–250 pg/mL), were run in duplicate. Absorbance (450-nm analytical read; 570-nm background correction read) was measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT), and the mean from the individual sample replicates was used for statistical analysis.

2.6 Liver and Brain Measurement of Mn and Fe

For analysis of tissue Mn and Fe, the liver median lobe and four to six random slices of the forebrain were weighed and digested in 1-mL concentrated nitric acid for 2 h at 70°C and then brought to a total volume of 5 mL with ddH_2O. An Inductively Coupled Argon Plasma-Axially Viewed Optical Emission Spectophotometry (ICP-AVOES) was used to determine Mn and Fe concentrations as in Dodd and Filipov (2011). Instrument values for Mn and Fe were reported as concentration in solution (ppm). The mean for each sample was expressed as μg Mn/g tissue and used for statistical analysis.

2.7 Statistical Analysis

All data were analyzed using Sigmaplot v12.5 (Systat Software, Inc., Chicago, IL); graphs were generated using GraphPad Prism v8.4.3 (San Diego, CA). Two-way repeated measures (RM) or three-way RM ANOVA (post-LPS) were used to assess behavior parameters across 5-min intervals for the OFTs. Body/organ weights, water intake, estrus staging, GT, EZM, and all ELISA data were analyzed by using two-way ANOVA. For all ANOVAs, the *F*-value is displayed as F (df, df) with degrees of freedom designated as (groups -1, total mice - groups). A Student–Newman–Keuls (SNK) post hoc comparison was run if a significant main effect or an interaction was detected, with statistical designation as (p-value, q-value). A p-value of ≤ 0.05 was considered significant.

3 Results

3.1 BW, Water Intake, Organ Weight, and Estrous Cycle

BWs were unaffected by Mn ($F(3,45) \leq 1.579$, $p \geq 0.1$), except for an end-of-study, male-specific, weight increase ($F(1,23)=5.498$, $p=0.028$; Figure 1A). Males in the Mn group gained about 20%, whereas control males' gain was 17% (Figure 1B). End female weight gain was about 20% in both groups (Figure 1B). Throughout the entire experimental period, water intake by both male and female Mn-exposed mice was similar to their respective controls (Figure 2).

There was a week-dependent sex effect, such that during Week 4 ($F(3,44)=6.417$, $p=0.028$), males consumed more water than females. Mn consumption did not affect estrous cycle ($F(1,22)\leq 0.600$, $p\geq 0.1$; Figures 3 and 4); both Mn-exposed and control female mice continued to cycle throughout the study. Eight weeks of Mn exposure also did not affect relative brain, kidney, liver, spleen, thymus, brown, or subcutaneous adipose tissue weights in either sex ($F(3,44)\leq 2.908$, $p\geq 0.1$; Table 1). Males, but not females, challenged with LPS had heavier livers (absolute; Table S1) and relative weights than vehicle controls ($F(3,20)=8.967$, $p=0.007$). In terms of relative organ weights, Mn-exposed males, with and without LPS, had heavier livers than male controls ($F(3,20)=7.096$, $p=0.015$; Table 1) and only the Mn + LPS female livers were heavier than the rest ($t=3.586$, $p=0.004$). Interestingly, LPS-challenged females had heavier spleens, both absolute (Table S1) and relative weight (Table 1) than saline controls ($F(3,20)=26.485$, $p<0.001$); this increased splenic weight was more pronounced in the females that were exposed to Mn ($q=4.562$, $p<0.001$; Table 1).

3.2 Behavioral Analysis

3.2.1 OFT

After 6 weeks of Mn DW exposure, a two-way ANOVA revealed an overall main effect of sex on locomotor activity with males being more active, with respect to total distance traveled (Figure 5A; $F(3,44)=11.039$, $p=0.002$), number of rearings (Figure 5B; $F(3,44)=14.334$, $p<0.001$), and grid crossings ($F(3,44)=9.037$, $p=0.004$; data not shown) regardless of DW treatment. Mn-exposed mice exhibited a significant ($F(3,44)=5.189$, $p=0.028$) decrease in vertical activity (number of rearings) during the first 5-min (Figure 5B) and also throughout the 30-min OFT ($F(3,44)=4.251$, $p=0.045$; data not shown). Mn-caused decrease of rearings was more pronounced in the females ($q=2.244$, $p=0.03$). Over the 30-min testing duration, locomotor activity decreased ($F(3,44)=88.547$, $p<0.001$) irrespective of Mn and sex, as all mice habituated to the arena over time (Figure 6); there was also a strong trend for Mn-exposed males to be more active early in the test (during the first 10 min; Figure 6; $t=1.989$, $p=0.059$).

Mn-treatment was associated with decreased anxiety/increased fearlessness, that is, Mn-exposed mice spent significantly more time in the center (Figure 5C; $F(3,44)=5.431$, $p=0.024$) and less time in the periphery (Figure 5D; $F(3,44)=4.129$, $p=0.048$) of the arena during the first 5-min exploration phase of the OFT. This effect was more prominent in the Mn-exposed males ($q=2.136$, $p=0.038$). There was also a main effect of sex, that is, female mice spent significantly more time in the center than male mice ($F(3,44)=12.958$, $p<0.001$).

3.2.2 GT

Mn-exposed mice, regardless of sex, took more steps to complete the test (Figure 7A; $F(3,44)=4.120$, $p=0.048$) and exhibited a significantly shorter right-to-left hind paw interstep distance (Figure 7C; $F(3,44)=4.623$, $p=0.037$), the distance between consecutive right to left steps (Figure S1), and stride length (Figure 7B; $F(3,44)=7.779$, $p=0.006$), the distance between two consecutive pawprints of the same paw (Figure S1). Two-way ANOVA revealed a main sex effect on fore/hind paw overlap, with females having a shorter overlap distance than males (Figure 7D; $F(3,44)=12.473$, $p<0.001$), the distance between a step's fore and hind paw (Figure S1).

3.2.3 EZM

In the EZM, Mn-exposed mice, specifically males, spent significantly more time in the open arms of the maze compared with controls (Figure 8A; $F(3,44)=4.946$, $p=0.031$), an indication of decreased anxiety/increased fearlessness. Latency to enter the closed arm was not significantly different between Mn treatments or sexes ($F(3,44)=0.0172$, $p=0.896$ and $F(3,44)=0.509$, $p=0.479$, respectively; data not shown). For SAP, one of the parameters used to assess risk assessment behavior, two-way ANOVA revealed the overall main effects of Mn and sex. Mn-exposed mice exhibited a decrease in the number of closed-arm SAP attempts than saline controls (Figure 8C; $F(3,44)=6.326$, $p=0.016$). Female mice, regardless of Mn treatment, attempted more closed-arm SAPs than males ($F(3,44)=30.620$, $p<0.001$). In terms of general

EZM activity, Mn-exposed mice displayed an increase in the number of closed and open arm entries, which was male driven (q=2.145, p<0.001; data not shown).

3.2.4 Post-LPS OFT

After 8 weeks of Mn DW exposure, a three-way ANOVA revealed the main effects of sex and LPS on locomotor activity. Males were more active than females, regardless of Mn treatment or LPS challenge, with respect to the total distance traveled (Figure 9A; F(3,20)=7.253, p=0.01). This main effect of sex persisted from the 6-week (pre-LPS) time point (i.e., Figure 5A). Post-LPS, the LPS-challenged mice, regardless of sex or Mn treatment, traveled less distance (Figure 9A; F(3,20)=5.352, p=0.026) and had decreased vertical activity during the first 5-min interval (Figure 9B; F(3,20)=7.888, p=0.008). Within sex, LPS-challenged females, but not males, made fewer rearings than saline controls, an effect that approached significance (F(3,20)=3.994, p=0.053). Over the 15-min testing duration, mice habituated and locomotor activity decreased irrespective of Mn, LPS, and sex (F(3,20)=5.079, p=0.03).

Interestingly, the Mn-induced decreased time in the periphery and increased time in the center, which were observed during the OFT at 6 weeks (Figure 5C,D, respectively), were still present after 8 weeks of Mn DW exposure in the males, but not the females (Figure 9C,D; periphery and center times, respectively). There were overall main effects of Mn and sex on the times spent in the periphery and center of the arena. Mn-exposed mice spent significantly more time in the center (Figure 9D; F(3,20)=9.838, p=0.003) and less time in the periphery (Figure 9C; F(3,20)=11.181, p=0.002) of the arena; this main effect was due to the Mn-exposed males and independent of LPS (Figure 9D; q=3.046, p=0.004).

3.3 Plasma Cytokine and Acute Phase Protein ELISAs

In the absence of LPS, plasma IL-6 and TNFα were undetectable, regardless of Mn treatment and sex (Figure 10A,B, respectively). Six hours after a single LPS administration, increased plasma IL-6 and TNFα in both Mn and control mice were noted. A two-way ANOVA revealed an overall main effect of sex, with females' plasma IL-6 levels being greater than in

males (Figure 10A; $F(3,20)=7.463$, $p<0.001$). Notably, within LPS, male mice that were treated with Mn had greater plasma IL-6 and TNFα levels compared with saline + LPS males (Figure 10A; $q=6.133$, $p=0.012$ and Figure 10B; $q=2.190$, $p=0.034$, respectively); in the females, Mn diminished the LPS effect on both inflammatory cytokines (Figure 10A; $q=6.133$, $p<0.001$ and Figure 10B; $q=4.125$, $p<0.001$, respectively). Baseline IL-10 (without LPS) was higher in the males than in the females (Figure 10C; $F(3,20)=17.816$, $p<0.001$), and it was unaffected by Mn ($F(3,20)=0.130$, $p=0.72$). After LPS, IL-10 in the plasma decreased in the males ($q=4.811$, $p=0.001$), but it increased significantly in the females (Figure 10C; $q=9.237$, $p<0.001$). As was the case for TNFα and IL-6, Mn-treated/LPS-challenged (Mn + LPS) female mice had lower IL-10 ($F(3,20)=7.219$, $p=0.01$) than LPS-treated control mice.

In both sexes, plasma CRP significantly increased after LPS administration (Figure 10D; $F(3,20)=216.234$, $p<0.001$), and this increase was unaffected by Mn ($F(3,20)=0.291$, $p=0.591$). A three-way ANOVA revealed an overall main effect of sex within the LPS-administered groups, that is, male's plasma CRP levels were greater than in females ($F(3,20)=10.026$, $p=0.002$). In the absence of LPS, plasma SAA was undetectable, regardless of DW treatment and sex; LPS administration increased plasma SAA in both DW treatment groups and sexes. There was an overall main effect of sex, with plasma SAA being higher in males (Figure 10E; $F(3,20)=24.306$, $p<0.001$). Although DW Mn exposure did not increase plasma SAA levels post-LPS compared with saline + LPS males, in females, similar to IL-6, TNFα, and IL-10, Mn diminished the LPS effects on SAA (Figure 10E; $F(3,20)=13.524$, $p<0.001$).

3.4 Liver and Brain Mn and Fe

After 8 weeks of Mn DW exposure, a three-way ANOVA revealed the overall main effects of Mn and sex on liver Mn. Liver Mn levels were higher in Mn-exposed mice, regardless of sex, in the absence of LPS (Figure 11A; $F(3,20)=106.033$, $p<0.001$).

Saline-challenged females had higher liver Mn regardless of DW treatment (Figure 11A; $F(3,20)=5.935$, $p=0.019$), however, this overall effect of sex was not seen among LPS-

challenged mice ($F(3,20)=0.482$, $p=0.492$). Three-way ANOVA revealed an overall LPS main effect of LPS; LPS-challenged mice had lower liver Mn levels than control mice ($F(3,20)=5.173$, $p=0.028$). This LPS effect was more pronounced in females, with Mn + LPS females having significantly lower Mn liver levels than Mn + saline females (Figure 11A; $q=5.822$, $p<0.001$). There were also overall main effects of sex and DW treatment on liver Fe levels; Fe was lower in the Mn-exposed mice (Figure 11C; $F(3,20)=19.170$, $p<0.001$). This overall Mn main effect was driven by the Mn-exposed females, which had significantly lower levels of liver Fe than controls ($F(3,20)=16.745$, $p < 0.001$); in the males, there was only a trend in the same direction ($F(3,20)=3.313$, $p=0.084$). Female mice, regardless of LPS challenge or DW treatment, had higher liver Fe than males (Figure 11C; $F(3,20)=60.045$, $p<0.001$).

In the brain, Mn-exposed mice, regardless of sex and LPS challenge, had higher levels of Mn than controls overall (Figure 11B; $F(3,20)=48.292$, $p<0.001$). Brain Fe levels were only significantly different within the female mice. Mn-exposed females had significantly higher brain Fe levels than controls overall (Figure 11D; $F(3,20)=5.49$, $p=0.03$), but this effect was because of the greater levels in the absence of LPS (Figure 11D; $q=4.101$, $p=0.009$).

4 Discussion

We explored the effects of DW Mn exposure on motor and mood-related behaviors of both male and female mice, with the female estrus cycle monitored throughout the study. Additionally, we examined sex differences and the effects of Mn on response to an LPS challenge. Previous rodent studies investigating neurobehavioral consequences of Mn exposure vary in Mn doses/concentrations, types of behavioral tests, strain, and age of mice utilized, as well as duration in Mn treatment prior to behavior testing and routes of exposure, including IP injection (Fleming et al., 2018; Liu et al., 2019), oral gavage (Moreno et al., 2009), subcutaneous osmotic pump (Sepúlveda et al., 2012), and DW (Avila et al., 2010; Krishna et al., 2014), the latter employed here as well. Although study variations might factor in the differences between behavioral outcomes reported, common behavioral domains, that is, locomotor, are

affected across multiple studies and exposure paradigms (Krishna et al., 2014; Liu et al., 2019; Moreno et al., 2009; Sepúlveda et al., 2012).

In the current study, Mn-exposed mice exhibited decreased vertical locomotor activity, shown by fewer rearing attempts during the OFT. DW Mn-exposed male mice here did not display as much early hyperactivity during the OFT, a DW Mn effect reported by Krishna et al. (2014), but there was a similar trend for the Mn-exposed males to be hyperactive early in the OFT. Moreno et al. (2009) and Liu et al. (2019) did not find overall alterations in horizontal locomotor activity caused by Mn exposure. However, these studies either did not monitor (Liu et al., 2019) or observe (Moreno et al., 2009) changes in rearing activity. Although the current study used the same Mn concentration, exposure route, and similar timeline as Krishna et al. (2014), the present mice were 2–3 months younger; age has been found to be a factor in Mn-induced neurological dysfunction (Moreno et al., 2009) and it might have contributed to the less robust hyperactivity effect observed here in the males.

Locomotor deficits induced by Mn were observed during gait testing. Mn-exposed mice exhibited decreased stride and a left-biased hind paw interstep distance. To compensate for these deficits, Mn-exposed mice took more, but shorter, steps to complete the GT. reported a similar result, also demonstrating a left-side decrease in stride. When Mn was delivered continuously via an osmotic pump (at 30 mg/kg BW/day) in Sepúlveda et al. (2012), Mn exposure resulted in increased stride and decreased fore/hind paw overlap 1 week after treatment. When Fleming et al. (2018) utilized a lower Mn concentration, as well as older mice, they did not report any gait alterations or sex differences. Liu et al. (2019) reported similar gait deficits, but sex differences during gait testing were not reported. In the current study, females had a smaller fore/hind paw overlap, likely due to them being smaller than males of this strain and age. During the EZM, conducted under minimally anxiogenic red lighting (Williams, 1971), there was an Mn-induced increase of general activity, driven more by males, evident by the increased number of closed and open arm entries. Under halogen light conditions, which are

markedly more stressful (Williams, 1971), among both sexes of Sprague–Dawley rats, such Mn effect was not observed (Amos-Kroohs et al., 2016).

In terms of anxiety/fearlessness-like behavior, our findings are consistent with previous OFT studies, where Mn exposure was via DW (Krishna et al., 2014) or oral gavage (Moreno et al., 2009). Namely, Mn-exposed male mice spent more time in the center than in the periphery of the OFT. In line with the current study's OFT results, Mn-exposed mice also spent more time in the open than closed arms of the EZM, a similar finding reported by Amos-Kroohs et al. (2016) in Mn-exposed Sprague–Dawley rats. In the current study, this effect was more pronounced among males than females. Interestingly, Mn exposure also reduced the number of SAP attempts, a type of risk assessment when the mouse elongates the body toward potential treatment sources (Grewal et al., 1997). The SAP reduction, together with the increased time in the center and decreased rearing attempts, another risk assessment behavior (Choleris et al., 2001), suggests that DW Mn causes increased fearlessness/risky behavior rather than decreased anxiety-like behavior.

These results, taken together, indicate that consequences of subchronic Mn DW consumption include both locomotor and mood alterations in male and female mice, with some sex bias. It is worth noting that Mn exposure paradigms using an oral (DW) route, including ours, found that Mn-exposed mice exhibited reduced anxiety-like/increased fearlessness-like behavior, fitting with reports of Mn-exposed children having increased defiant/oppositional behaviors (Bouchard et al., 2007; Bowler et al., 2003; Laohaudomchok et al., 2011; Sahni et al., 2007).

In mice, LPS challenge results in decreased locomotion and behavioral alterations in the mood domain (Dockman et al., 2022; Krishna et al., 2016). In the present study, we confirmed that, in vivo, LPS exposure results in sickness behaviors in both sexes (i.e., less activity and increased anxiety-like behavior). When we examined sex differences between LPS-induced sickness behaviors, we found that males exposed to LPS were generally more active than

females, regardless of Mn exposure. Although prior exposure to Mn did not influence post-LPS locomotor activity among males, they did spend more time in the center, which is similar to reports of mood alterations/compulsive behavior exhibited by children exposed to Mn via DW (Bouchard et al., 2007; Bowler et al., 2003; Bowler et al., 2006; Laohaudomchok et al., 2011; Sahni et al., 2007). Indeed, both Mn + saline and Mn + LPS-treated males spent more time in the open area of the OFT. Thus, Mn-induced risky/oppositional behavior, in males specifically, is likely to persist despite other behavior-modifying immune stimuli being present.

In order to investigate potential peripheral inflammatory alterations associated with the post-LPS behaviors, we assessed selected plasma cytokine and APP levels. Mn alone did not influence plasma cytokine or APP levels. However, when in conjunction with LPS, Mn-exposed males had a greater inflammatory response (increased IL-6 and TNFα). By contrast, LPS, both with and without Mn, induced a significant increase in plasma CRP and SAA of both sexes. Interestingly, females displayed a more robust innate immune response to LPS than males, which relates to the observed greater display of sickness behavior among females. That said, females within the Mn + LPS group had significantly lower levels of IL-6, TNFα, and IL-10 than females in the saline + LPS group. It is interesting to note that plasma cytokine levels detected in male Mn + LPS and saline + LPS were opposite the cytokine levels produced in females of the respective treatment groups, with Mn potentiating the production of proinflammatory cytokines in the males while diminishing their levels among females. The increased cytokine and APP production in response to LPS, combined with the increased liver weight, suggests that DW Mn is potentiating the inflammatory response in male mice, in vivo*,* as it does in vitro (Filipov et al., 2005).

Rodent studies investigating sex differences in inflammatory response to LPS have reported varied results including males with a stronger peripheral cytokine response (Kuo, 2016), females responding with higher IL-6 and TNFα and with decreased IL-10 (Cai et al., 2016), and females with increased IL-6 and IL-10, but with decreased TNFα (Erickson et al.,

2018). These differing reports may be due to mice strain (IL-10+/+ and IL-10−/−, CD-1, or C57BL/6) and mice age (8–10 weeks and 12–14 months), as well as LPS source (*E. coli* serotypes 0127:B8, O26:B6, or 0111:B4 and *Salmonella typhimurium*), LPS dose (0.2–5 mg/kg BW), or timing of post-LPS challenge plasma collection (6 h, 1 week, or 28 h post-LPS challenge) (Cai et al., 2016; Dockman et al., 2022; Erickson et al., 2018; Kuo, 2016). It has been reported that females possess smaller infectious loads and stronger overall immunity compared to males and that females have had increased inflammation after ovariectomy (Iwasa et al., 2014; Mabley et al., 2005). However, females also tend to exhibit lower levels of inflammatory cytokines, which is influenced by the effects of estrogen (Bouman et al., 2005; Darnall & Suarez, 2009). Though saline-administered mice did not have detectable levels of proinflammatory cytokines in either DW group, males with continual Mn exposure did produce a more robust innate immune response to LPS. This is in line with in vitro studies that have found Mn potentiates proinflammatory cytokine production in LPS-treated microglia (Filipov et al., 2005) and in vivo when Mn exposure is combined with the parkinsonian toxicant MPTP (Hammond et al., 2020). Although females, regardless of DW treatment, displayed typical sickness behaviors in response to LPS, Mn + LPS males continued to exhibit risky behavior suggesting the possibility that males may be more susceptible to and/or affected for a longer duration by Mn. Dockman et al. (2022) reported that females had a more robust response to LPS than males, with increases in IL-6, TNFα, and IL-10, similar to the current study, where female plasma cytokines after equivalent LPS dose and timing were greater than males, and in line with human data reporting a more robust female immune response to the same inflammatory stimuli compared with males (Bouman et al., 2005). The anti-inflammatory cytokine IL-10 is important for rebalancing the immune response to a homeostatic state, and estrogen has been previously found to help accelerate this by inducing IL-10 action (Norden et al., 2016). Oztan et al. (2019) and Kresovich et al. (2018) have previously reported that adult men who have been exposed to excess Mn via occupational/dietary routes have higher levels of

proinflammatory cytokines in their blood than those in the control groups; our data, with the males, suggest that this is the case in nonoccupational DW exposures when Mn exposure is interfaced with another inflammatory challenge.

The liver is important in regulating body burden, distribution, and elimination of Mn, and chronic exposure can result in excess Mn being distributed to other organs instead of removal (Chen et al., 2018; Huang et al., 2011). In the current study, Mn exposure, via DW, significantly increased levels of Mn in both the brain and liver of both sexes. Mn-exposed mice had significantly increased liver and brain Mn levels, as well as decreased female-biased liver iron levels; Mn exposure did not affect brain iron levels, in agreement with results by Alsulimani et al. (2015) and Huang et al. (2011), as well as with reports that Mn is an antagonist of iron (Garcia et al., 2006). Mn-exposed males did have heavier liver weight; when challenged with LPS, there was an increase in liver Mn levels as well as increased levels of plasma cytokines, but no change in liver iron levels. Although Mn-exposed females also had higher levels of Mn in the liver, when challenged with LPS, their liver Mn, but not brain Mn, decreased. Although Mn-exposed females did have similar elevated levels of Mn in the brain as males, Mn-exposed males displayed sustained behavioral dysfunction, possibly suggesting that the Mn-induced behavioral effects of Mn within females may be more inconspicuous. Previous studies have indicated that human females have exhibited lower intelligence scores and slower visuospatial learning (Rechtman et al., 2020; Riojas-Rodríguez et al., 2010), which may suggest parallels with the more subtle nature of the effects observed in Mn-exposed females compared with males. Because the lasting male-specific behavioral alterations do not appear to be due to greater Mn deposition in the brain or liver, in the future, we will extend this research to enclose the contribution of brain neurochemistry, as well as neuroinflammation, to investigate the sex differences of the effects of Mn observed in the current study. Because Mn is known to disrupt dopaminergic, glutamatergic, and GABAergic systems within the brain (Lin et al., 2020; Soares et al., 2020), we will focus on brain monoamine and amino acid neurotransmitters.

In conclusion, the current study characterized sex differences in behavioral deficits in Mn-exposed mice and expanded the existing literature on potential risks of DW Mn overexposure. We have shown that Mn exposure through DW for a subchronic period induces behavioral alterations in mice. Although both sexes demonstrated neurobehavioral dysfunction, our results suggest that males may be more susceptible to continuous Mn exposure and exhibit persistent deficits. Males exposed to Mn via DW also displayed an augmented response to an inflammatory challenge, which suggests a male bias in this domain. Females, on the other hand, exhibited diminished cytokine response, which could impede the immune system's defense against pathogens. These sex differences in cytokine responses and certain behavioral deficits further suggest that Mn effects, at least in the context of DW exposure, are sex-biased.

Author Contributions

N.M.F. conceived and designed the study. N.M.F., H.D.L., and J.M.C. assisted in the investigation and sample collection. H.D.L. performed sample and data analysis. N.M.F. and H.D.L. wrote the manuscript. All authors read/contributed editorially and approved the final manuscript version.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Figures and Tables

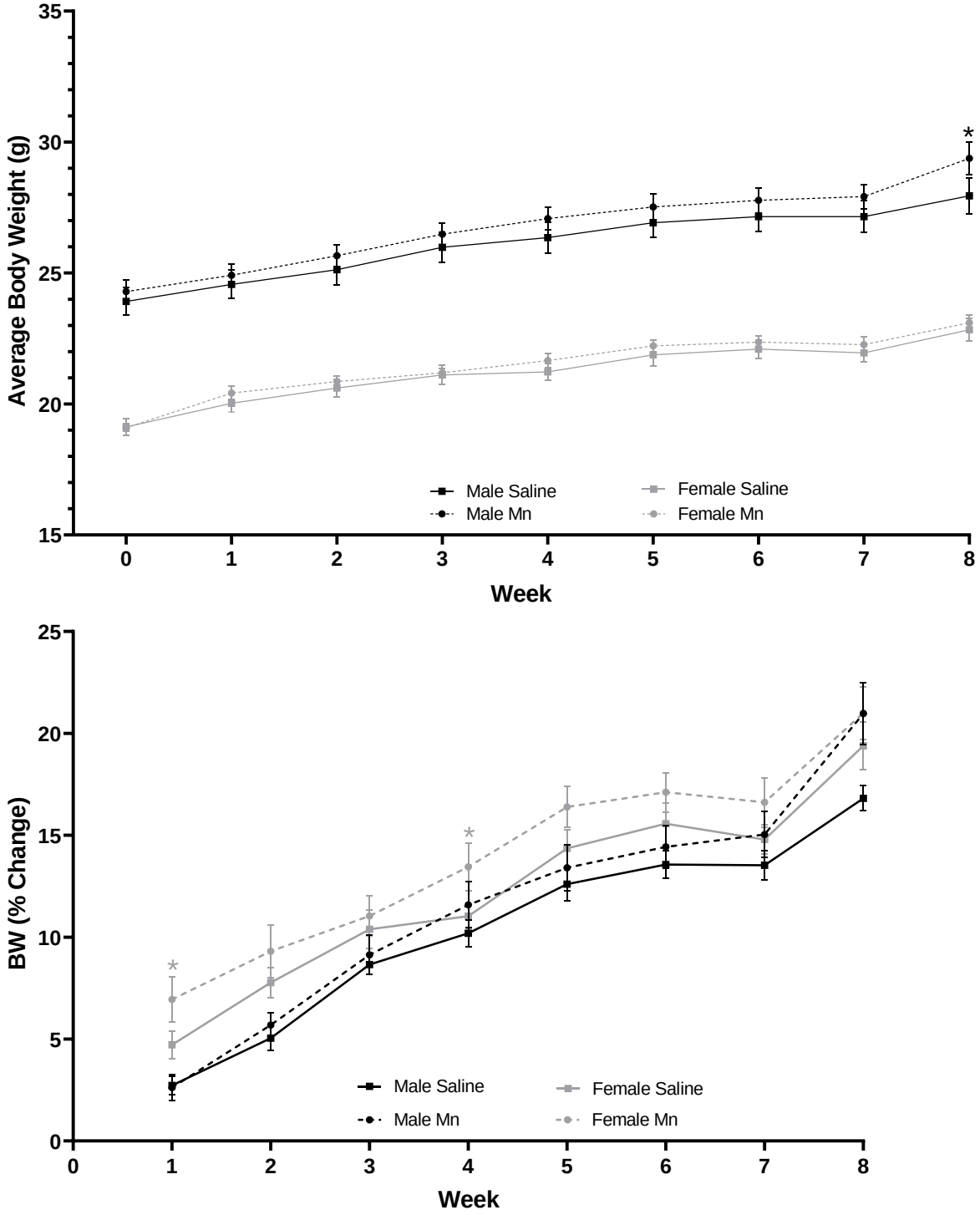

Figure 2.1. Effect of Mn DW (0.4 g/L) exposure on body weight (BW) and on percent change in BW. (A) Average body weight; (B) percent change in BW. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; $n=12$/group.

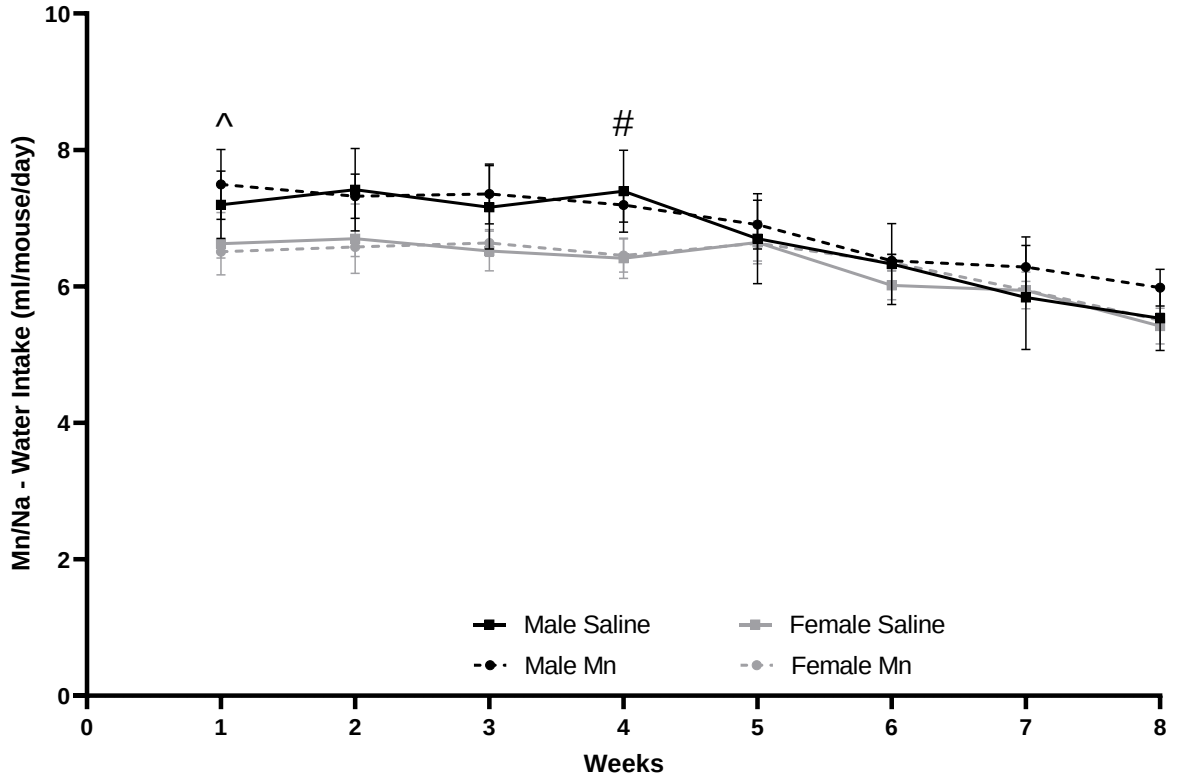

Figure 2.2. Effect of Mn DW (0.4 g/L) on water intake (mL/mouse/day) during the 8 weeks of treatment duration. Water intake is presented as mean ± SEM. # Indicates a significant sex difference $p<0.05$, and ^ indicates a trending sex difference $0.05<p<0.10$; $n=12$/group.

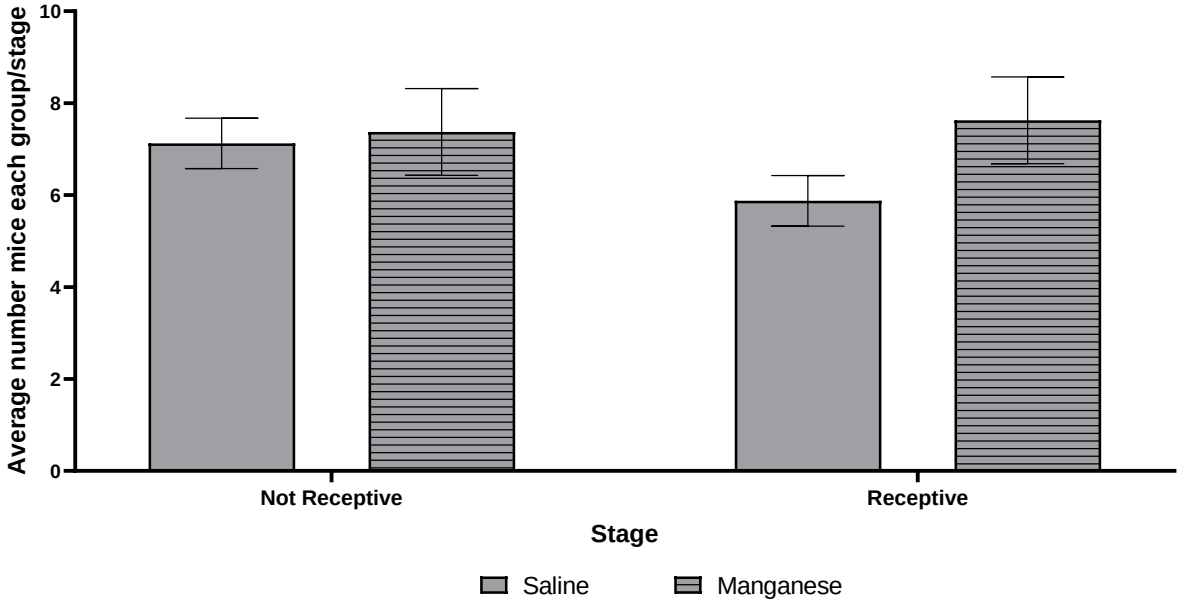

Figure 2.3. Average number of female mice in receptive (estrus and metestrus) or not receptive (diestrus and proestrus) stage of the estrous cycle.

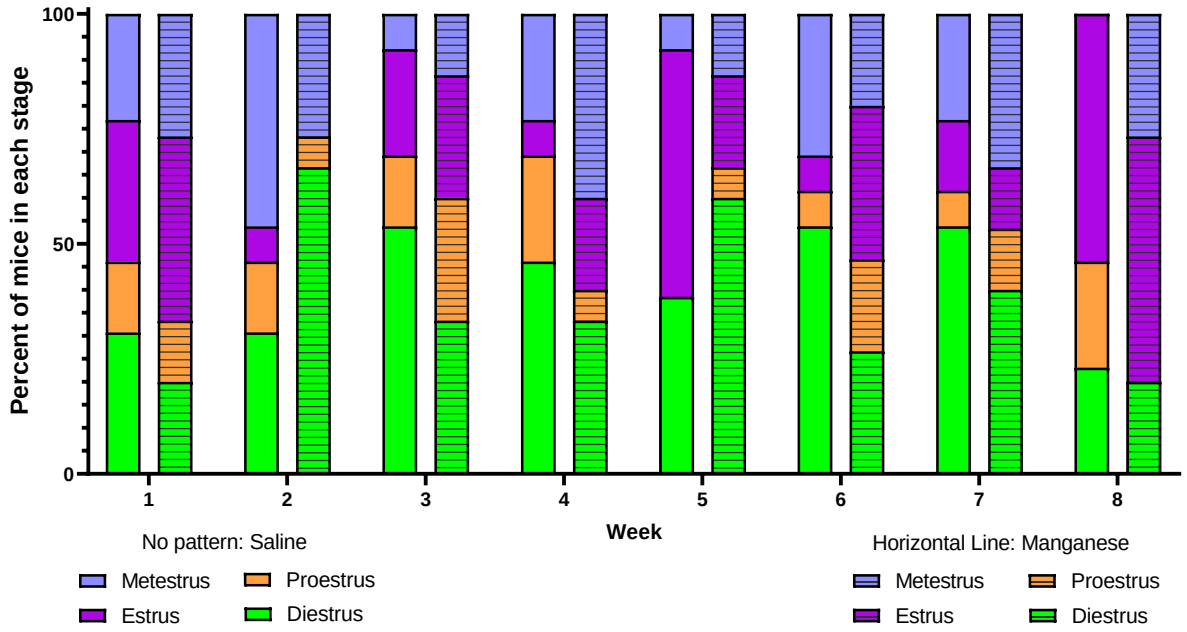

Figure 2.4. Percent of female mice in each stage of the estrous cycle throughout the 8-week period (*n* = 12/group).

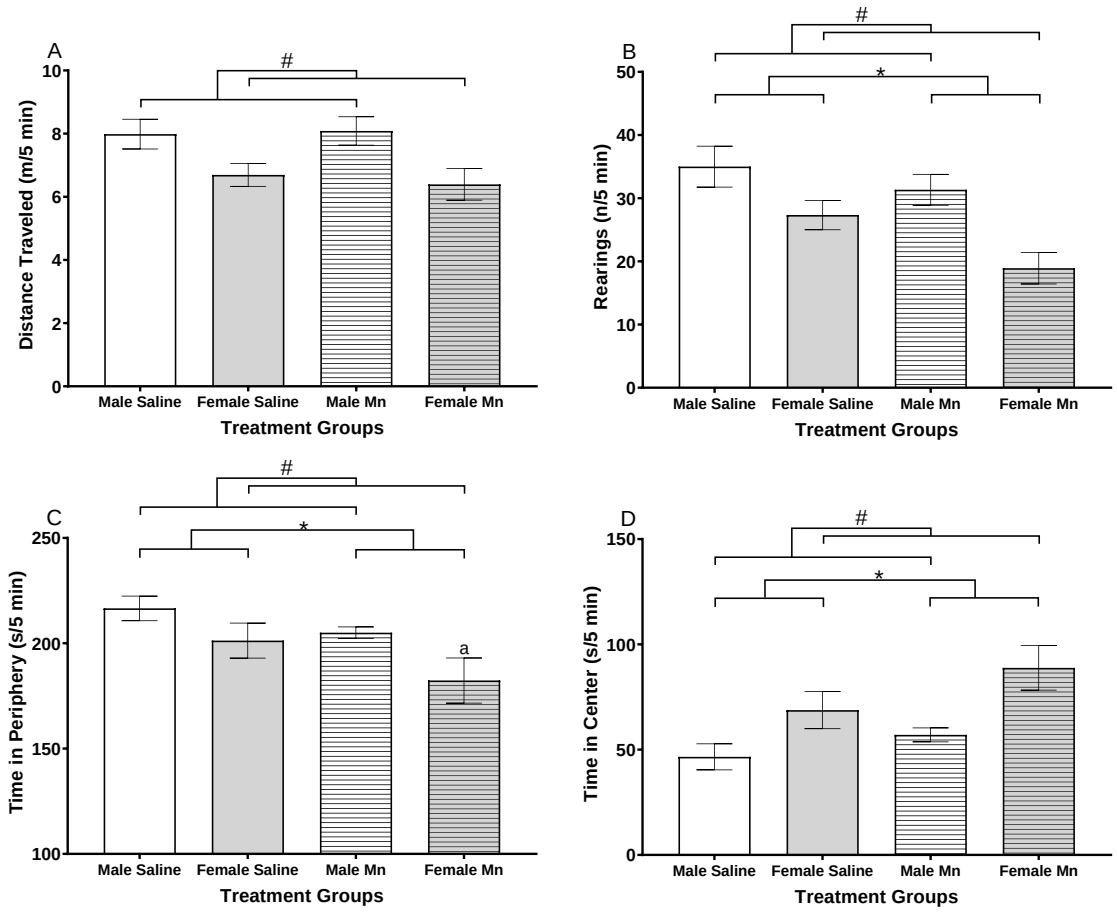

Figure 2.5. Open field test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or sex differences on distance traveled (A), rearing activity (B), time spent in the periphery (C), or time spent in the center (D) of the open field arena (first 5 min). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$. [a]Indicates $p<0.05$ between saline and Mn within sex. $n=12$/group.

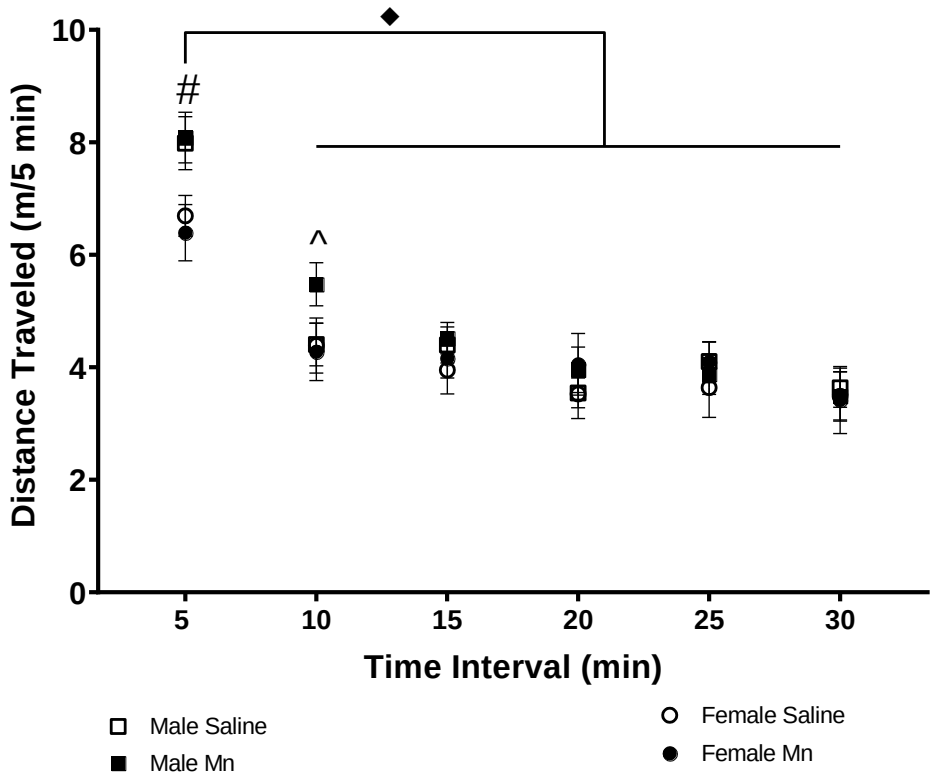

Figure 2.6. Open field test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure on total distance traveled per 5-min interval in the open field arena. Data are presented as mean ± SEM. ♦Indicates a significant effect of time; that is, habituation $p<0.05$; # indicates a significant effect of sex $p<0.05$; ^ indicates a trending Mn effect $0.05<p<0.10$; $n=12$/group.

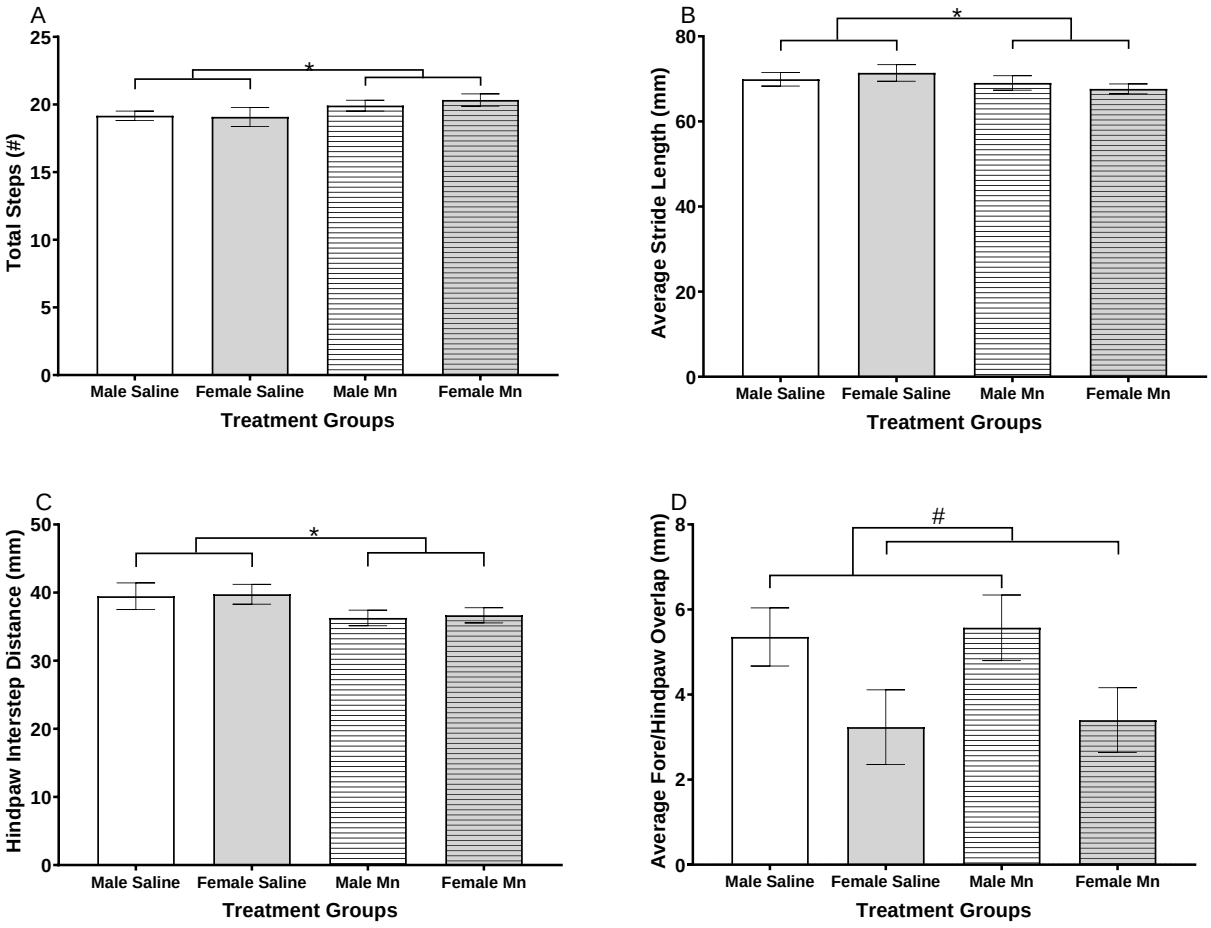

Figure 2.7. Gait test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or sex differences on the total number of steps taken (A), average stride length (B), hind paw interstep distance (C), and average fore/hind paw overlap distance (D). Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; $n=12$/group.

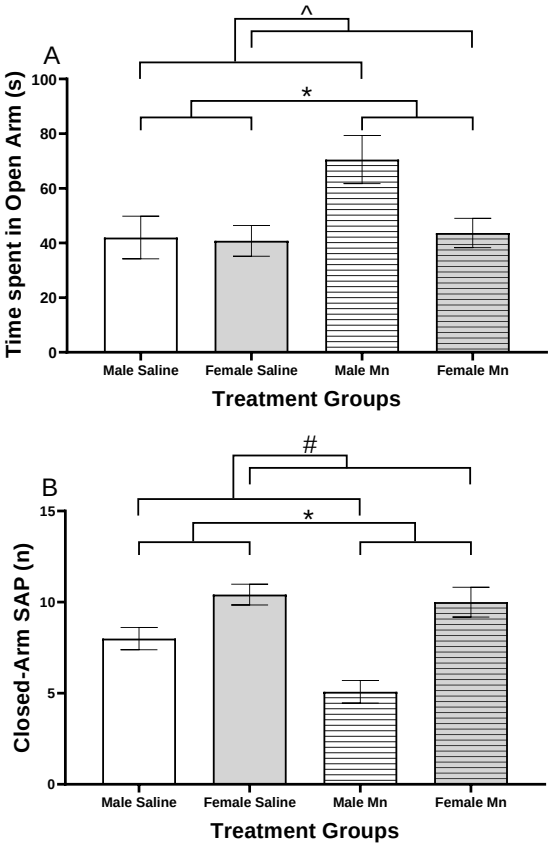

Figure 2.8. Elevated zero maze. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or sex differences on time spent in the open arm(s) (A) and number of closed-arm stretch attend posture (SAP) attempts (B). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; ^ indicates a trending effect of sex $0.05<p<0.10$; $n=12$/group.

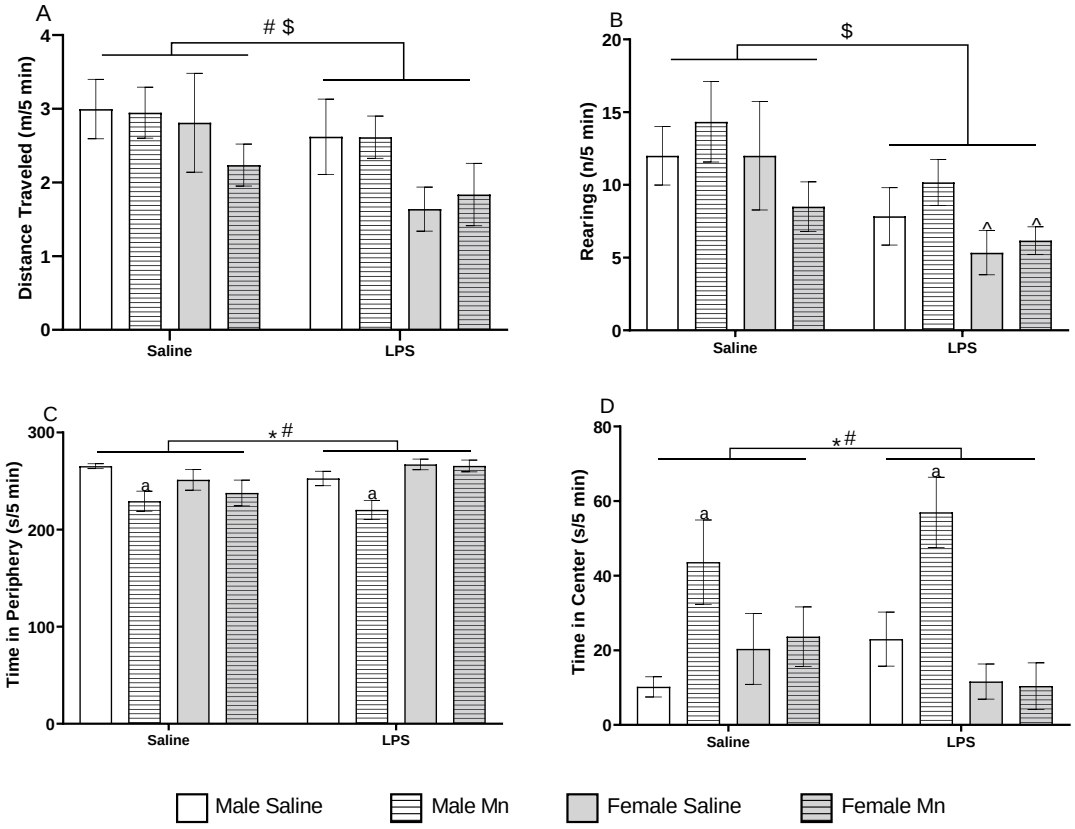

Figure 2.9. Post-LPS open field test. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or sickness behavior, four hours after a single dose of either saline or LPS (0.3 mg/kg BW), on distance traveled (first 5 min) (A), rearing activity (B), and times spent in the periphery (C) or in the center (D) of the open field arena. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; $ indicates a significant effect of LPS, $p<0.05$; ^ a trend within female mice $0.05<p<0.10$. $^{a}p<0.05$ for Mn effect within males challenged with saline or LPS, respectively; $n=6$/group.

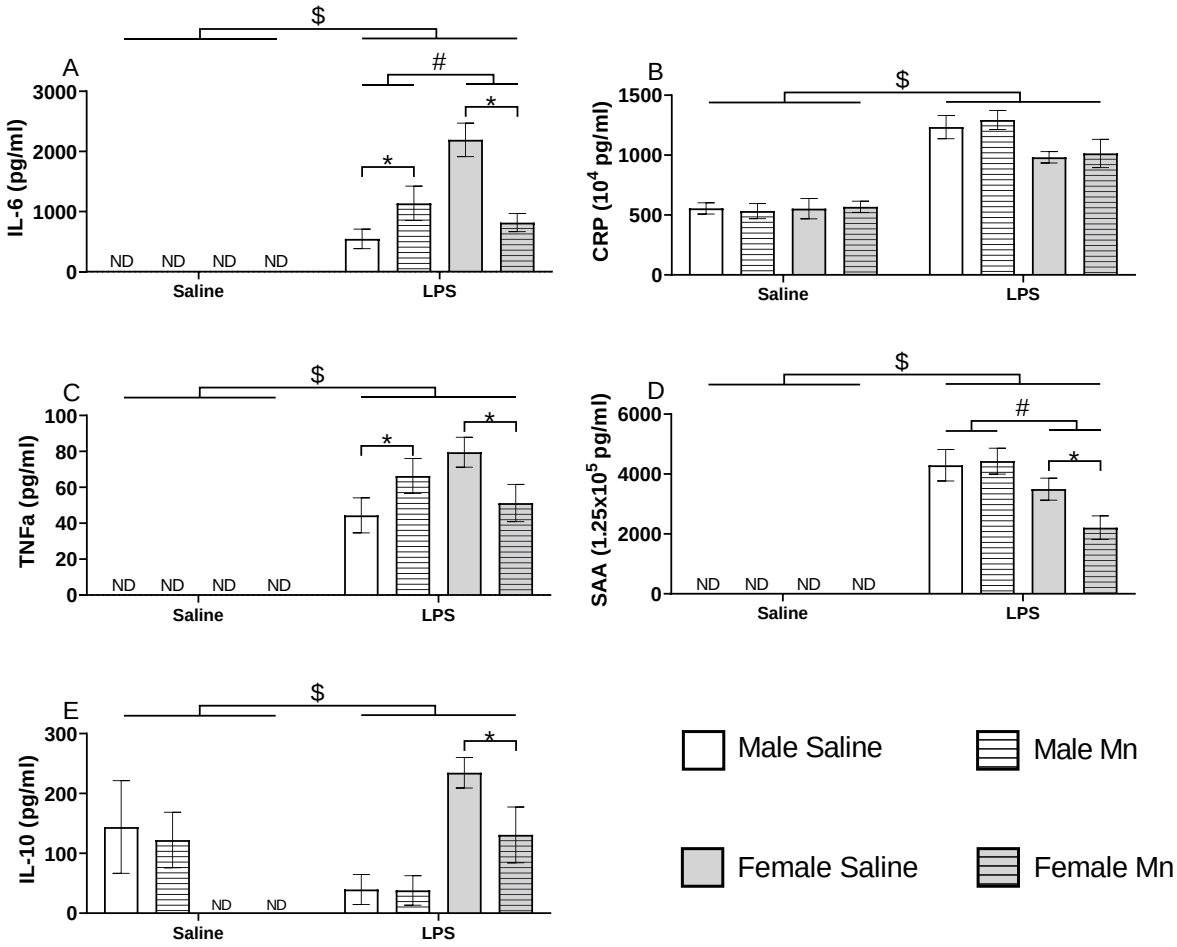

Figure 2.10. Plasma cytokine and acute phase protein (APP) levels. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg BW) on levels of plasma cytokines and APPs 6 h after LPS administration, (A) IL-6, (B) CRP, (C) TNFα, (D) SAA, and (E) IL-10. Data are presented as mean ± SEM. $^{\#}$ Indicates a significant effect of sex $p<0.05$; * indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS $p<0.05$; not detected (ND); $n=6$/group.

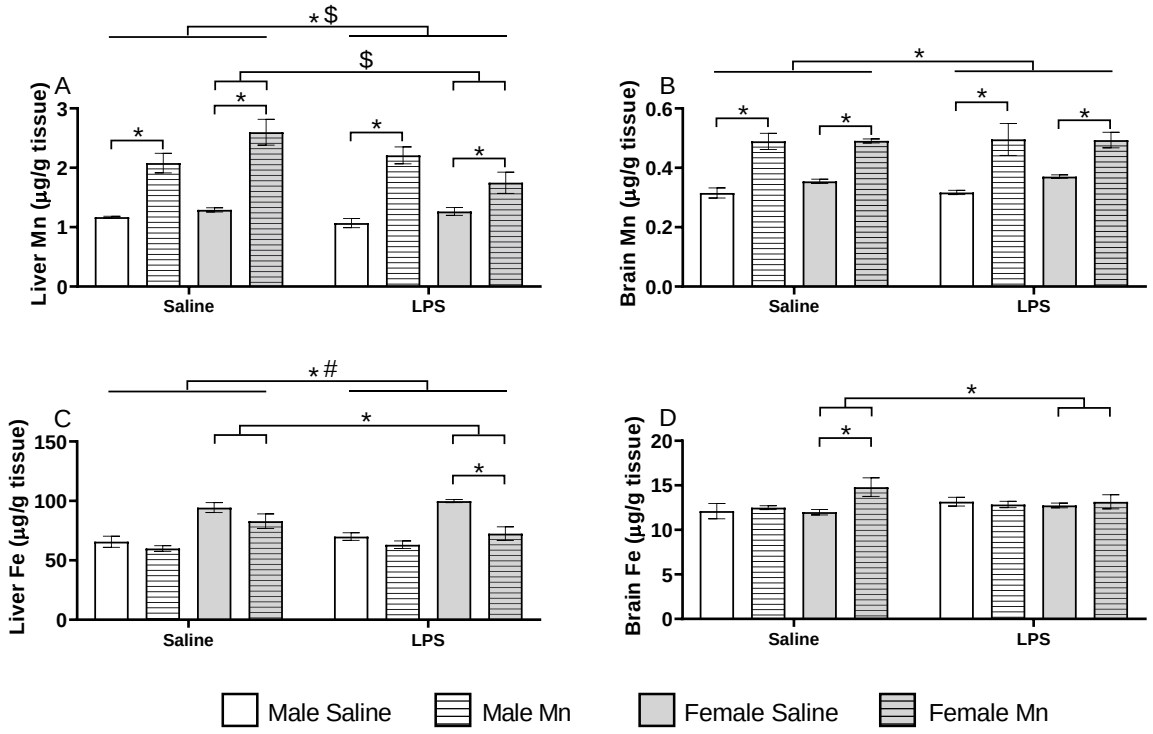

Figure 2.11. Liver and brain Mn and Fe levels. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg BW) on Mn and Fe levels in brain and liver tissues, 6 h after LPS administration, (A) liver Mn levels, (B) brain Mn levels, (C) liver Fe levels, and (D) brain Fe levels. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; $indicates a significant effect of LPS $p<0.05$; $n=6$/group.

Table 2.1. Relative tissue weight (mg/kg BW) for each treatment group after 8 weeks of Mn/saline DW exposure and 6 hours post LPS challenge. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge			
Sex	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Female	Brain	21.6 ± 0.40	22.3 ± 0.72	21.0 ± 0.69	20.8 ± 0.64
	Kidney	12.6 ± 0.12	13.5 ± 0.35	12.5 ± 0.20	13.3 ± 0.25
	Liver	46.8 ± 0.94	46.5 ± 2.43	47.1 ± 1.28	**51.4 ± 0.86**
	Spleen	3.4 ± 0.21	**4.5 ± 0.21**	3.1 ± 0.23	**4.9 ± 0.34**
	Thymus	2.2 ± 0.09	2.2 ± 0.12	1.9 ± 0.24	1.9 ± 0.54
	BAT	3.9 ± 0.26	3.9 ± 0.17	3.8 ± 0.31	4.7 ± 0.60
	SQ	3.2 ± 0.21	3.7 ± 0.60	3.5 ± 0.71	3.1 ± 0.49
	OT	9.3 ± 1.30	9.9 ± 0.98	8.0 ± 1.49	8.3 ± 0.71
	RT	2.5 ± 0.41	3.2 ± 0.40	2.0 ± 0.35	2.9 ± 0.48
Male	Brain	18.0 ± 0.33	17.5 ± 0.52	16.7 ± 0.47	17.0 ± 0.41
	Kidney	13.6 ± 0.32	13.6 ± 0.44	13.0 ± 0.52	13.3 ± 0.31
	Liver	41.3 ± 2.37	**45.5 ± 0.72**	**44.0 ± 1.15**	**48.3 ± 0.86**
	Spleen	2.6 ± 0.11	2.6 ± 0.54	2.4 ± 0.18	3.1 ± 0.26
	Thymus	1.1 ± 0.15	1.1 ± 0.16	1.2 ± 0.07	1.1 ± 0.05
	BAT	3.5 ± 0.27	2.9 ± 0.13	2.3 ± 0.22	3.5 ± 0.27
	SQ	2.5 ± 0.29	2.5 ± 0.29	3.2 ± 0.23	2.6 ± 0.39
	ET	10 ± 0.55	10.5 ± 1.22	13.1 ± 2.23	13.1 ± 2.13
	RT	3.3 ± 0.48	3.3 ± 0.36	4.9 ± 1.11	5.3 ± 1.29

Tissue abbreviations: BAT, brown adipose tissue; SQ, subcutaneous adipose tissue; ET, epididymal adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue.

Supporting Information

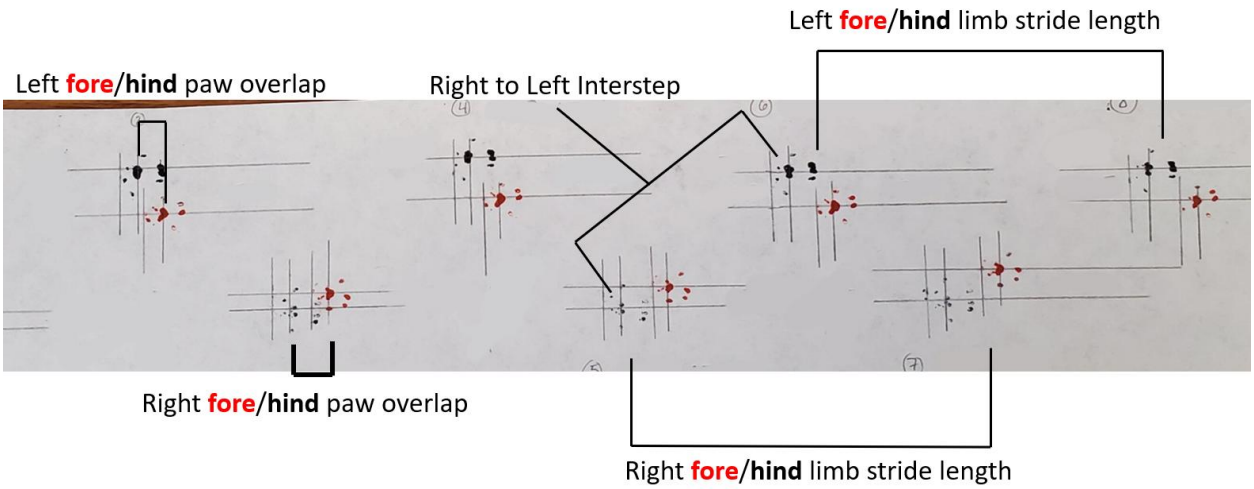

Figure S2.1. Gait Test paw placement designations on runway. Fore and hind paws were painted with a non-toxic red and black ink, respectively. Parameter definitions: fore/hindpaw overlap is the distance between a step's fore and hind paw; hind paw interstep distance (aka step length) is the distance between consecutive right to left steps; stride length is the distance between two consecutive pawprints of the same paw.

Table S2.1. Absolute tissue weight (g) for each treatment group after 8 weeks of Mn/saline DW treatment and 6 hours post LPS challenge. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge			
Sex	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Female	Brain	0.477 ± 0.0034	0.484 ± 0.0039	0.474 ± 0.0121	0.458 ± 0.0111
	Kidney	0.278 ± 0.0050	0.293 ± 0.0063	0.284 ± 0.0076	0.294 ± 0.0075
	Liver	1.040 ± 0.0403	1.012 ± 0.0562	1.067 ± 0.0217	1.131 ± 0.0223
	Spleen	0.075 ± 0.0039	**0.097 ± 0.0044**	0.070 ± 0.0059	**0.107 ± 0.0078**
	Thymus	0.048 ± 0.0021	0.047 ± 0.0032	0.043 ± 0.0062	0.052 ± 0.0106
	BAT	0.087 ± 0.0064	0.085 ± 0.0044	0.086 ± 0.0063	0.104 ± 0.0132
	SQ	0.072 ± 0.0058	0.082 ± 0.0147	0.079 ± 0.0157	0.070 ± 0.0110
	OT	0.209 ± 0.0329	0.218 ± 0.0240	0.184 ± 0.0373	0.184 ± 0.0172
	RT	0.057 ± 0.0098	0.072 ± 0.0115	0.045 ± 0.0086	0.064 ± 0.0111
Male	Brain	0.474 ± 0.0056	0.472 ± 0.0059	0.474 ± 0.0060	0.479 ± 0.0052
	Kidney	0.357 ± 0.0070	0.376 ± 0.0159	0.372 ± 0.0208	0.376 ± 0.0159
	Liver	1.086 ± 0.0609	**1.238 ± 0.0639**	**1.251 ± 0.0500**	**1.366 ± 0.0426**
	Spleen	0.069 ± 0.0026	0.086 ± 0.0071	0.067 ± 0.0052	0.086 ± 0.0071
	Thymus	0.029 ± 0.0041	0.028 ± 0.0043	0.034 ± 0.0017	0.033 ± 0.0018
	BAT	0.092 ± 0.0069	0.080 ± 0.0053	0.092 ± 0.0073	0.100 ± 0.0109
	SQ	0.067 ± 0.0077	0.068 ± 0.0074	0.067 ± 0.0083	0.075 ± 0.0139
	ET	0.264 ± 0.0152	0.284 ± 0.0302	0.380 ± 0.0733	0.377 ± 0.0755
	RT	0.088 ± 0.0124	0.088 ± 0.0101	0.142 ± 0.0348	0.153 ± 0.0439

Tissue abbreviations: BAT, brown adipose tissue; SQ, subcutaneous adipose tissue; ET, epididymal adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue.

CHAPTER 3

INFLUENCE OF MANGANESE EXPOSURE VIA DRINKING WATER AND AN LPS CHALLENGE ON NEUROTRANSMITTER DYNAMICS AND GLIAL ACTIVATION IN ADULT MALE AND FEMALE MICE

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Abstract

While manganese (Mn) is an essential metal, overexposure to it is neurotoxic and leads to adverse neurological consequences. Consumption of Mn-contaminated drinking water (DW) is the primary source of non-occupational Mn-exposures. Behavioral alterations, i.e., reduced locomotor activity and decreased fearless/anxiety-like behavior and sex differences in immune responses after DW Mn-exposure, as we reported previously, suggest potential disruption in brain neurochemistry with possible neuroinflammatory underpinnings, underscoring the need of further investigation into major neurotransmitter systems and neuroinflammation status in the context of DW exposure to Mn. When male and female mice, with GFP tagged microglia, were exposed to Mn DW for 8-weeks and then challenged with lipopolysaccharide (LPS), striatal levels of GABA and GLU, ventral hippocampal (vHIP) levels of GLN and GABA, and prefrontal cortex (PFC) levels of 5-HT, NE, 5-HIAA, GLN, and GABA were all influenced by Mn differently (decreases or increases in neurotransmitter/metabolite levels), depending on region, sex, and neurotransmitter type. Across various hippocampal areas, females had more microglia and astrocytes; Mn exposure increased dHIP microglia activation while LPS increased vHIP microglia activation (manifested by increased GFP intensity), more so in the females. Mn exposure also led to a higher number of dHIP microglia present. Mn-exposed males had less TH+ neurons in the substantia nigra, less microglia in the VTA, and higher microglia activation in the ventromedial striatum, while Mn did not affect TH+ neurons in females. Overall, these data suggest that the previously reported behavioral deficits, are due to increased striatal and hippocampal inhibition (GABA), increased PFC 5-HT in males, and increased PFC GLN in females, and that the hallmark striatal DAergic dysfunction is not yet present.

Keywords: Manganese | Neurotransmitter Homeostasis | Neuroinflammation | Sex Differences | Microglia & Astrocyte Activation | Tyrosine Hydroxylase

1 Introduction

Manganese (Mn) is a vital trace metal that plays a crucial role in various physiological processes, including regulation of blood glucose, cellular energy, and macronutrient metabolism and homeostasis (Chen et al., 2018; Erikson et al., 2007; IOM, 2001). Mn is also a biological cofactor in several enzymatic processes (Judy L. Aschner & Michael Aschner, 2005). Despite its essential role, excessive exposure to Mn leads to neurotoxicity. The impact of Mn on the brain health is influenced by exposure level, route, and duration of exposure (Chen et al., 2018; Lucchini & Tieu, 2023; Lucchini et al., 2009). Excess Mn exposure to the public through Mn contaminated drinking water has become a concern (Gonzalez-Cuyar et al., 2014; Laohaudomchok et al., 2011; Utembe et al., 2015). Studies have previously linked Mn exposure via drinking water, but not diet, to intellectual impairment in children, with effects including memory deficits and lower intelligence scores (Bouchard et al., 2011; Brna et al., 2011; Hafeman et al., 2007; Wasserman et al., 2006). In the brain, Mn predominantly affects the basal ganglia, leading to symptoms resembling parkinsonism (Racette et al., 2017) characterized by symptoms such as speech difficulties, postural instability, tremors at rest, rigidity, gait abnormalities (Bowler et al., 2007; Park et al., 2006; Sahni et al., 2007). Furthermore, overexposure to Mn can lead to mood disturbances, including irritability, depression, compulsive behaviors, and anxiety in both humans and laboratory animals (Bouchard et al., 2007; Bowler et al., 2011; Dodd et al., 2005; Krishna et al., 2014; Laohaudomchok et al., 2011; Liu et al., 2019; Ludwig et al., 2024).

These Mn-induced locomotor and mood impairments have been previously shown to be linked with alterations in several neurotransmitter systems (Fordahl & Erikson, 2014; Gwiazda et al., 2002; Lin et al., 2020; Soares et al., 2020; Tamar et al., 2021), with disruption of dopamine (DA), especially in the basal ganglia, affecting motor function. Excess Mn can impact both dopaminergic and serotonergic systems in the basal ganglia, particularly in the striatum. Changes in DA and serotonin (5-HT) levels in this region have been implicated in the

development of motor and behavioral impairments (Balachandran et al., 2020). Previous studies have demonstrated that Mn can inhibit tyrosine hydroxylation (TH: the rate limiting step in DA biosynthesis), inhibit dihydroxyphenylalanine (DOPA) formation, and decrease 5-HT levels while increasing 5-HT utilization (Liu & Ju, 2023; Pittman, 2005). Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter and can be influenced by Mn. Mn effect on the GABAergic system has had contrasting reports including elevated GABA in the thalamus and adjacent basal ganglia (Dydak et al., 2011), decreased uptake by astrocytes (Fordahl & Erikson, 2014), or even increased extracellular concentrations of GABA (Anderson et al., 2008) by Mn exposure. Glutamate is the brain's most prevalent excitatory neurotransmitter and is highly sensitive to changes in energy supply. The imbalance of GABAergic and glutamatergic neurotransmission is implicated in cognitive deficits, anxiety, and mood disturbances in regions including the hippocampus and prefrontal cortex. Mn-induced mitochondrial dysfunction and the subsequent reactive oxygen species generation could directly inhibit the uptake of GLU and increase its concentration in the extracellular environment (Gunter et al., 2012; Pajarillo et al., 2021; Soares et al., 2020).

We previously reported that exposure to Mn via the drinking water increased Mn levels in both the brain and liver, and it induced behavior changes, in both sexes, but not in the same manner (Ludwig et al., 2024). Our behavioral and peripheral cytokine analysis suggested that males were more affected by Mn exposure in terms of mood and immune response compared to females. Specifically, Mn exposed males showed heightened fearlessness while females did not exhibit these effects to the same extent and males had potentiated peripheral inflammatory cytokine production after an immunogen challenge whereas the reverse was seen in females. LPS administration increases proinflammatory cytokines in the periphery as well as in the brain (Perry, 2004) and alters brain neurotransmitter systems, including 5-HT and DA, which are integral to mood regulation and immune responses (Dodd & Filipov, 2011; Krishna et al., 2016; Rezaei et al., 2024). Previous low level subchronic Mn DW exposure study, by Krishna et al.

(2014), suggested that a subtle serotonergic imbalance is the driving influence of Mn-induced behavior impairments, with increased astrocytic activation in the substantia nigra pars reticulata and increased reactive oxygen species implying an increase of neuroinflammation in Mn-exposed male mice – leaving Mn exposure neurological impacts on females still understudied. Thus, the present study sought to (i) determine the effects of Mn overexposure on regional brain monoamine and amino acid neurotransmitters, (ii) determine if the Mn effects on neurotransmitters are sex-specific, (iii) assess how Mn-altered neurotransmitters impacted observed behavioral alterations, and (iv) determine the effects of Mn overexposure, with and without an inflammagen, on neuroinflammation.

2 Materials and Methods

2.1 Reagents

All chemicals, unless otherwise stated, including manganese ($MnCl_2 \cdot 4H_2O$) and LPS (*Escherichia coli* serotype 0111: B4), were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Animals

All procedures that involved handling animals were approved in advance by the University of Georgia Institutional Animal Care and Use Committee (IACUC) and were in compliance with the latest National Institutes of Health and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Homozygous male and female CX_3CR1^{GFP} mice, on a C57BL/6 background (Jackson Labs, stock 005582), were used for all experiments. Throughout the study mice were housed in a AAALAC accredited facility, same-sex mice were group-housed (2-5 per cage) in an environmentally controlled room (22-24°C) with a relative humidity of 50-70% and maintained on a 12 h light/dark cycle. PicoLab Rodent Diet 20 (LabDiet, 5053) was available *ad libitum.* Mice were acclimated to using water bottles for three weeks prior to the start of the study, were randomly assigned to drinking water (DW) treatment groups, and were 2 months old at the onset of treatment.

2.3 Animal Treatment & Tissue Collection

Twelve males and 13 females mice were exposed to vehicle control (NaCl; 0.4 g Na/L) and 13 males and 15 females were exposed to $MnCl_2$ (0.4 g Mn/L) in deionized water for eight weeks. The water bottles were replaced weekly with freshly prepared solutions of the respective treatments. Weekly measurements included body weight, water intake, and estrus cycle stages (for females only). After six weeks of treatment, behavioral tests were conducted (with 12 mice per group) while continuing the respective treatment regimens. At the end of the eight-week exposure, a random subset of mice that underwent behavior testing from each sex and treatment group received an intraperitoneal injection of either saline vehicle or lipopolysaccharide (LPS; *Escherichia coli* serotype 0111: B4 $1.5x10^{12}$ EU or 0.3 mg/kg BW). Four hours after the injection, sickness behavior was evaluated in an open field test. Outcomes of these behavior tests are reported in (Ludwig et al., 2024). The mice were sacrificed six hours post injections (two hours after the open field test). The brains were extracted, weighed, and rinsed in ice cold HEPES-buffered Hank's saline solution (pH 7.4). Then the brains were divided longitudinally into two hemispheres; one half was fresh frozen on dry ice while the other half was immersion fixed in 4% paraformaldehyde and transferred to 30% sucrose before flash freezing in 2-methylbutane. Organs, adipose tissue, and plasma were additionally collected; plasma was used for cytokine analysis. Both brain halves were stored at -80°C until further processing. Behavioral data, peripheral cytokine analysis, and brain Mn data were published recently (Ludwig et al., 2024).

2.4 Neurochemical Analysis

Fresh frozen half-brains were sliced at 500 µm thickness and were individually placed on glass microscope slides and were frozen on dry ice. Micropunches (1.5 mm diameter) of the prefrontal cortex, ventral hippocampus, and the striatum were acquired from the frozen brain slices that contained these regions. Frozen punches were then homogenized via sonication in 100 µL of 0.2N perchloric acid, then centrifuged (13,200 x G at 4°C for 10 minutes). An aliquot

of the supernatant (20 µL) was injected into an HPLC (Waters Arc HPLC System™) with a Waters™ 3465 electrochemical detector (ECD) for detection of: 1) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT); 2) serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); 3) norepinephrine and its metabolite3-methoxy-4-hydroxyphenylglycol (MHPG); 4) gamma-aminobutyric acid (GABA); 5) glutamate (GLU); and 6) glutamine (GLN). The analytes were separated on a C18 column, with a 3.5 µm diameter, using a flow rate of 1 mL/min. The monoamine mobile phase was made as in Coban and Filipov (2007). The amino acid mobile phase, with final pH of 4.5 (adjusted with 1 M phosphoric acid (Fisher Scientific, Hampton, NH) consisted of EDTA (Fisher Scientific, Hampton, NH), monosodium phosphate (Fisher Scientific, Hampton, NH), and 25% HPLC-grade methanol (Millipore, Burlington, MA) as an organic modifier. For amino acid analysis, samples were derivatized prior to HPLC analyses as in Pencheva et al. (2023) with minor modifications. Standard solutions of GABA, GLU, and GLN were prepared with deionized water. For derivatization, 4 µL of the sample (either standard or prepared brain sample) and 996 µL of dd water were mixed with 10 µL of derivatizing agent and were allowed to sit at room temperature for 10 minutes before HPLC analysis. The derivatizing agent was prepared by mixing o-Phthalaldehyde, HPLC-grade absolute ethanol, 1 M sodium sulfite, and 0.1 M tetraborate buffer (Acros Organics [now Thermo Fisher Scientific], Waltham, MA). Prior to statistical analysis, brain neurotransmitter and metabolite data were normalized on a per mg protein basis using the Bradford method as in (Krishna et al., 2014).

2.5 Immunohistochemistry

Fixed half-brains were coronally sectioned (40 µm thick) as in Carpenter et al. (2022) on a cryostat (Leica CM1950, Leica Biosystems, Deer Park, IL), collected, and stored in 0.02 M phosphate buffer in 48-well plates at 4°C until staining. Free-floating sections containing the dorsal hippocampus (dHIP) and ventral hippocampus (vHIP), which also contained the substantia nigra pars compacta and reticulata, were stained for glial fibrillary acidic protein

(GFAP) a marker for astrocytes. Additionally, the sections containing the substantia nigra pars compacta and reticulata, and the striatum (STR) were stained for tyrosine hydroxylase (TH). Sections were incubated, as appropriate, with the following primary antibodies diluted in 0.1% TritonX-100 in PBS for 48 h at 4°C: 1) 1:3000 chicken anti-GFAP (Aves Labs, Davis, CA); or 2) 1:2000 rabbit anti-TH (Millipore, Burlington, MA). Following primary antibody incubation, the sections were washed and incubated with the appropriate secondary red fluorescent antibody (GFAP: 1:1000 goat anti-chicken 594, Abcam, Cambridge, UK; TH: 1:1000 goat anti-rabbit 594, Abcam) for 2 h in the dark at room temperature followed by a 5-minute incubation with a nuclear stain (Hoechst 33258, Invitrogen, Waltham, MA). On all sections, GFP (CX_3CR1) for microglia were identified in the green fluorescent channel. After the final wash, the sections were mounted to glass microscope slides and coverslipped with VectaMount (Vector Labs, Newark, GA).

Images were taken using a BZ-X800 Fluorescence Microscope (Keyence, Itasca, IL). All regions of interest were captured at 20x magnification including the striatum, substantia nigra, and the hilus region, CA1, CA3, and molecular layer of the dentate gyrus of both dorsal and ventral hippocampus. Signal intensity (GFP, GFAP, and TH) and relative cell count (microglia, astrocytes and TH+ neurons) were analyzed using the ImageJ software and the Cell counter plugin as in (Carpenter et al., 2021).

2.6 Statistical analysis

All data were analyzed using SigmaPlot v12.5 (Systat Software, Inc., Chicago, IL) and all graphs were generated using GraphPad Prism v8.4.3 (San Diego, CA). A three-way analysis of variance (ANOVA) or two-way ANOVA (within sex), were used to determine main effects of Mn, LPS, sex, treatment and/or sex interactions. For all ANOVAs, the F-value is displayed as $F(df, df)$ with the degrees of freedom designated as (number of groups-1, total mice-number of groups). A p-value of ≤0.05 was considered significant. If an ANOVA was significant for a main effect or interaction, then a Student-Newman-Keuls (SNK) *post-hoc* comparison was performed, with statistical designation as (p-value, q-value).

3 Results

3.1 Neurochemical Analysis

3.1.1 Striatum

Three-way ANOVA revealed main effects of sex and LPS, but not of 8-week Mn exposure, on striatal monoamine neurotransmitters (Figure 3.1). While sex did not affect DA itself (Figure 3.1A and B), males had greater levels of both DA's metabolites DOPAC and HVA than females (Figure 3.1A and B; $F(7,40)=5.348$, $p=0.026$ and; $F(7,40)=13.695$, $p<0.001$ respectively). Consequently, the DOPAC/DA, HVA/DA, and DOPAC+HVA/DA ratios in the males were higher than females: $F(7,40)=3.129$, $p=0.085$; $F(7,40)=17.054$, $p<0.001$; and $F(7,40)=5.957$, $p=0.019$; respectively. Females, on the other hand, had more striatal 5-HT than males (Figure 3.1A and B; $F(7,40)=9.021$, $p=0.005$). Post LPS, HVA, 5-HIAA, and 5-HT (Figure 3.1A and B; $F(7,40)=14.824$, $p<0.001$; $F(7,40)=64.5$, $p<0.001$; $F(7,40)=10.307$, $p=0.003$, respectively) all increased, with the effect of LPS on 5-HT being higher in the females ($F(7,40)=7.274$, $p=0.014$). Interestingly, in females, but not males, LPS lowered striatal DA levels (Figure 3.1A and B; $F(7,40)=5.204$, $p=0.034$). This LPS effect was driven by the Mn+LPS females which had lower DA levels than saline or Mn+saline females ($p=0.025$, $q=3.435$).

Mn did have effects on striatal amino acid neurotransmitters. Three-way ANOVA revealed main effects of sex and Mn on GABA levels; females had higher striatal GABA than males (Figure 3.1A and B; $F(7,40)=6.821$, $p=0.013$) and GABA levels in the striatum of Mn-exposed mice were higher than controls (Figure 1A and B; $F(7,40)=14.999$, $p<0.001$). The latter effect was driven by the Mn-exposed males ($F(3,20)=17.665$, $p<0.001$). There was an overall main effect of Mn on the GLU/GABA ratio, which was higher in the Mn-exposed mice (Figure 3.1A and B; $F(7,40)=24.299$, $p<0.001$). In the males, striatal GLU/GABA ratio was greater in the Mn-exposed mice ($F(3,20)=29.327$, $p<0.001$). Females that were exposed to Mn, (Figure 3.1A and

B; $F(3,20)=6.921$, $p=0.016$), or females that were challenged with LPS had lower levels of GLU than controls (Figure 3.1A and B; $F(3,20)=4.921$, $p=0.038$).

3.1.2 Ventral Hippocampus

In terms of monoamine effects, a three-way ANOVA revealed that females, regardless of DW treatment or LPS challenge, had higher vHIP levels of NE, 5-HT, MHPG, 5-HI!!, and DOPAC (Figure 3.1C and D; $F(7,40)=5.245$, $p=0.027$; $F(7,40)=16.098$, $p<0.001$; $F(7,40)=26.993$, $p<0.001$; $F(7,40)=17.824$, $p<0.001$; $F(7,40)=17.849$, $p<0.001$ respectively) than males. Mice challenged with LPS had higher levels of 5-HIAA than controls (Figure 3.1C and D; $F(7,40)=13.481$, $p<0.001$), and this effect was driven by the LPS-challenged females ($F(3,20)=9.782$, $p=0.005$). There were also overall main effects of Mn, sex, and LPS on vHIP amino acid neurotransmitters. Mn exposed mice had a higher GLU/GABA ratio than saline controls (Figure 3.1C and D; $F(7,40)=5.692$, $p=0.022$), and this Mn effect was more pronounced in males ($F(3,20)=10.753$, $p=0.004$). Male mice had higher levels of GLN and GLU than females (Figure 3.1C and D; $F(7,40)=12.933$, $p<0.001$; $F(7,40)=9.897$, $p=0.003$ respectively), as well as a higher GLU/GABA ratio (Figure 3.1C and D; $F(7,40)=4.258$, $p=0.046$). LPS challenge lowered GLN levels overall (Figure 3.1C and D; $F(7,40)=12.805$, $p<0.001$), but this effect was male-driven ($F(3,20)=8.763$, $p=0.008$). Additionally, LPS challenged mice, regardless of sex or DW treatment, had lower levels of GLU and GABA than controls (Figure 3.1C and D; $F(7,40)=5.083$, $p=0.030$ and $F(7,40)=9.201$, $p=0.004$; respectively).

3.1.3 Prefrontal Cortex

In the prefrontal cortex (PHC), Mn, LPS, and/or sex affected multiple neurotransmitters and/or metabolites. Mn-exposed mice had higher levels of 5-HT in the PFC overall (Figure 3.1E and F; $F(7,40)=28.650$, $p<0.001$) and this effect was more pronounced in the males ($F(7, 40)=5.440$, $p=0.030$). Mn exposed females, but not males, had higher 5-HIAA levels than controls (Figure 3.1E and F; $F(3,20)=8.068$, $p=0.010$). Males had higher levels of NE than females (Figure 3.1E and F; $F(7,40)=21.533$, $p<0.001$), due in part to the LPS induced, female-

specific decrease of NE (F(3,20)=7.190, p=0.014) and the having lower NE in female control mice (F(3,20)=8.918, p=0.007). LPS challenged mice had lower DOPAC levels than controls (Figure 3.1E and F; F(7,40)=11.696, p=0.001) without affecting DA levels (data not shown). This effect was more pronounced amongst the LPS challenged males (F(3,20)=8.817, p=0.008). While Mn exposed females tended to have more DOPAC than controls, but this effect did not reach significance (F(3,20)=4.211, p=0.053). LPS challenged mice had higher PCF 5-HIAA, but not 5-HT, levels than controls (Figure 3.1E and F; F(7,40)=5.058, p=0.030). This effect was most pronounced in the LPS challenged females (F(3,20)=8.020, p=0.010). Three-way ANOVA revealed main effects of Mn, sex, and LPS on amino acid neurotransmitters within the PFC. Mn exposed mice had higher PFC levels of GLN and GABA (Figure 3.1E and F; F(7,40)=17.608, p<0.001 and F(7,40)=15.936, p<0.001, respectively) and a resultant lower GLU/GABA ratio than controls (Figure 3.1E and F; F(7,40)=27.591, p<0.001). Additionally, females had higher levels of GABA (Figure 3.1E and F; F(7,40)=48.685, p<0.001), while males had a higher GLU/GABA ratio (Figure 3.1E and F; F(7,40)=207.029, p<0.001). Finally, LPS challenged mice had lower PCF GLN levels (Figure 3.1E and F; F(3,20)=5.371, p=0.026), an effect driven mostly by the LPS challenged males (F(3,20)=6.159, p=0.023).

3.2 Effects of Mn and/or LPS on Tyrosine hydroxylase immunoreactivity

In the ventral tegmental area (VTA), ventromedial substantia nicra pars compacta (SNpc), and lateral SNpc there were no significant main treatment effects for tyrosine hydroxylase (TH) optical density in either sex. However, Mn exposure decreased the number of TH+ neurons in the ventromedial SNpc within the males (Figure 3.2B; F(3,20)= 6.377, p=0.027) Mn did not affect the number of TH+ neurons within females. Figures 3.3 and 3.4 are representative images of the substantia nigra, depicting microglia (CX_3CR1^{GFP}+ cells) and TH+ neurons, for all treatment groups in females and males respectively.

In the ventromedial striatum (VM STR) and dorsolateral (DL) STR Mn DW exposure did not affect TH optical density, however, it did increase GFP (microglia) optical density within

males significantly (Figure 3.5A; p=0.014, q=2.986). LPS challenged males, but not females, had an increase in TH optical density in both the VM STR (Figure 3.5B; p=0.020, q=3.586) and in the DL STR (Figure 3.5C; p=0.018, q=3.674). Mn exposed and saline challenged males (Mn+saline) had a higher number of microglia present in the VM STR than controls (Figure 3.6A; p=0.041, q=3.107). LPS challenged females, regardless of DW treatment, had a lower number of microglia present in the DL STR than controls (Figure 3.6B; p=0.022, q=3.526). Figures 3.7 and 3.8 are representative images of the striatum, depicting microglia (CX_3CR1^{GFP}+ cells) and TH+ immunoreactivity of axonal projections, for all treatment groups in both sexes.

3.3 Effects of Mn and/or LPS on Microglia and Astrocytes

3.3.1 Dorsal Hippocampus

In the hilar region of the dorsal hippocampus, three-way ANOVA revealed main effects of Mn, LPS, and sex on microglia signal intensity (GFP) and a main effect of LPS on astrocyte signal intensity (GFAP). Mn exposed mice had a higher microglia intensity than controls (Figure 3.9A; F(7,40)=4.809, p=0.034), with the intensity being most predominant among females exposed to Mn (F(3,20)=7.855, p=0.011). Similarly, LPS challenged mice, more specifically Mn+LPS, had higher microglia intensity than controls (F(7,40)=4.627, p=0.038). Females also had higher microglia intensity than males (Figure 3.9A; F(7,40)=7.079, p=0.011). Astrocyte signal intensity were significantly higher among mice challenged with LPS than saline controls (Figure 3.9B; F(7,40)=5.184, p=0.028), and this effect was only a trend among males and not significant within females. In the CA1, CA3, and molecular layer of the dentate gyrus, three-way ANOVA revealed a main effect of sex for both GFAP and GFP (Table S3.1).

Three-way ANOVA of microglia (CX_3CR1^{GFP}+/Hoechst+) in the hilar region revealed that overall Mn exposed mice had a higher number of microglia present than controls (Figure 3.9C; F(7,40)=5.184, p=0.028). There was a greater number of microglia present among Mn exposed females (F(3,20)=4.694, p=0.043 and more specifically among Mn+LPS females (p=0.020, q=3.582). Males challenged with LPS had a higher number of microglia than controls

(F(3,20)=4.832, p=0.040), and LPS did not significantly affect the number of microglia in the hilar region among females. When the intensity per cell was analyzed in the dorsal hippocampus, three-way ANOVA revealed a main effect of sex of microglia intensity in the hilar region, molecular layer of the dentate gyrus as well as in the CA3 (Table S3.3). Three-way ANOVA revealed a main effect of sex in intensity/astrocyte in the hilar region, CA1, CA3, and the molecular layer of the dentate gyrus (Table S3.3).

3.3.2 Ventral Hippocampus

A three-way ANOVA revealed a main effect of sex, but not of Mn exposure nor LPS, on signal intensity in both microglia and astrocytes within the ventral hippocampus. Female mice regardless of Mn exposure or LPS challenge had higher microglia signal intensity than males in the hilar region (Figure 3.9C; F(7,40)=14.653, p<0.001). Females had higher microglia and astrocyte signal intensity in CA1, CA3, and in the molecular layer of the dentate gyrus than males (Table S3.2). Two-way ANOVA, within females, revealed main effects of Mn and/or LPS with females having a higher signal intensity than males in CA1, CA3 and lower intensity in the molecular layer of the dentate gyrus (Table S3.2). Figures 3.10 and 3.11 are representative images of the hippocampus, depicting microglia (CX_3CR1^{GFP}+ cells) and astrocytes (GFAP+ cells), for all treatment groups in females and males respectively.

Three-way ANOVA of microglia (CX_3CR1^{GFP}+/Hoechst+) in the molecular layer of the dentate gyrus revealed that overall LPS challenged mice had a higher number of microglia present than controls (Table S3.2; F(7,40)=6.892, p=0.013). This overall main effect of LPS was driven by the females that were challenged with LPS having a higher number of microglia present than saline challenged females (F(3,20)=11.287, p=0.003). Additionally, saline+LPS females had a higher number of microglia present than saline+saline females (p=0.007, q=4.228). Two-way ANOVA, within females, revealed that LPS challenged females had a higher number of microglia present than saline controls in the hilar region (Figure 3.9E; F(3,20)=6.478, p=0.019), as well as in the CA1 and CA3 (Table S3.2). Three-way ANOVA revealed an overall

effect of sex on the number of astrocytes (GFAP+/Hoechst+) in the ventral hippocampus (Table S3.2). Two-way ANOVA, within males, revealed main effects of Mn and LPS. Males exposed to Mn and challenged with saline (Mn+saline) had a higher number of astrocytes present than saline controls in both the hilar region (Figure 3.9F; p=0.015, q=3.904) and in the molecular layer of the dentate gyrus (Table S3.2; p=0.029, q=3.400). Mn+LPS males had a lower number of astrocytes present than Mn+saline males in the hilar region (Figure 3.9F; p=0.032, q=3.353) as well as in the molecular layer of the dentate gyrus (Table S3.2; p=0.043, q=3.117).

When the intensity per cell was analyzed in the ventral hippocampus, three-way ANOVA revealed a main effect of sex of intensity/microglia in the hilar region, in the CA3, and in the molecular layer of the dentate gyrus (Table S3.4).

3.3.3 Substantia nigra

In the substantia nigra pars reticulata (SNpr) and substantia nigra pars compacta (SNpc), three-way ANOVA a main effect of sex, but not of Mn or LPS. Females had a higher microglia signal intensity in the SNpr (Figure 3.12A; F(7,40)=7.413, p=0.010) and in the SNpc (Figure 3.12C; F(7,40)=7.085, p=0.011) than males. Additionally, females had a higher astrocyte signal intensity in the SNpr (Figure 3.12B; F(7,40)=9.669, p=0.004) and in the SNpc (Figure 3.12D; F(7,40)=4.254, p=0.046) than males. Males that were exposed to Mn had lower microglia signal intensity in the SNpr than control males (Figure 3.12A; F(3,20)=4.841, p=0.042). In the SNpc, Mn exposed females had higher microglia signal intensity than saline control (Figure 3.12C; F(3,20)=5.955, p=0.024) while the opposite was observed in Mn exposed males (F(3,20)=4.581, p=0.047). Males that were exposed to Mn had lower astrocyte signal intensity in the SNpr than control males (Figure 3.12B; F(3,20)=5.135, p=0.0037). Three-way ANOVA of astrocytes (GFAP+/Hoechst+) in the SNpr revealed that females had a higher number of astrocytes present than males (Figure 3.12E; F(7,40)=6.876, p=0.013). While the number of microglia in the SNpr or astrocytes present in the SNpr and SNpc were not significant, there were significant effects when the intensity per cell was analyzed (Table S3.5).

In the ventral tegmental area (VTA), Mn exposure resulted in a decrease in the number of microglia in the VTA within males (Figure 3.2C; $F(3,20)=6.947$, $p=0.021$). Mn did not affect the number of microglia within females however, LPS challenged females had more microglia in the ventral medial SNpc than controls (Figure 3.2A; $F(3,20)=11.023$, $p=0.003$).

4 Discussion

Mn-induced neurotransmitter alterations have been connected with impairments related to locomotion and mood (Krishna et al., 2014; Lin et al., 2020; Moreno et al., 2009). Therefore, we conducted this study to expand upon the impact of Mn exposure via drinking water on the behavioral alterations that were previously observed in adult male and female mice (Ludwig et al., 2024). In the current study, Mn drinking water exposure led to significant changes in brain amino acid and monoamine neurochemistry. These changes were rather region and somewhat sex-specific, and they might explain some of the behavioral alterations that were observed previously.

The striatum, a region that contains a high concentration of dopamine neuron axonal projections and involved with motor control, with appropriate dopamine function is essential for coordinating movements. The Mn induced behavioral alterations, that we previously reported in Ludwig et al. (2024) occurred in the absence of a measurable effect on striatal DA homeostasis (measured two weeks after behavior testing). Very similar results were observed by Krishna et al. (2014) and Witholt et al. (2000) who demonstrated neurobehavioral function alterations without changes in striatal DA following subchronic Mn exposure. In general, DA is considered the neurotransmitter that predominantly controls locomotor and emotional function and alterations in its homeostasis have been associated with locomotor and emotional dysfunction (Sotnikova et al., 2005). Tissue levels of the neurotransmitters may vary from when they are released due to tissues storing the neurotransmitters vs once released they rapidly diffuse across the synaptic cleft and bind to receptors. This indicates the complexity of neurotransmitter regulation, where changes in neurotransmitter release and receptor activity may precede

changes in tissue concentrations. In this context, the increased GABA levels and the altered GLU/GABA ratio in the males and the lower GLU levels in the females, could have resulted in motor control issues, playing a role in the altered stride length and interstep distances. While behavioral alterations observed in our previous study were not due to Mn-induced changes in tissue striatal DA levels (or other striatal monoamines), Mn and LPS exposures combined did reduce striatal DA levels in females. The increased 5-HT and 5-HIAA among females, particularly after LPS challenge, could indicate that there is increased serotonin activity or turnover in the striatum of these females. LPS challenged males on the other hand had increased levels of 5-HIAA in the striatum with no change in 5-HT levels indicating that there could be an increase of neuronal activity in the region.

The cortex is associated with decision making, emotion, learning, as well as for regulation of stress responsivity and Mn has been found in the frontal cortex in humans (Lucchini et al., 2014). In our previous study, the mice displayed a decreased fear or increased risky type of behavior across multiple behavior tests and testing timepoints, especially among the males. The Mn-induced increase in GABA suggests an increase in inhibitory control within the PFC which could result in reduced motor activity and promote reduced anxiety. The Mn-exposed mice had a decreased GLU/GABA ratio, especially among the males, could result in the increase impulsivity or risky type of behavior that was observed during open field testing. The Mn-induced increase in GLN could indicate that there is a disruption in the synthesis of excitatory or inhibitory neurotransmitters in the PFC. Surprisingly, the PFC was the only region tested that had Mn-induced perturbations in monoamine neurotransmitters. Mn-exposed males had significantly lower levels of 5-HT, which has been linked with difficulties with mood regulation, cognitive function and impulsivity (Garcia-Garcia et al., 2017; Goodfellow et al., 2009; Jacobsen et al., 2012). This could be related to the increased risky behaviors exhibited by the Mn-exposed males that we previously reported. Mn-exposed females had an increase in 5-HIAA levels, without impacting 5-HT levels, indicating that there may be an overactivation of the serotonergic

system, which could lead to an increase in anxiety in cases of long term elevated 5-HIAA levels. It is interesting that in Krishna et al. (2014), the cortex measured with the least Mn deposition, in the MRI, in adult mice exposed to the same concentration of Mn through drinking water for the same duration as the current study. Interestingly, the differential effects of Mn exposure on the cortex and PFC in males and females could be further influenced by sex hormones like estrogen, which are known to play a neuroprotective role in many neurotoxic contexts. The modulation of glial activation and neurotransmitter systems by estrogen may contribute to the observed sex differences in neurochemical changes and behaviors.

The hippocampus is associated with learning and memory, and Mn toxicity can result in cognitive impairments, memory deficits, and behavioral changes, such as decreased fear or increase in risky behaviors that were observed in the male mice of the current study. Males exposed to Mn had a decrease in GABA levels, which resulted in an increase in the GLU/GABA ratio. This alteration in the GABAergic system could contribute to manic-like behaviors, such as an increase in impulsive behaviors, if the excitatory drive becomes dominant. In Tamar et al. (2021), male rats exposed to Mn via drinking water had a higher accumulation of Mn in the hippocampus as well as an increase in anxiety-like behavior than their female counterparts. The vHIP was another region that LPS challenged females had elevated 5-HIAA levels. While this may suggest enhanced 5-HT turnover, which could be a sign of increased anxiety, it can also be indicative of overactivity in the serotonergic system. Hormones, such as estrogen, may influence Mn accumulation in the brain or even have a protective role against manganese neurotoxicity.

As the hippocampus is important for learning and memory, and these aspects have been reported to be impacted by Mn exposure (Bouchard et al., 2011; Brna et al., 2011; Hafeman et al., 2007; Sahni et al., 2007; Wasserman et al., 2006; Woolf et al., 2002), it was also important to investigate microglia and astrocytes in several areas of the dHIP and vHIP, in addition to measuring hippocampal neurotransmitters. As microglia are the immune cell of the brain, they

monitor and respond to changes in their environment including injury, infection, and neurodegeneration. Astrocytes support neuronal function and when they become reactive, they can influence disease progression. Both cells are involved with synaptic plasticity and help to regulate synaptic activity in the hippocampus by maintaining the environment and neuronal communications (Chowen & Garcia-Segura, 2021; Franklin et al., 2021). In the dHIP, Mn-exposure led to higher GFP optical density (OD; indicating greater microglia activation) in females, specifically in Mn+LPS females in the hilar region. Mn-exposed and LPS challenged (Mn+LPS) females as well as LPS challenged males had a higher number of microglia present in the dHIP hilar region, while Mn-exposed males had a higher number of microglia present in the dHIP CA3. Astrocyte numbers in the dHIP were not affected by Mn exposure. Similarly, in the vHIP, Mn-exposure only affected females' OD; there was greater microglia activation in the CA1, CA3, and molecular layer of the dentate gyrus and astrocyte activation in the CA3. Within the substantia nigra pars reticulata (SNpr), Mn-exposed males show reduced OD of both GFP and GFAP, indicative of lower microglia and astrocyte activity. The reverse was seen in Mn-exposed mice regarding their astrocytic activation in the SNpr that was reported by Krishna et al. (2014). Mn-exposure in the females on the other hand resulted in increased GFP OD within the substantia nigra pars compacta. Taken together, this indicates that there may be a sex-dependent response to Mn, with females exhibiting a potentially exaggerated glial activation compared to males in response to Mn exposure. In the dHIP, vHIP, SNpr, and SNpc females typically had higher OD for both microglia and astrocytes than males. Even though males had higher number of astrocytes present in dHIP, the females had higher levels of astrocyte activation with the lower number of astrocytes. Within the vHIP, females had higher number of astrocytes than males, but both sexes had similar levels of astrocyte activation per cell. While microglial numbers didn't differ between sexes, female microglia were more active than, and it is interesting that LPS challenged females had a similar level of microglial activity as the microglia within males. The observed alterations in glial cells and neurotransmitter within the

hippocampus may provide insight into the more nuanced effects of Mn exposure on females as we previously suggested. Prior research has shown that Mn-exposed human females tend to exhibit slower learning and lower intelligence scores (Rechtman et al., 2020; Riojas-Rodríguez et al., 2010), which could be indicative of underlying changes in hippocampal function.

In conclusion, the current study characterized sex differences in neurotransmitters as well as glial cells involved with neuroinflammation in Mn-exposed mice, expanding on the literature highlighting the risks associated with Mn DW overexposures. Our previously reported observations regarding the increased fearlessness/reduced anxiety-like behavior exhibited by Mn-exposed males, as well as the disturbances in gait in both sexes, could be attributed to the changes in glutamatergic and GABAergic neurotransmitter systems as an early consequence to low-level Mn exposure prior to alterations in striatal DA. While both sexes demonstrated changes in neurotransmitter systems, our results further suggest that the effects of Mn exposure are sex biased, with the effects on females being subtler and the neurobehavioral dysfunction in males beginning with changes in glutamatergic and GABAergic systems rather than with changes occurring first within the DAergic system. Furthermore, these findings could inform the development of gender-specific therapeutic approaches and highlight the potential for interventions aimed at reducing the neurotoxic impact of Mn.

Author contributions

NMF conceived and designed the study. NMF and HDL assisted in the sample collection. HDL performed sample and data analysis. NMF and HDL wrote the manuscript. All authors read/contributed editorially and approved the final manuscript version.

Conflict of Interest

The authors declare they have no potential conflicts of interest.

Data availability

Data will be made available on reasonable request.

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Figures and Tables

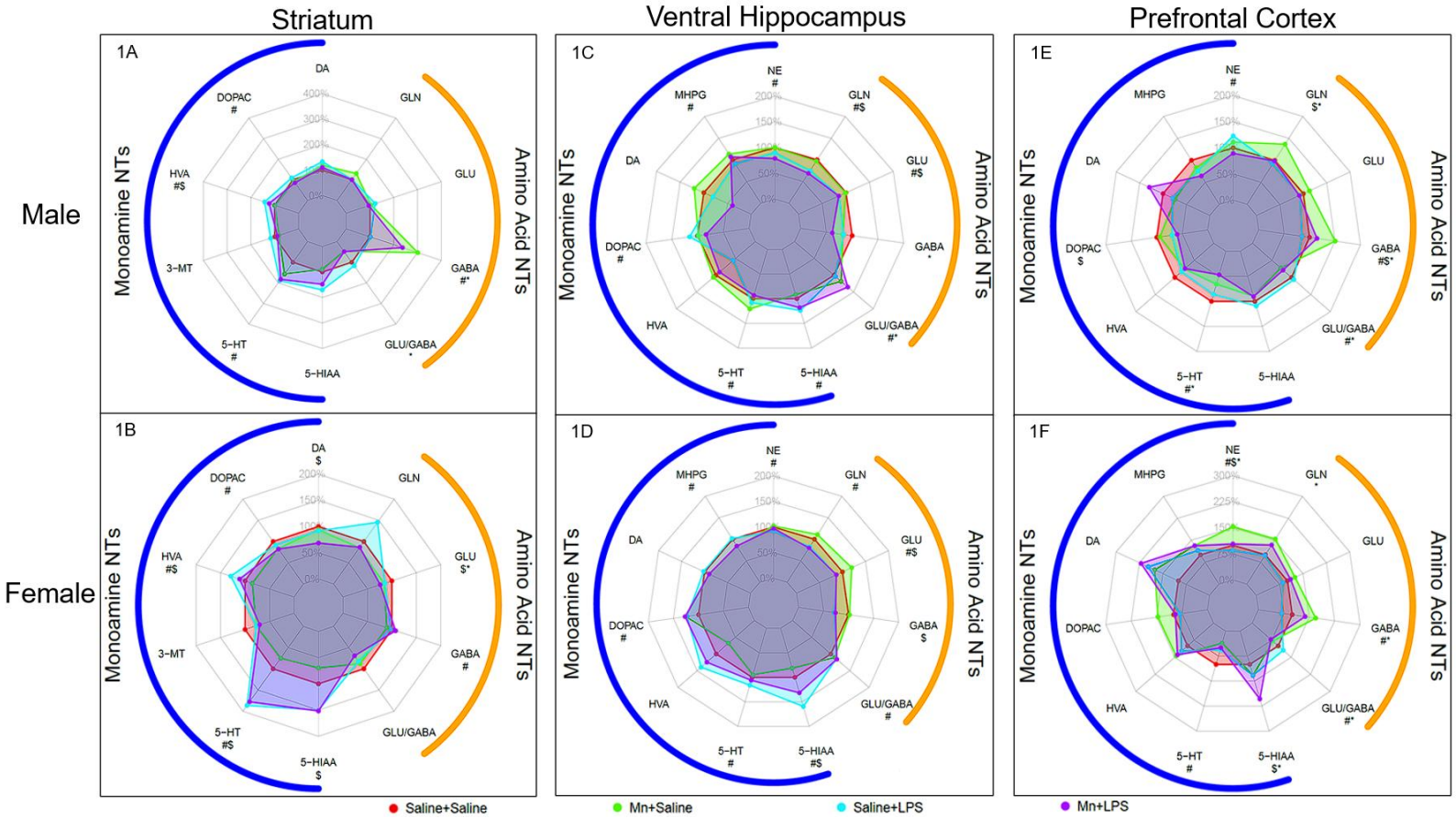

Figure 3.1. Summary of key monoamine and amino acid neurotransmitter findings. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg) on regional brain neurotransmitter levels six hours after LPS administration. STR males (A), STR females (B), vHIP males (C), vHIP females (D), PFC males (E), PFC females (F). The radar plots highlights the effect of Mn/LPS/sex-differences on neurotransmitter levels in different brain regions. Higher and lower percentage values from the Saline+Saline (red) group indicate deviations from the control group. Data are presented as a percentage; *Indicates a significant effect of Mn $p \leq 0.05$; $^{\#}$indicates a significant effect of sex $p \leq 0.05$; $^{\$}$indicates a significant effect of LPS $p \leq 0.05$; *n*=6/group. Data used for the generation of the radar plots is in Tables S3.6 and S3.7.

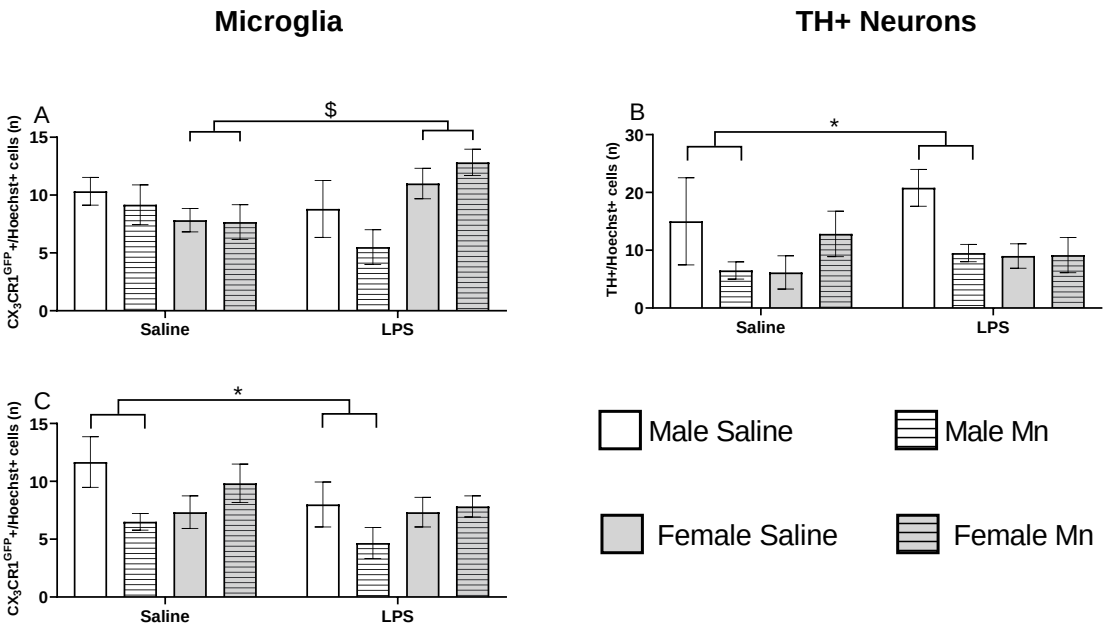

Figure 3.2. Microglia (CX_3CR1^{GFP}) and Tyrosine hydroxylase (TH) cell count in the ventral medial substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). CX_3CR1^{GFP}+/Hoechst+ and TH+/Hoechst+ count of ventral medial SNpc microglia (A), ventral medial SNpc TH+ neurons (B), and VTA microglia (C). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS, $p<0.05$; n=6/group.

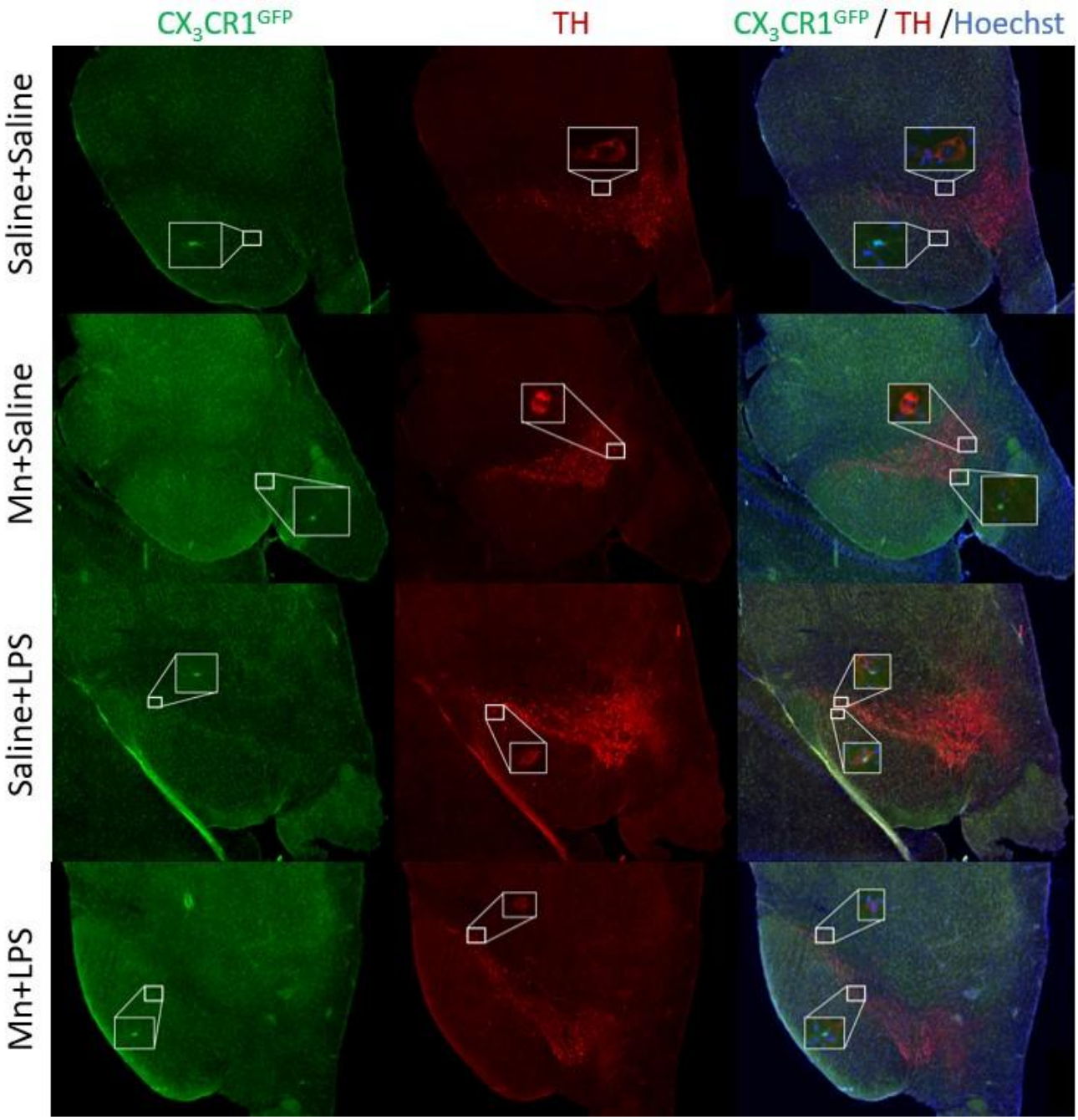

Figure 3.3. CX_3CR1^{GFP} and TH immunoreactivity in the substantia nigra after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in female mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), TH+ neurons (TH; column 2), and CX_3CR1^{GFP} and TH overlay with nuclear staining (Hoechst; column 3) in the substantia nigra at 20x magnification.

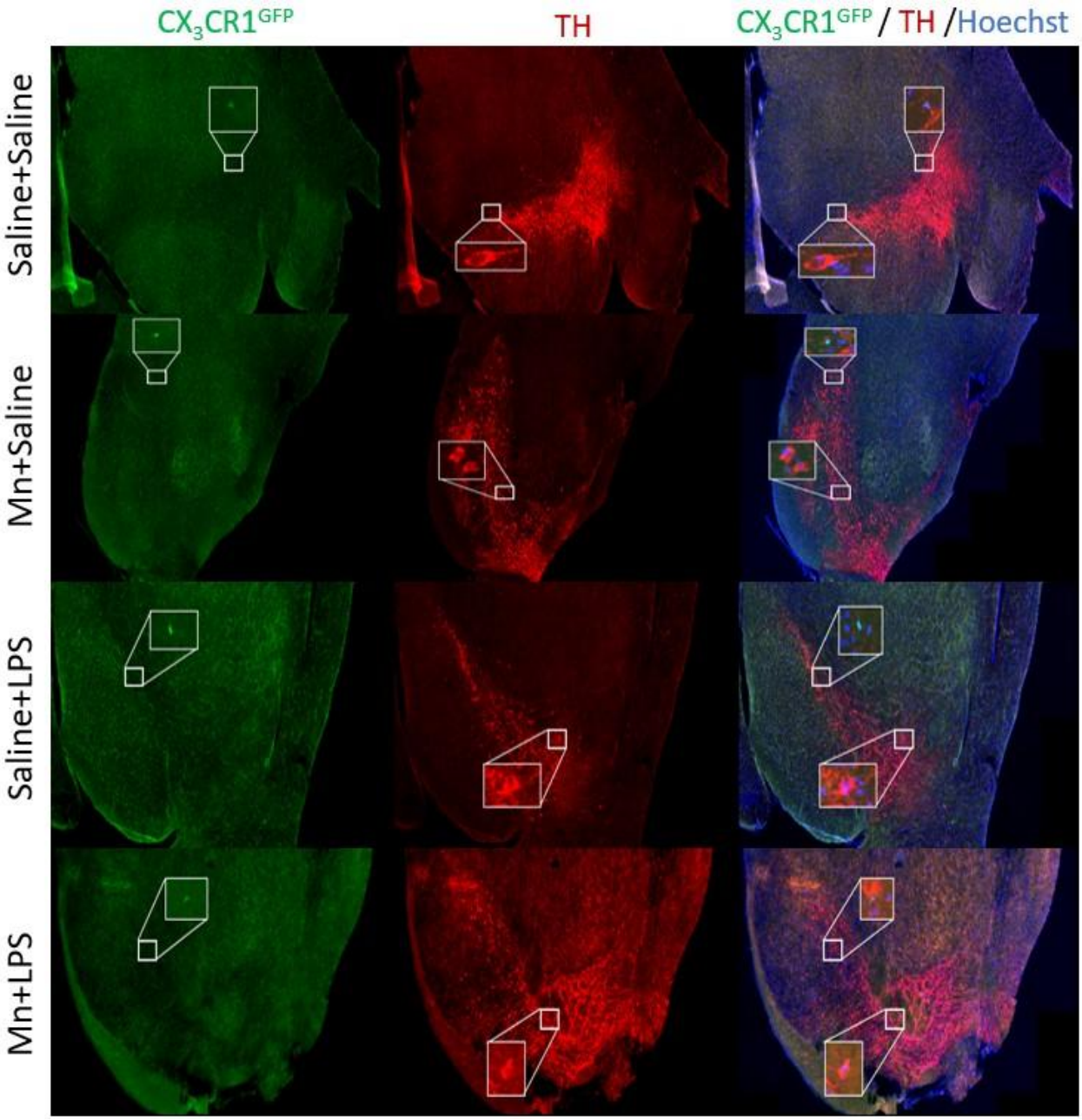

Figure 3.4. CX_3CR1^{GFP} and TH immunoreactivity in the substantia nigra after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in male mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), TH+ neurons (TH; column 2), and CX_3CR1^{GFP} and TH overlay with nuclear staining (Hoechst; column 3) in the substantia nigra at 20x magnification.

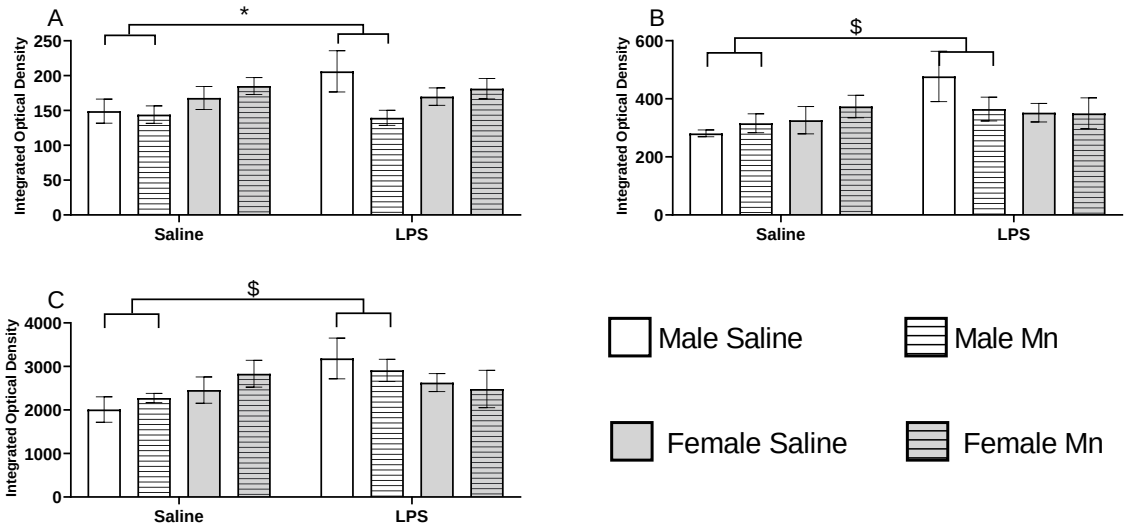

Figure 3.5. Microglia (CX_3CR1^{GFP}) and Tyrosine hydroxylase (TH) immunoreactivity in the ventromedial striatum (VM STR) and dorsolateral striatum (DL STR) after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Semi-quantitative analysis (integrated optical density) of VM STR microglia (A), VM STR TH+ neurons (B), and DL STR TH+ neurons (C). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; $^{\$}$indicates a significant effect of LPS, $p<0.05$; $n=6$/group.

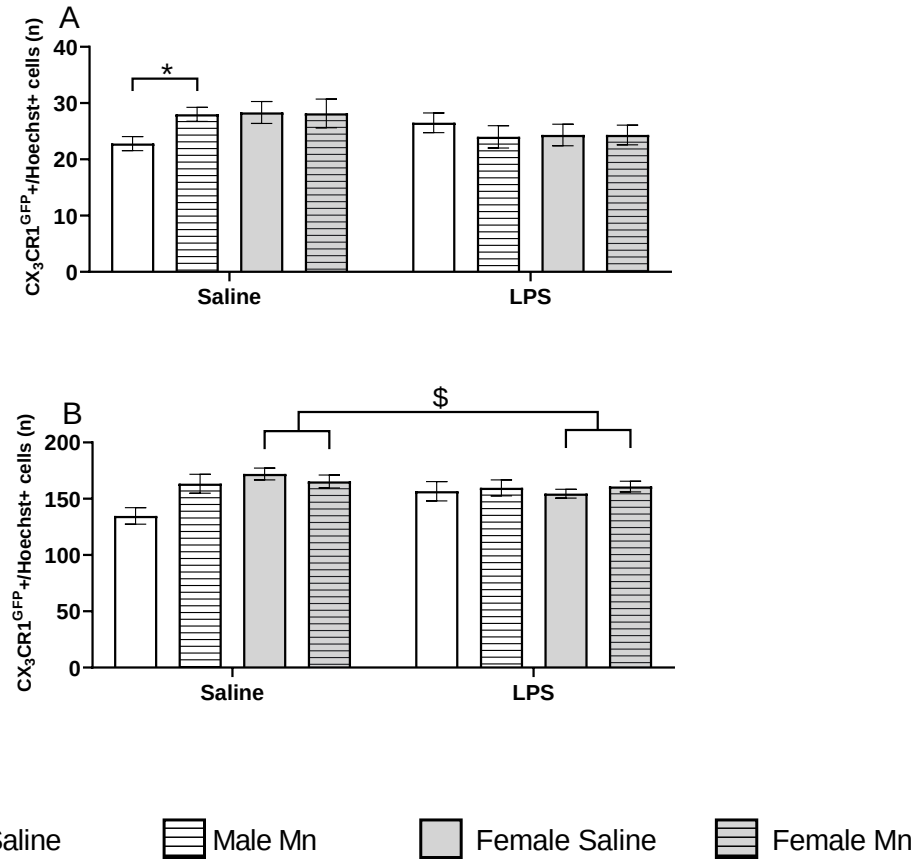

Figure 3.6. Microglia (CX_3CR1^{GFP}) cell count in the ventromedial striatum (VM STR) and dorsolateral striatum (DL STR) after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). CX_3CR1^{GFP}+/Hoechst+ and count of VM STR microglia (A) and DL STR microglia(B). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS, $p<0.05$; $n=6$/group.

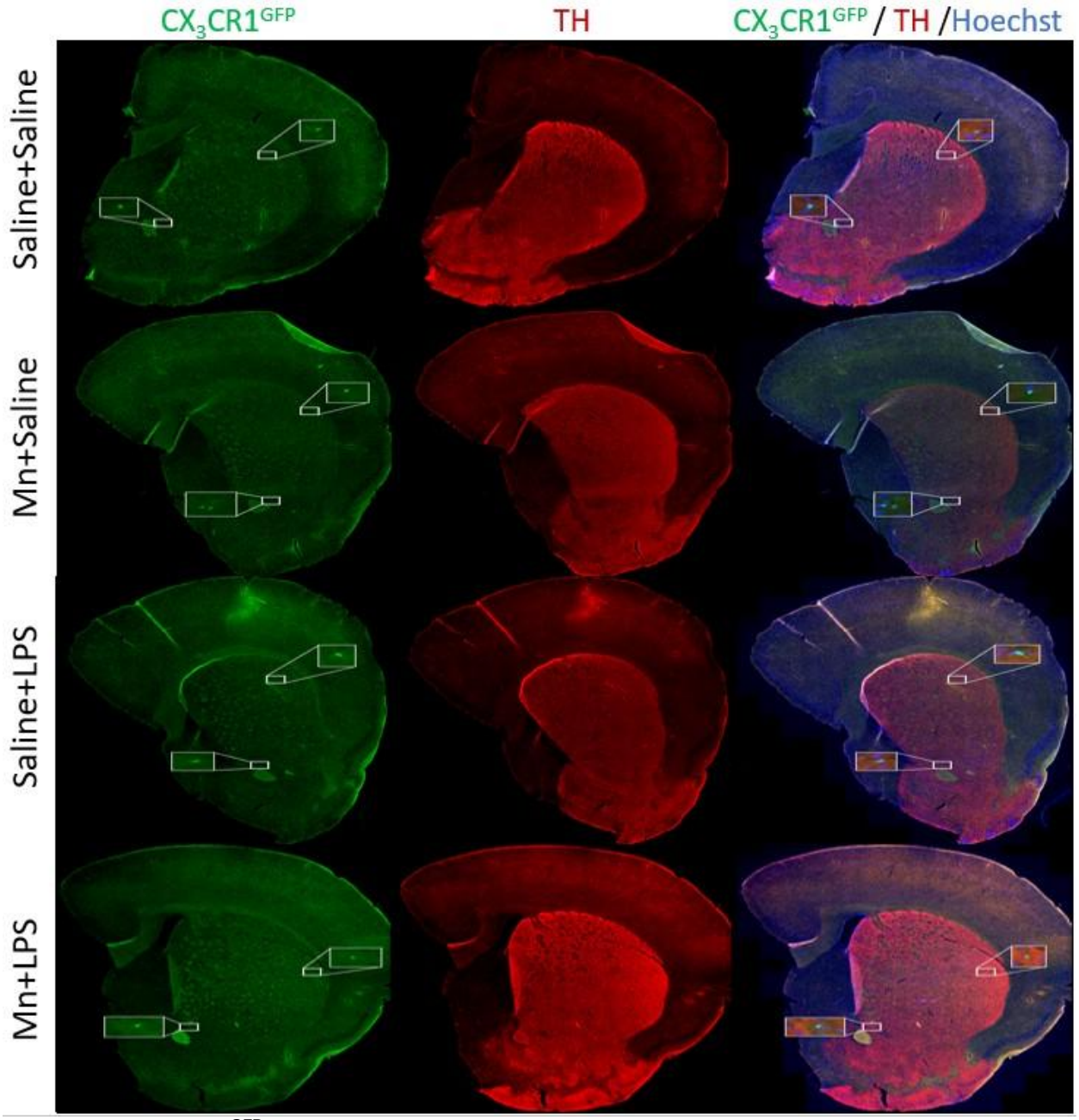

Figure 3.7. CX_3CR1^{GFP} and TH immunoreactivity in the striatum after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in female mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), TH+ immunoreactivity (TH; column 2), and CX_3CR1^{GFP} and TH overlay with nuclear staining (Hoechst; column 3) in the striatum at 20x magnification.

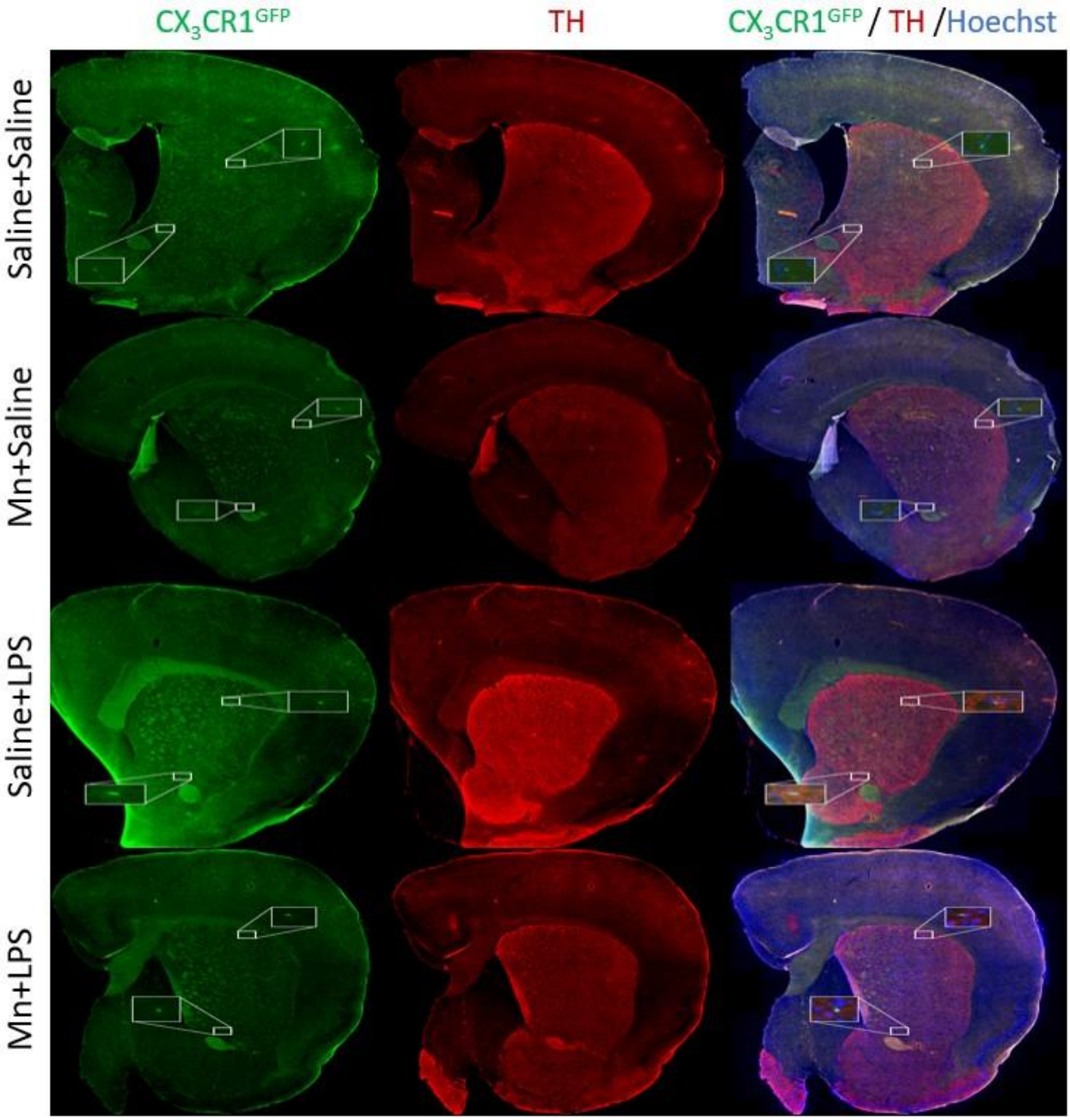

Figure 3.8. CX_3CR1^{GFP} and TH immunoreactivity in the striatum after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in male mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), TH+ immunoreactivity (TH; column 2), and CX_3CR1^{GFP} and TH overlay with nuclear staining (Hoechst; column 3) in the striatum at 20x magnification.

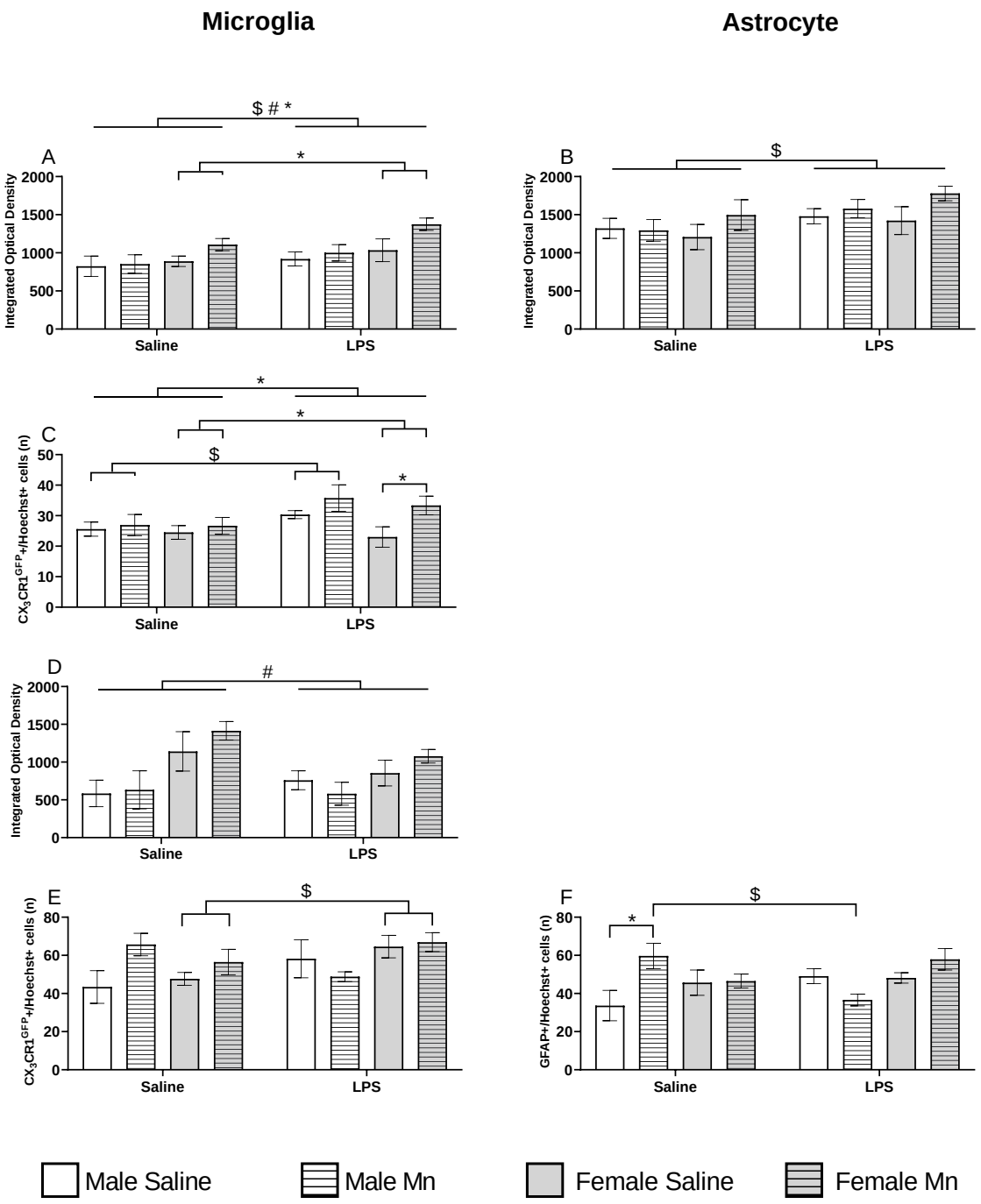

Figure 3.9. CX_3CR1^{GFP} and glial fibrillary acidic protein (GFAP) immunoreactivity and cell count in the hilar region dorsal and ventral hippocampus (dHIP and vHIP respectively) after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Semi-quantitative analysis (integrated optical density) of dHIP microglia intensity (A), dHIP astrocyte intensity (B), dHIP microglia cell count (C), vHIP microglia intensity (D), vHIP microglia cell count (E), and vHIP astrocyte cell count (F). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; $ indicates a significant effect of LPS, $p<0.05$; $n=6$/group.

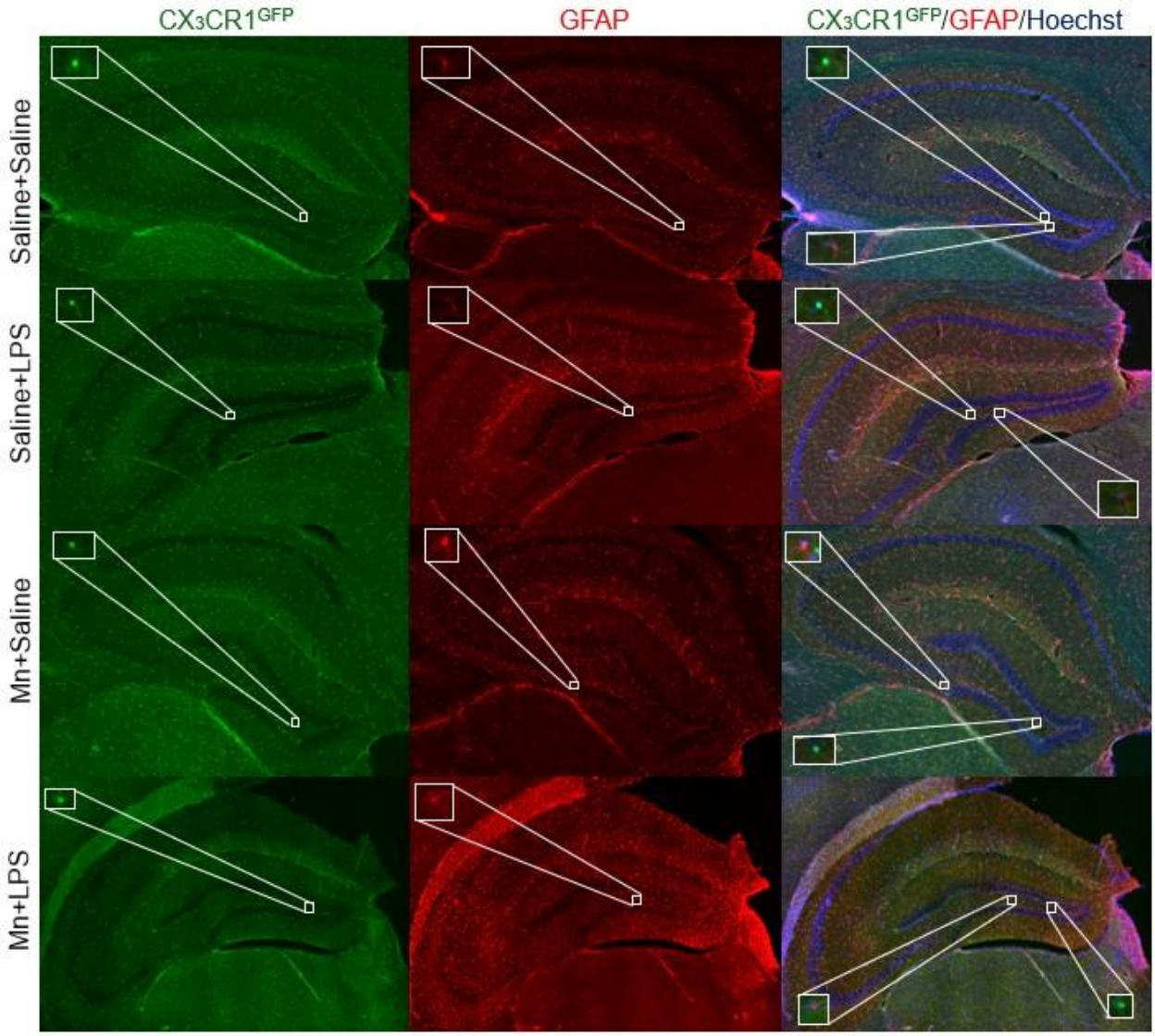

Figure 3.10. CX_3CR1^{GFP} and GFAP immunoreactivity in the hippocampus after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in female mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), astrocytes (GFAP; column 2), and CX_3CR1^{GFP} and GFAP overlay with nuclear staining (Hoechst; column 3) in the dorsal hippocampus at 20x magnification.

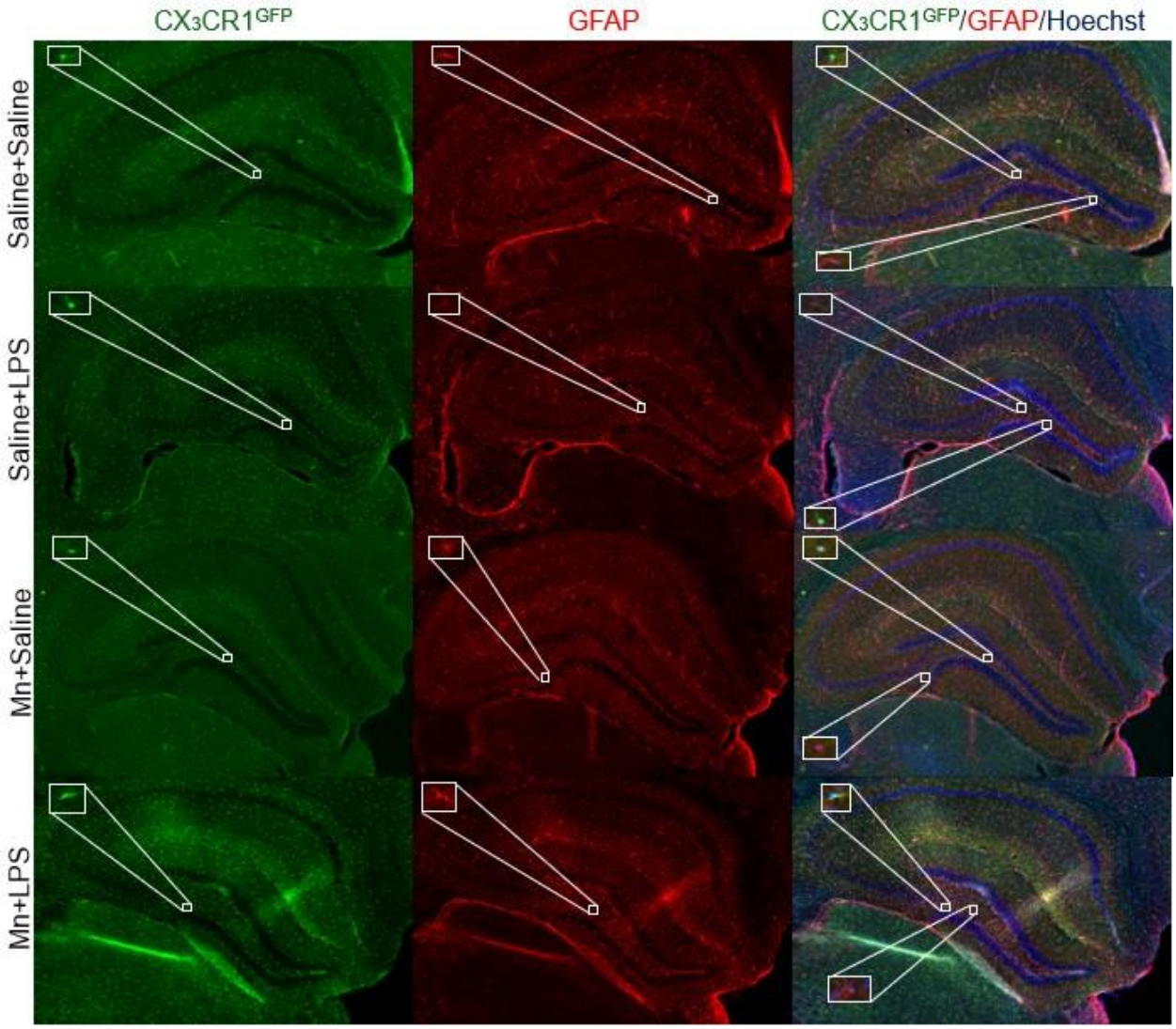

Figure 3.11. CX_3CR1^{GFP} and GFAP immunoreactivity in the hippocampus after 8 weeks of Mn DW (0.4 g/L) and/or acute LPS administration (0.3 mg/kg body weight) in male mice. Representative images depict microglia (CX_3CR1^{GFP}; column 1), astrocytes (GFAP; column 2), and CX_3CR1^{GFP} and GFAP overlay with nuclear staining (Hoechst; column 3) in the dorsal hippocampus at 20x magnification.

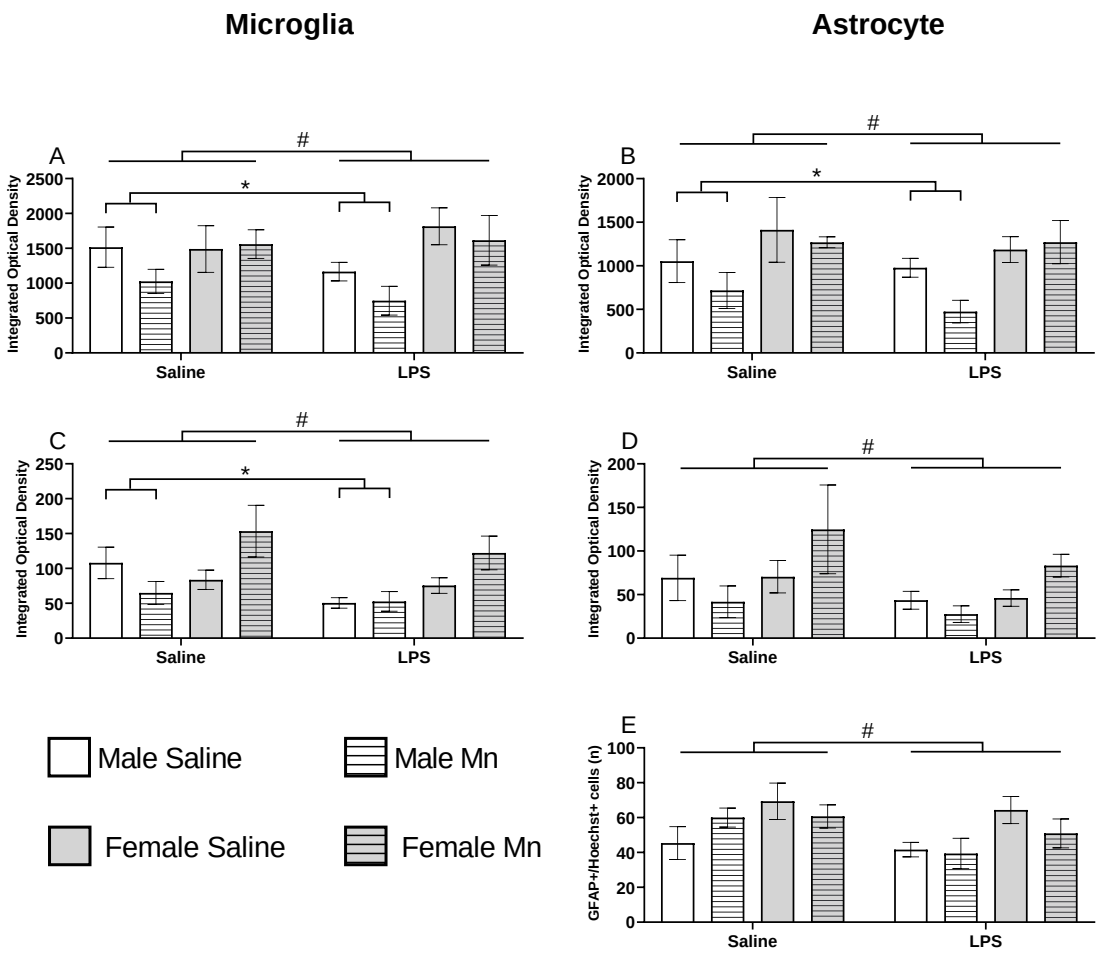

Figure 3.12. CX_3CR1^{GFP} and glial fibrillary acidic protein (GFAP) immunoreactivity in the substantia nigra pars reticulata (SNpr) and substantia nigra pars compacta (SNpc), and GFAP cell count in the SNpr after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Semi-quantitative analysis (integrated optical density) of microglia SNpr (A), astrocyte SNpr (B), microglia SNpc (C), astrocyte SNpc (D), astrocyte cell count SNpr. Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; $^{\#}$ indicates a significant effect of sex $p<0.05$; $^{\$}$ indicates a significant effect of LPS, $p<0.05$; n=6/group.

Table S3.1. CX_3CR1^{GFP} (microglia) and glial fibrillary acidic protein (GFAP; astrocyte) immunoreactivity and cell count in the dorsal hippocampus after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

			DW Treatment - LPS Challenge			
Sex	Hippocampus Region	Measurement	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Male	CA1	GFP intensity	217.4 ± 22.74	227.6 ± 21.33	264.3 ± 45.06	205.7 ± 15.12
	CA1	GFAP intensity	199.3 ± 25.48	237.7 ± 10.90	216.4 ± 13.02	233.6 ± 24.21
	CA3	GFP intensity	261.6 ± 37.72	238.5 ± 11.57	254.8 ± 15.43	227.7 ± 17.88
	CA3	GFAP intensity	247.5 ± 40.30	256.5 ± 9.56	285.5 ± 43.12	230.7 ± 24.30
	Dentate Gyrus	GFP intensity	278.4 ± 35.67	288.6 ± 19.40	326.6 ± 43.88	265.3 ± 12.30
	Dentate Gyrus	GFAP intensity	275.1 ± 32.07	304.8 ± 18.30	333.3 ± 30.90	290.6 ± 24.90
	CA1	CX_3CR1^{GFP}+ cell	7.5 ± 0.52	7.4 ± 0.49	7.2 ± 0.59	6.7 ± 0.31
	CA1	GFAP+ cell	5.8 ± 0.81	6.6 ± 0.35	7.7 ± 0.31	8.1 ± 0.35
	CA3	CX_3CR1^{GFP}+ cell	**6.9 ± 0.83**	7.6 ± 0.58	**9.4 ± 0.70**	**8.0 ± 0.74**
	CA3	GFAP+ cell	5.7 ± 0.21	5.8 ± 0.70	6.3 ± 0.56	5.2 ± 0.46
	Dentate Gyrus	CX_3CR1^{GFP}+ cell	5.3 ± 0.46	6.6 ± 0.68	6.4 ± 0.35	5.4 ± 0.40
	Dentate Gyrus	GFAP+ cell	5.3 ± 0.36	5.3 ± 0.54	5.8 ± 0.56	5.3 ± 0.44
Female	CA1	GFP intensity	237.9 ± 16.61	286.2 ± 36.17	301.4 ± 17.54	289.1 ± 29.63
	CA1	GFAP intensity	275.3 ± 21.13	313.7 ± 31.98	321.0 ± 31.14	315.2 ± 21.77
	CA3	GFP intensity	255.3 ± 17.41	311.2 ± 35.29	362.8 ± 42.06	362.1 ± 52.13
	CA3	GFAP intensity	252.7 ± 11.25	328.1 ± 36.11	360.1 ± 54.71	347.1 ± 34.80
	Dentate Gyrus	GFP intensity	302.0 ± 9.76	338.8 ± 39.04	361.2 ± 15.70	392.9 ± 64.11
	Dentate Gyrus	GFAP intensity	313.5 ± 10.90	367.4 ± 38.39	393.5 ± 45.67	384.8 ± 17.62
	CA1	CX_3CR1^{GFP}+ cell	7.8 ± 0.60	8.2 ± 0.44	7.8 ± 0.28	6.7 ± 0.40
	CA1	GFAP+ cell	7.1 ± 0.72	7.5 ± 0.58	7.1 ± 1.08	7.3 ± 0.38
	CA3	CX_3CR1^{GFP}+ cell	7.3 ± 0.53	7.4 ± 0.62	7.1 ± 0.44	8.4 ± 0.65
	CA3	GFAP+ cell	4.3 ± 0.60	4.8 ± 0.40	4.8 ± 0.53	4.3 ± 0.77
	Dentate Gyrus	CX_3CR1^{GFP}+ cell	4.0 ± 0.50	4.8 ± 0.60	5.1 ± 0.65	4.3 ± 0.46
	Dentate Gyrus	GFAP+ cell	4.2 ± 0.48	**4.6 ± 0.15**	5.3 ± 0.56	**3.7 ± 0.40**

In all three areas, the signal intensity of both microglia and astrocytes were significantly higher among females than males (Table S3.1: GFP CA1: $F(7,40)=6.633$, $p=0.014$; GFAP CA1: $F(7,40)=25.773$, $p<0.001$; GFP CA3: $F(7,40)=11.709$, $p=0.001$; GFAP CA3: $F(7,40)=7.318$, $p=0.010$; GFP dentate gyrus: $F(7,40)=5.740$, $p=0.021$; GFAP dentate gyrus: $F(7,40)=9.414$, $p=0.004$). Within the CA3 region, Mn exposed males, but not females, had significantly higher number of microglia present ($F(3,20)=11.080$, $p=0.003$). In the molecular layer of the dentate gyrus, Mn+LPS females had lower number of astrocytes than Mn+saline females (Table S3.1; $p=0.016$, $q=3.719$). Three-way ANOVA of astrocytes (GFAP+/Hoechst+) in the CA1, CA3, and molecular layer of the dentate gyrus of the hippocampus revealed that males had a higher number of astrocytes than females, regardless of Mn or LPS treatments (Table S3.1: GFAP CA1: $F(7,40)=25.773$, $p<0.001$; GFAP CA3: $F(7,40)=7.318$, $p=0.010$; GFAP dentate gyrus: $F(7,40)=9.414$, $p=0.004$).

Table S3.2. CX_3CR1^{GFP} (microglia) and glial fibrillary acidic protein (GFAP; astrocyte) immunoreactivity and cell count in the ventral hippocampus after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

			DW Treatment - LPS Challenge			
Sex	Hippocampus Region	Measurement	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Male	CA1	GFP intensity	124.2 ± 40.38	101.4 ± 11.10	39.5 ± 38.11	95.3 ± 21.05
	CA1	GFAP intensity	80.3 ± 22.86	79.1 ± 25.50	59.0 ± 45.67	93.8 ± 27.82
	CA3	GFP intensity	155.9 ± 39.05	99.5 ± 24.51	110.3 ± 36.83	134.7 ± 29.54
	CA3	GFAP intensity	119.1 ± 36.33	94.2 ± 25.61	118.3 ± 46.69	112.7 ± 24.98
	Dentate Gyrus	GFP intensity	149.8 ± 33.95	146.2 ± 18.40	68.0 ± 38.20	117.8 ± 24.10
	Dentate Gyrus	GFAP intensity	98.3 ± 25.92	111.0 ± 17.39	81.2 ± 44.27	113.6 ± 23.74
	CA1	CX_3CR1^{GFP}+ cell	8.8 ± 2.03	7.3 ± 1.49	8.1 ± 1.39	7.7 ± 1.18
	CA1	GFAP+ cell	7.6 ± 0.92	7.0 ± 0.96	7.6 ± 1.14	6.3 ± 0.77
	CA3	CX_3CR1^{GFP}+ cell	8.3 ± 1.20	8.8 ± 0.96	8.8 ± 1.18	9.3 ± 1.24
	CA3	GFAP+ cell	4.2 ± 0.65	5.4 ± 1.09	5.9 ± 0.83	4.9 ± 0.98
	Dentate Gyrus	CX_3CR1^{GFP}+ cell	4.9 ± 0.68	5.3 ± 0.72	6.9 ± 1.01	4.5 ± 0.92
	Dentate Gyrus	GFAP+ cell	**3.3 ± 0.38**	**5.4 ± 0.78**	**5.8 ± 1.25**	4.1 ± 0.56
Female	CA1	GFP intensity	**157.1 ± 18.59**	170.0 ± 26.32	**234.9 ± 15.36**	177.4 ± 15.91
	CA1	GFAP intensity	120.0 ± 26.51	130.4 ± 27.25	176.2 ± 24.02	142.5 ± 19.37
	CA3	GFP intensity	**172.4 ± 21.40**	195.7 ± 17.18	**238.9 ± 7.96**	**180.8 ± 16.57**
	CA3	GFAP intensity	**133.2 ± 22.64**	152.8 ± 12.75	**228.0 ± 27.27**	**145.3 ± 28.77**
	Dentate Gyrus	GFP intensity	**191.2 ± 26.28**	178.7 ± 26.01	**262.6 ± 20.90**	208.1 ± 12.14
	Dentate Gyrus	GFAP intensity	182.0 ± 39.33	**132.5 ± 27.39**	223.3 ± 24.29	**157.0 ± 13.98**
	CA1	CX_3CR1^{GFP}+ cell	6.7 ± 0.60	**11.5 ± 1.51**	8.8 ± 1.28	**9.8 ± 0.99**
	CA1	GFAP+ cell	5.1 ± 0.69	8.9 ± 0.52	7.2 ± 1.39	8.0 ± 0.72
	CA3	CX_3CR1^{GFP}+ cell	7.2 ± 0.78	**11.8 ± 0.61**	8.9 ± 1.72	**11.7 ± 0.96**
	CA3	GFAP+ cell	7.7 ± 0.95	6.9 ± 0.69	6.3 ± 0.86	8.7 ± 0.61
	Dentate Gyrus	CX_3CR1^{GFP}+ cell	6.9 ± 1.38	**5.8 ± 0.89**	6.5 ± 0.99	**7.8 ± 1.12**
	Dentate Gyrus	GFAP+ cell	5.7 ± 0.48	5.2 ± 0.31	6.2 ± 0.61	6.3 ± 0.56

Female mice regardless of Mn exposure or LPS challenge had higher microglia signal intensity than males in the CA1, CA3, and molecular layer of the dentate gyrus (Table S3.2; $F(7,40)=28.233$, $p<0.001$; $F(7,40)=16.150$, $p<0.001$; $F(7,40)=24.213$, $p<0.001$, respectively). Additionally, female mice regardless of Mn exposure or LPS challenge had higher astrocyte signal intensity than males in the CA1, CA3 and molecular layer (Table S3.2; $F(7,40)=11.355$, $p=0.002$; $F(7,40)=7.068$, $p=0.012$; $F(7,40)=13.364$, $p<0.001$, respectively). Two-way ANOVA, within females, revealed main effects of Mn and/or LPS. Females exposed to Mn had higher microglia signal intensity in the CA1 and molecular layer (Table S3.2; $F(3,20)=16.150$, $p<0.001$ and $F(3,20)=5.208$, $p=0.034$ respectively). In the CA3, Mn+saline females had higher microglia signal intensity than saline+saline females ($F(3,20)=4.022$, $p=0.01$) and Mn+LPS females had a lower microglia signal intensity than Mn+saline females ($F(3,20)=3.516$, $p=0.022$). In the CA3, Mn+saline females had higher astrocyte signal intensity than saline+saline females ($F(3,20)=4.001$, $p=0.011$) and Mn+LPS females had a lower microglia signal intensity than Mn+saline females ($F(3,20)=3.490$, $p=0.023$). In the molecular layer of the dentate gyrus, females challenged with LPS, regardless of Mn exposure, had lower astrocyte signal intensity than saline challenged mice ($F(3,20)=4.357$, $p=0.050$). Females had a higher number of astrocytes present in both the CA1 and molecular layer ($F(7,40)=14.680$, $p<0.001$ and $F(7,40)=7.890$, $p=0.008$ respectively) than males did for either region. LPS challenged females had a higher number of microglia present than saline controls in the CA1 and CA3 ($F(3,20)=6.502$, $p=0.019$ and $F(3,20)=6.823$, $p=0.017$ respectively).

Table S3.3. Dorsal Hippocampus signal intensity per cell. CX_3CR1^{GFP} (microglia) and glial fibrillary acidic protein (GFAP; astrocyte) immunoreactivity per cell in the dorsal hippocampus after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Semi-quantitative analysis (integrated optical density) per cell. Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

			DW Treatment - LPS Challenge			
Sex	Hippocampus Region	Measurement	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Male	Hilar Region	GFP	32.1 ± 3.62	30.3 ± 2.84	31.3 ± 2.36	29.1 ± 3.43
	CA1	GFP	30.3 ± 4.68	31.3 ± 3.42	38.2 ± 6.82	30.8 ± 1.46
	CA3	GFP	49.2 ± 9.71	36.6 ± 2.23	**33.8 ± 3.11**	**28.1 ± 1.44**
	Dentate Gyrus	GFP	40.6 ± 3.52	39.2 ± 3.87	35.4 ± 4.73	34.7 ± 3.53
	Hilar Region	GFAP	49.6 ± 1.40	59.1 ± 5.00	58.6 ± 6.76	68.1 ± 6.13
	CA1	GFAP	35.1 ± 4.28	44.8 ± 7.13	36.3 ± 5.09	45.7 ± 4.24
	CA3	GFAP	44.5 ± 5.78	41.6 ± 5.20	45.3 ± 7.16	42.6 ± 2.74
	Dentate Gyrus	GFAP	53.2 ± 6.83	61.8 ± 10.02	61.4 ± 8.94	55.1 ± 3.98
Female	Hilar Region	GFP	37.1 ± 2.67	46.1 ± 4.37	43.1 ± 3.64	**42.6 ± 3.83**
	CA1	GFP	31.2 ± 2.54	35.8 ± 5.40	**38.9 ± 1.97**	**42.9 ± 2.26**
	CA3	GFP	37.4 ± 2.92	41.7 ± 3.89	**55.3 ± 6.70**	**49.6 ± 6.76**
	Dentate Gyrus	GFP	41.9 ± 2.45	45.4 ± 2.89	52.3 ± 4.56	47.8 ± 7.46
	Hilar Region	GFAP	65.9 ± 8.69	63.5 ± 4.82	60.6 ± 3.66	83.1 ± 6.77
	CA1	GFAP	67.6 ± 7.86	67.3 ± 6.70	68.2 ± 5.41	81.7 ± 10.51
	CA3	GFAP	66.3 ± 5.37	73.6 ± 13.07	72.1 ± 7.49	85.2 ± 9.39
	Dentate Gyrus	GFAP	80.0 ± 8.70	81.4 ± 10.74	75.4 ± 4.19	113.3 ± 16.25

Females had higher intensity/microglia than males did regardless of treatment in all three of these regions (Table S3.3; GFP hilar region: $F(7,40)=22.851$, $p<0.001$; GFP CA3: $F(7,40)=5.832$, $p=0.020$; GFP dentate gyrus: $F(7,40)=9.223$, $p=0.004$). In the CA3, Mn exposed males had lower intensity/microglia than controls (Table S3.3; $p=0.035$, $q=3.208$). Interestingly, the opposite was seen in Mn exposed females (Table S3.3; $p=0.026$, $q=3.413$). Within the CA1, only Mn exposed females were affected and they had higher

intensity/microglia than controls (Table S3.3; $F(3,20)=4.944$, $p=0.038$). Females had a higher intensity/astrocyte in all four areas than males did regardless of treatment (Table S3.3; GFAP hilar region: $F(7,40)=5.336$, $p=0.026$; GFAP CA1: $F(7,40)=42.053$, $p<0.001$; GFAP CA3: $F(7,40)=32.785$, $p<0.001$; GFAP dentate gyrus: $F(7,40)=19.655$, $p<0.001$). Three-way ANOVA also revealed a main effect of LPS within the hilar region, LPS challenged mice had a higher intensity/astrocyte than saline controls (Table S3.3; $F(7,40)=5.732$, $p=0.021$).

Table S3.4. Ventral Hippocampus signal intensity per cell. CX_3CR1^{GFP} (microglia) and glial fibrillary acidic protein (GFAP; astrocyte) immunoreactivity per cell in the ventral hippocampus after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Semi-quantitative analysis (integrated optical density) per cell. Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

			DW Treatment - LPS Challenge			
Sex	Hippocampus Region	Measurement	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Male	Hilar Region	GFP	11.4 ± 3.44	13.8 ± 1.71	10.2 ± 4.16	12.1 ± 3.15
	CA1	GFP	14.5 ± 3.11	24.2 ± 13.09	5.3 ± 5.20	14.1 ± 3.61
	CA3	GFP	19.4 ± 2.87	17.1 ± 5.38	15.1 ± 6.09	25.3 ± 9.29
	Dentate Gyrus	GFP	18.2 ± 2.69	17.6 ± 2.92	7.8 ± 4.84	15.4 ± 5.53
	Hilar Region	GFAP	20.9 ± 5.86	15.9 ± 1.28	14.5 ± 5.91	22.0 ± 6.12
	CA1	GFAP	22.8 ± 6.76	14.7 ± 6.59	8.3 ± 8.12	28.9 ± 14.28
	CA3	GFAP	29.3 ± 10.34	19.6 ± 6.10	19.2 ± 7.07	33.8 ± 13.64
	Dentate Gyrus	GFAP	32.7 ± 8.71	20.9 ± 1.77	10.9 ± 6.64	30.2 ± 7.80
Female	Hilar Region	GFP	23.2 ± 3.85	12.7 ± 1.88	**26.0 ± 2.57**	**16.1 ± 0.88**
	CA1	GFP	25.3 ± 4.75	16.6 ± 3.54	**28.2 ± 2.87**	**18.7 ± 2.18**
	CA3	GFP	39.7 ± 10.16	22.1 ± 2.13	**39.4 ± 7.06**	**22.8 ± 1.24**
	Dentate Gyrus	GFP	29.3 ± 6.62	15.2 ± 2.02	**34.9 ± 7.39**	**18.5 ± 2.13**
	Hilar Region	GFAP	22.9 ± 4.54	15.9 ± 3.24	30.5 ± 3.74	17.7 ± 1.94
	CA1	GFAP	16.2 ± 4.19	19.1 ± 3.35	**32.0 ± 7.90**	**16.7 ± 1.93**
	CA3	GFAP	26.3 ± 8.70	29.2 ± 4.38	44.4 ± 13.67	19.0 ± 2.49
	Dentate Gyrus	GFAP	32.4 ± 6.39	27.0 ± 6.79	36.6 ± 2.79	26.5 ± 4.58

Females had higher intensity /microglia than males did regardless of treatment in the hilar region (Table S3.4; $F(7,40)=13.948$, $p<0.001$), CA3 (Table S3.4; $F(7,40)=6.925$, $p=0.012$), and in the dentate gyrus (Table S3.4; $F(7,40)=8.230$, $p=0.007$). Females challenged with LPS had lower intensity/microglia than saline controls in the hilar region (Table S3.4; $F(3,20)=16.140$, $p<0.001$), in the CA1 (Table S3.4; $F(3,20)=6.893$, $p=0.016$), in the CA3 (Table S3.4; $F(3,20)=7.355$, $p=0.013$), and in the molecular layer of the

dentate gyrus (Table S3.4; $F(3,20)=8.655$, $p=0.008$). Three-way ANOVA revealed a sex x Mn x LPS effect of intensity /astrocyte in the CA1. Males that were exposed to Mn and challenged with LPS had a higher intensity /astrocyte (Table S3.4; $F(7,40)=5.357$, $p=0.026$). Within the molecular layer of the dentate gyrus, Mn+saline males had lower intensity/astrocyte (Table S3.4; $F(3,20)=4.618$, $p=0.047$).

Table S3.5. Substantia nigra intensity per cell. CX_3CR1^{GFP} (microglia) and glial fibrillary acidic protein (GFAP; astrocyte) immunoreactivity per cell in the substantia nigra pars reticulata (SNpr) and substantia nigra pars compacta (SNpc) after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

			DW Treatment - LPS Challenge			
Sex	Hippocampus Region	Measurement	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Male	SNpr	GFP	**14.1 ± 4.56**	7.8 ± 1.31	**6.1 ± 0.90**	6.4 ± 1.41
	SNpc	GFP	35.6 ± 27.10	12.0 ± 3.65	9.8 ± 2.90	7.3 ± 1.24
	SNpr	GFAP	**23.1 ± 2.22**	23.6 ± 1.58	**12.1 ± 3.52**	11.5 ± 1.21
	SNpc	GFAP	13.8 ± 2.31	11.1 ± 1.97	9.1 ± 3.80	5.5 ± 0.95
Female	SNpr	GFP	9.4 ± 0.85	9.1 ± 0.82	11.2 ± 1.37	13.0 ± 3.59
	SNpc	GFP	12.2 ± 1.71	15.6 ± 3.70	14.8 ± 1.62	19.0 ± 5.07
	SNpr	GFAP	19.7 ± 2.41	19.6 ± 3.00	22.8 ± 3.58	26.3 ± 3.09
	SNpc	GFAP	**14.6 ± 1.74**	16.1 ± 3.67	**24.7 ± 7.76**	23.8 ± 4.12

Males exposed to Mn had lower intensity /microglia in the SNpr than controls (Table S3.5; $p=0.047$, $q=2.908$). Three-way ANOVA revealed a main effect of sex of intensity/astrocyte. Females had a higher intensity /astrocyte than males in the SNpr (Table S3.5; $F(7,40)=5.097$, $p=0.030$) and in the SNpc (Table S3.5; $F(7,40)=11.746$, $p=0.002$). Males exposed to Mn had a lower intensity /astrocyte in the SNpr than controls (Table S3.5; $F(3,20)=21.533$, $p<0.001$).

Table S3.6. Brain neurotransmitters and their metabolites in male CX_3CR1^{GFP} mice after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Data are presented as mean ± SEM. Bold indicates $p<0.05$; n=6/group. Abbreviations: PFC: prefrontal cortex; vHIP: ventral hippocampus; STR: striatum; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; 3-MT: 3-methoxytyramine; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; GLN: glutamate; GLU: glutamine; GABA: gamma-aminobutryic acid.

Brain Region	Neurotransmitter or Metabolite	Treatment			
		Saline+Saline	Mn+Saline	Saline+LPS	Mn+LPS
STR	DA	97.79 ± 17.08	116.29 ± 15.39	130.55 ± 7.01	107.96 ± 5.42
	DOPAC	28.71 ± 5.84	26.44 ± 3.31	31.47 ± 3.68	24.72 ± 3.51
	HVA	12.38 ± 2.35	12.27 ± 1.28	17.65 ± 0.72	15.31 ± 1.08
	3-MT	7.83 ± 1.88	6.45 ± 0.69	9.19 ± 0.50	6.97 ± 1.62
	5-HT	1.58 ± 0.29	2.48 ± 0.75	3.00 ± 0.51	2.89 ± 0.29
	5-HIAA	2.92 ± 0.55	2.60 ± 0.34	4.97 ± 0.19	4.32 ± 0.26
	GLN	8.92 ± 1.87	11.68 ± 3.81	9.11 ± 0.98	9.02 ± 0.62
	GLU	10.76 ± 1.82	11.27 ± 1.59	13.19 ± 1.33	10.26 ± 0.68
	GABA	1.94 ± 0.39	5.86 ± 1.42	1.99 ± 0.24	4.61 ± 0.81
	GLU/GABA	5.80 ± 0.51	2.73 ± 0.84	6.79 ± 0.41	2.79 ± 0.76
vHIP	NE	5.95 ± 0.61	5.97 ± 0.75	5.35 ± 0.38	4.70 ± 0.73
	MHPG	2.74 ± 0.41	3.11 ± 0.21	2.49 ± 0.18	2.93 ± 0.30
	DA	1.40 ± 0.90	1.69 ± 0.64	1.13 ± 0.35	0.56 ± 0.20
	DOPAC	2.35 ± 0.23	2.32 ± 0.28	2.71 ± 0.28	1.96 ± 0.28

	HVA	0.79	±	0.62	0.84	±	0.33	0.44	±	0.20	0.71	± 0.12
	5-HT	5.73	±	1.20	6.91	±	0.82	6.19	±	0.49	5.34	± 1.01
	5-HIAA	6.72	±	0.80	6.15	±	0.45	8.31	±	0.90	7.93	± 1.17
	GLN	15.07	±	1.70	14.62	±	1.76	11.32	±	0.82	10.39	± 0.79
	GLU	16.49	±	1.45	16.37	±	2.43	14.09	±	0.91	14.00	± 1.28
	GABA	6.18	±	0.76	5.07	±	0.75	5.16	±	0.45	3.79	± 0.24
	GLU/GABA	2.73	±	0.14	3.23	±	0.14	2.83	±	0.27	3.70	± 0.24
PFC	NE	6.83	±	1.03	7.59	±	1.13	8.38	±	1.30	6.11	± 0.96
	MHPG	8.18	±	1.38	6.78	±	0.81	6.25	±	0.76	5.26	± 0.47
	DA	0.49	±	0.15	0.36	±	0.08	0.39	±	0.12	0.63	± 0.20
	DOPAC	3.70	±	0.62	3.47	±	0.13	2.60	±	0.40	2.22	± 0.27
	HVA	0.71	±	0.27	0.53	±	0.09	0.59	±	0.18	0.53	± 0.19
	5-HT	1.42	±	0.32	0.94	±	0.16	1.23	±	0.14	0.67	± 0.23
	5-HIAA	3.09	±	0.60	2.77	±	0.39	3.38	±	0.37	2.80	± 0.44
	GLN	8.66	±	0.68	11.84	±	0.56	7.93	±	0.80	8.53	± 1.07
	GLU	14.88	±	1.54	16.83	±	1.88	13.75	±	1.34	13.60	± 1.64
	GABA	3.20	±	0.32	4.81	±	0.32	2.73	±	0.26	3.67	± 0.46
	GLU/GABA	4.76	±	0.47	3.45	±	0.21	5.05	±	0.16	3.74	± 0.15

Table S3.7. Brain neurotransmitters and their metabolites in female CX_3CR1^{GFP} mice after 8 weeks of Mn DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg). Data are presented as mean ± SEM. Bold indicates $p<0.05$; $n=6$/group. Abbreviations: PFC: prefrontal cortex; vHIP: ventral hippocampus; STR: striatum; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; 3-MT: 3-methoxytyramine; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; GLN: glutamate; GLU: glutamine; GABA: gamma-aminobutryic acid.

Brain Region	Neurotransmitter or Metabolite	Treatment			
		Saline+Saline	Mn+Saline	Saline+LPS	Mn+LPS
STR	DA	121.34 ± 8.84	113.64 ± 7.88	111.35 ± 4.49	83.20 ± 12.39
	DOPAC	25.08 ± 1.42	20.95 ± 1.51	23.07 ± 1.45	20.63 ± 3.14
	HVA	10.37 ± 1.06	8.82 ± 0.60	13.40 ± 0.51	11.52 ± 1.64
	3-MT	8.19 ± 1.02	6.31 ± 0.58	6.32 ± 0.34	5.75 ± 0.97
	5-HT	3.22 ± 0.23	2.41 ± 0.30	6.00 ± 0.55	5.72 ± 2.16
	5-HIAA	3.32 ± 0.15	2.31 ± 0.17	4.99 ± 0.26	5.04 ± 0.62
	GLN	7.57 ± 0.32	6.47 ± 0.31	11.00 ± 2.93	6.55 ± 0.48
	GLU	12.55 ± 0.57	10.51 ± 0.53	10.77 ± 0.67	9.55 ± 0.68
	GABA	2.22 ± 0.23	1.99 ± 0.11	2.16 ± 0.14	2.39 ± 0.29
	GLU/GABA	6.10 ± 0.93	5.36 ± 0.40	5.06 ± 0.38	4.23 ± 0.45
vHIP	NE	6.59 ± 0.27	6.74 ± 0.44	6.04 ± 0.72	6.42 ± 0.60
	MHPG	4.66 ± 0.43	3.88 ± 0.57	4.75 ± 0.53	3.93 ± 0.43
	DA	1.42 ± 0.31	1.27 ± 0.17	1.45 ± 0.25	1.28 ± 0.26
	DOPAC	2.68 ± 0.21	3.36 ± 0.24	3.30 ± 0.42	3.40 ± 0.28

	HVA	0.59 ± 0.17	0.40 ± 0.07	0.82 ± 0.27	0.74 ± 0.39
	5-HT	8.80 ± 0.67	8.32 ± 0.88	10.21 ± 1.80	9.34 ± 1.36
	5-HIAA	9.53 ± 0.78	7.75 ± 0.94	15.20 ± 2.66	12.53 ± 1.62
	GLN	10.27 ± 1.05	11.40 ± 1.42	8.50 ± 1.36	8.22 ± 0.93
	GLU	11.84 ± 1.43	14.21 ± 1.84	10.26 ± 1.83	10.26 ± 1.29
	GABA	5.04 ± 0.63	5.14 ± 0.69	3.58 ± 0.45	3.72 ± 0.64
	GLU/GABA	2.51 ± 0.35	2.77 ± 0.04	2.87 ± 0.30	2.90 ± 0.30
PFC	NE	4.00 ± 0.48	6.13 ± 0.46	3.37 ± 0.54	4.14 ± 0.45
	MHPG	5.18 ± 0.60	6.82 ± 0.71	5.95 ± 1.24	6.81 ± 0.87
	DA	0.30 ± 0.05	0.86 ± 0.34	0.59 ± 0.08	0.66 ± 0.19
	DOPAC	3.08 ± 0.29	4.50 ± 0.47	2.49 ± 0.68	2.91 ± 0.61
	HVA	0.36 ± 0.12	0.53 ± 0.11	0.45 ± 0.10	0.51 ± 0.09
	5-HT	0.55 ± 0.22	0.20 ± 0.10	0.31 ± 0.12	0.28 ± 0.14
	5-HIAA	2.52 ± 0.36	3.35 ± 0.43	3.35 ± 0.52	5.14 ± 0.51
	GLN	7.12 ± 0.74	10.99 ± 0.66	7.01 ± 1.22	9.53 ± 0.77
	GLU	12.44 ± 1.17	15.43 ± 2.11	10.64 ± 1.98	13.68 ± 1.35
	GABA	8.07 ± 1.36	13.67 ± 3.23	5.55 ± 1.00	11.16 ± 0.70
	GLU/GABA	1.75 ± 0.27	1.39 ± 0.29	2.09 ± 0.29	1.26 ± 0.15

CHAPTER 4

BEHAVIORAL EFFECTS OF MANGANESE DRINKING WATER EXPOSURE AND A LPS CHALLENGE IN ADULT MIRNA-155 KNOCKOUT MICE WITH A COMPARATIVE BEHAVIORAL ANALYSIS TO WILD TYPE MICE

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Abstract

Manganese (Mn) is an important essential metal; however, overexposures have been shown to influence a variety of adverse physiological and behavioral outcomes. Excessive microglia activation, with resulting increase in proinflammatory cytokine production, has been implicated in Mn neurotoxicity. During inflammation, expression of the proinflammatory microRNA-155 (miR155) is increased overall, but particularly in the brain. While we explored how the nervous and immune systems of males and females respond to excessive Mn in the drinking water (DW), in this study we investigated behavioral and sex differences in response to subchronic Mn DW treatment and a lipopolysaccharide (LPS) challenge of adult miR155 knockout (KO) and wild-type (WT) mice. After 6 weeks on Mn DW, in motor function tests, Mn exposure resulted in reduced activity in both strains, but the WT mice were impacted more. In tests of mood (open field test [OFT]/elevated zero maze), Mn-exposed WT mice, but not KO mice, exhibited fearlessness-like behaviors. Two weeks after behavioral assessment, mice were challenged with LPS and both strains showed typical sickness behavior during post-LPS OFT. Peripheral cytokines and acute phase proteins were increased in both sexes and strains, and Mn alone did not affect peripheral cytokine output in either sex/strain. In WT mice, Mn-exposed/LPS-challenged males had potentiated plasma cytokine output, and this was only observed for TNFα of Mn-exposed/LPS-challenged KO females. Together, these findings indicate that some locomotor deficits and the mood alterations observed in Mn-exposed WT mice are ameliorated with miR155 is missing. This study provides insight into the complex interactions between genetics, sensitivity to innate immune stimuli, and sex in susceptibility to metal (Mn in this case) overexposures.

Keywords: Acute Phase Proteins | miRNA-155 | Cytokines | LPS | Manganese | Sex Differences

1 Introduction

Manganese (Mn) is a naturally occurring, ubiquitous essential metal (ATSDR, 2012; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Filipov et al., 2005; Krishna et al., 2014). Even though Mn is an essential metal and an important cofactor for multiple biological processes (J. L. Aschner & M. Aschner, 2005; ATSDR, 2012; Chen et al., 2018; IOM, 2001), overexposure to Mn is neurotoxic (Chen et al., 2018; Erikson et al., 2007) through mechanisms including oxidative stress, disruption of neurotransmitter systems, and mitochondrial dysfunction (Dodd & Filipov, 2011; Fernandes et al., 2023; Gunter et al., 2012; Krishna et al., 2014; Pajarillo et al., 2021; Tuschl et al., 2013). Prolonged low-level exposure to Mn can result in mood disturbances such as irritability, depression, compulsive behavior, and anxiety, as demonstrated in both clinical and preclinical studies (Bouchard et al., 2007; Bowler et al., 2011; Dodd et al., 2005; Krishna et al., 2014; Laohaudomchok et al., 2011; Liu et al., 2019; Ludwig et al., 2024). Mn overexposure causes activated microglia, the immune cell of the brain, to overproduce cytokines and other proinflammatory mediators (Crittenden & Filipov, 2008; Crittenden & Filipov, 2011; Filipov & Dodd, 2012; Zhao et al., 2009); the Mn-inflammagen, such as lipopolysaccharide (LPS), interaction is concentration dependent (Filipov et al., 2005).

microRNAs (miRNAs) are short, single-stranded, 18-25 nucleotides in length, non-coding conserved RNAs that can silence or express target genes, at the post-transcriptional level, by binding to complementary RNA sequences (Gaudet et al., 2018; Grogg et al., 2016; Mashima, 2015; Yin et al., 2017). miRNA-155 (miR-155) has a regulatory role in the signaling pathways of various cells including dendritic cells, T cells, B cells, and macrophages (Mashima, 2015). Within macrophages and, by extension, microglia, miR-155 expression is regulated by toll like receptor ligands (TLR), such as poly(I:C) and LPS (TLR3 and TLR4 ligands respectively) (Thounaojam et al., 2013). Inflammatory cytokines and chemokines, namely interferon gamma (IFN- γ) and interferon beta (IFN-β) also regulate miR-155 expression (Thounaojam et al., 2013). miR-155 is considered largely a proinflammatory miRNA; it is upregulated in both

inflammatory and neurological disorders and that when miR-155 is upregulated there is an increase in proinflammatory cytokine production (Gaudet et al., 2018). It has been reported that insoluble Mn exposure increase proinflammatory cytokines TNF-α and IL-6, as seen with soluble Mn, but this was associated with a decrease in miR-155 expression (Grogg et al., 2016). This suggests that Mn particles interact with innate immune cells very differently than inflammagens and soluble Mn. For example, miR-155 expression increases in cells that have been exposed to LPS (Yin et al., 2017). The down-regulation of miR-155 in this study (Yin et al., 2017) also deactivated the mitogen activated protein kinase (MAPK)/nuclear factor kappa-B (NF-κB) pathway; both of these pathways are activated by manganese (as $MnCl_2$) exposure (Crittenden & Filipov, 2008; Crittenden & Filipov, 2011; Filipov et al., 2005). Since soluble Mn enhances MAPK and NF-κB activity both individually and particularly when paired with LPS (Filipov et al., 2005), the effects of soluble Mn found in drinking water might be like the effects of LPS or synergistic to it when it comes to miR-155 expression. Therefore, it is probable that when microglia are pathologically activated with a bacterial stimulus, such as LPS, as well as by exposure to elevated levels of soluble Mn, miR-155 is upregulated and involved in the inflammatory and perhaps behavioral (Ludwig et al., 2024) effects of Mn.

Combining the knowledge that manganese accumulates in brain regions associated with motor and cognitive function (Krishna et al., 2014) and induces stress responses/neurotoxicity (Chen et al., 2018; Erikson et al., 2007; Dodd & Filipov, 2011), it becomes crucial to investigate the impact of Mn exposure in the presence and absence of miR-155. miR-155, especially in non-neuronal cells in the brain or periphery, may modulate neuronal responses to Mn toxicity, potentially altering the progression of Mn-induced neurological dysfunction. Specific focus on how miR-155 influences the inflammatory pathways could reveal mechanistic roles of miR-155 in Mn-induced behavioral alterations. Moreover, understanding the role of miR-155 in modulating neuroinflammatory responses could aid in identifying potential therapeutic targets, such as microRNA based therapies to counteract the neurotoxic effects of Mn exposure.

Therefore, this study aims at exploring how the absence of miR-155 impacts the behavior in male and female mice when exposed to subchronic low levels of Mn via the drinking water, with the addition of an LPS challenge. This study also investigates whether Mn-induced behavioral changes are present in the absence of miR-155 and whether these effects are more or less severe than when miR-155 is present, across both male and female subjects.

2 Materials and Methods

2.1 Reagents

All chemicals utilized, unless otherwise stated were purchased from Sigma Aldrich (St. Louis, MO), including LPS (*Escherichia coli* serotype 0111: B4) and manganese ($MnCl_2 \cdot 4H_2O$).

2.2 Animals, Treatment, and Tissue Collection

All procedures that involved handling animals were approved in advance by the University of Georgia Institutional Animal Care and Use Committee (IACUC) and were in accordance with the latest National Institutes of Health and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. In total, 55 mice (males: 27 and females: 28) that were homozygous for B6.Cg-Mir^{tm1Rsky}/J mice (*lacZ*-polyA::*loxP*-hUBC-neo-polyA-*loxP* [miR155KO]), on a C57BL/6 background (Jackson Labs, stock 007745) and 53 CX_3CR1^{GFP} (wild type [WT]; Jackson Labs, stock 005582) (males: 25, females: 28) were used for all experiments with behavior tests, brain Mn levels, and cytokine levels WT mice reported in Ludwig et al. (2024). Same-sex mice were group-housed (2-5 per cage) with PicoLab Rodent Diet 20 (LabDiet, 5053) available ad libitum and housed in an AAALAC accredited facility throughout the study, in an environmentally controlled room (22°C–24°C) with a relative humidity of 50%–70% and maintained on a 12-hour light/dark cycle. Mice were acclimated to using water bottles 3 weeks prior to the start of the study. Then, they were randomly assigned to drinking water treatment groups and were 2 months old at treatment onset.

The mice were exposed to vehicle control (NaCl; 0.4 g Na/L) or $MnCl_2$ (0.4 g Mn/L) (n = 12–15/group) in deionized water for 8 weeks. The water bottles were changed weekly with

freshly prepared control or Mn solutions. Body weight (BW), water intake, and estrus cycle stages (females only) were recorded weekly. The Mn DW concentration that was utilized in this study was chosen based on previous works that had resulted in significant and human-relevant brain Mn deposition (Avila et al., 2010; Krishna et al., 2014) as well as behavioral alteration in C57BL/6. After 6 weeks of DW treatment, behavior testing was carried out (n = 12/group) while maintaining the mice on their respective DW treatments. At the end of the 8-week exposure period, mice within each sex and treatment group were randomly selected to receive an intraperitoneal (IP) injection of either LPS (*E. coli* serotype 0111: B4 1.5×10^{12} EU or 0.3 mg/kg BW) or saline vehicle (n = 6/group). Four hours post LPS/vehicle treatment, sickness behavior was assessed in an open field test (OFT) and 2 hours later (6 hours post LPS/vehicle treatment) mice were sacrificed. Brains were extracted, weighed, washed in ice-cold HEPES—buffered Hank's saline solution (pH 7.4), and separated longitudinally into two hemispheres. One half was fixed in 4% paraformaldehyde, cryoprotected, and stored at −80°C, while the other half was fresh frozen on dry ice and were also stored at −80°C for future analyses. Additionally, organs (kidneys, liver, lymph nodes, spleen, and thymus) and fats (epididymal/ovarian, retroperitoneal, subcutaneous, and brown adipose tissue) were collected, weighed, and stored at −80°C; plasma was collected and stored at −80°C for enzyme-linked immunosorbent assays as described later.

2.3. Behavior Testing

The behavior tests utilized in this study were to assess locomotion and mood behaviors, which are known to be affected by Mn in rodents (Conrad et al., 2011; Krishna et al., 2014; Sepúlveda et al., 2012) as well as in humans exposed to Mn (Bouchard et al., 2007). Male and female mice were tested separately, with all equipment thoroughly cleaned between testing sessions for the different sexes. All mice underwent the tests in the same order, as presented below, and were naïve to the testing apparatuses before testing began. All of the tests were conducted and analyzed by an experimenter who was blind to the DW and LPS treatment

conditions as well as strain of mice. The behavior tests were carried out in a designated room for behavior testing, which was located in the same facility and near the animal housing room.

2.3.1 Open Field Test (OFT)

An open field area (25 cm × 25 cm × 40 cm, divided into a 16 square grid; Coulborn Instruments, Whitehall, PA) was used for the 30 min testing period, as in (Dockman et al., 2022; Krishna et al., 2016; Krishna et al., 2014) to evaluate locomotor function and anxiety-like behavior. Limelight tracking software (Actimetrics, Wilmette, IL) was used to monitor mice and record a video of each test. ANY-maze software (Stoelting Co., Wood Dale, IL) was used to assess and score locomotor activity per 5-minute interval and for the total 30-minute test. Parameters of interest include the total distance traveled (locomotor activity), time spent in the center versus time spent in the periphery (evaluating anxiety-like behavior), and the number of rearings (vertical locomotor activity). The same OFT arena, but ran for 15-minute duration, was used to assess sickness behavior post-LPS/vehicle treatment (further detailed in Section 2.3.4).

2.3.2 Gait Test (GT)

Motor function was evaluated by using a GT as in Carpenter et al. (2021). An 82 cm × 5.5 cm × 8 cm (l × w × h) runway, lined with white paper, with an empty cage containing home cage bedding placed at the end of the runway. Two trials, 5 minutes apart, were conducted - trial one was a training pre-trail and trial two was used for statistical analyses. The front and hind paws were freshly painted with nontoxic red and black ink, respectively (Office Depot, item #839994 and #839967 respectively), prior to each trial, and the mouse was allowed to walk across the runway. Between each trail, the runway was cleaned with 70% ethanol, dried, and lined with a new strip of paper. The gait parameters that were assessed include stride length, base width, interstep/intrastep distance, stride variability, total number of steps and cadence as described in (Carpenter et al., 2021; Mulherkar et al., 2013; Wang et al., 2017).

2.3.3 Elevated Zero Maze (EZM)

To further assess anxiety-like behaviors, an EZM apparatus (50-cm diameter; Stoelting Co., Wood Dale, IL) was used as in Carpenter et al. (2021), with locomotor activity monitored as well. Mice were placed at the center of an open quadrant facing inward, at the start of the test, and allowed to freely explore the maze during the 5-minute testing duration. Parameters of interest included: latencies to enter/exit a closed area for the first time, time spent in open and closed arms, number of head dips, and number of stretch attend posture attempts, as in Grewal et al. (1997). A mouse was considered to be in a zone when at least 70% of the body was in that zone. The parameters of the EZM were tracked and scored using ANY-Maze software (Stoelting).

2.3.4 Post-LPS Challenge (Post-LPS OFT)

At the end of the 8-week Mn exposure period, mice were randomly selected to receive either saline vehicle or LPS (0.3-mg/kg BW) IP injection, within each sex and treatment group. This dose has been previously found to stimulate peripheral and central inflammatory response and sickness associated behavioral changes in both adult and middle-aged mice on C57BL/6 background (Dockman et al., 2022; Krishna et al., 2016). Four hours after LPS/vehicle treatment, the mice underwent a 15 minute OFT, with the same parameters of interest as the novel OFT monitored and scored.

2.4 ELISA

Plasma concentrations of interleukin 6 (IL-6), interleukin 10 (IL-10), and tumor necrosis factor alpha (TNFα) were analyzed 6 hours post-LPS/vehicle administration using mouse-specific ELISA kits (Bio-Techne, Minneapolis, MN) following the manufacturer's directions. The samples and standards were ran in duplicate, IL-6 (1000-15.625 pg/mL), TNFα (2000-31.25 pg/mL), IL-10 (2000-31.25 pg/mL), CRP (1500–23.4 pg/mL), and SAA (16,000–250 pg/mL). The absorbance (450-nm analytical read; 570-nm background correction read) was

measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT), and the mean from the individual sample replicates was used for statistical analysis.

2.5 Statistical Analysis

All data were analyzed using Sigmaplot v12. (Systat Software, Inc., Chicago, IL) all graphs were created using GraphPad Prism v8.4.3 (San Diego, CA). Two-way ANOVA was used to assess behavior parameters, pre-LPS, and two-way repeated measure ANOVA was used to assess behavior parameters across 5-minute intervals for the OFT. Three-way ANOVA was used to assess post-LPS OFT behavior parameters and two-way ANOVA within sex. All Body/organ weights, water intake, estrus staging and ELISA data were analyzed by using two-way ANOVA. For all ANOVAs, the F-value is displayed as $F(df, df)$ with the degrees of freedom designated as (number of groups-1, total mice-number of groups). A p-value of ≤ 0.05 was considered significant. A Student-Newman-Keuls (SNK) post hoc comparison was run if significant main effects or treatment/sex interactions were detected, with the statistical designation as (p-value, q-value).

3 Results – Male vs Female miR155KO

3.1 Body Weight (BW), Water Intake, Organ Weight, and Estrus Cycle

BWs were unaffected by Mn (Figure 4.1A; $F(3,45)<0.0152$, $p>0.1$) for either sex throughout the entire study. Males in the Mn DW group gained about 26% and males in the saline DW group gained about 23% by the end of the 8-week period. During weeks 1, 6, and 7 Mn-exposed males did have a higher percent BW change than control males (Figure 4.1B; $F(1, 23)>5.342$, $p<0.031$) and during week 8 there was only a trend for Mn-exposed males to have a higher percent BW change than controls ($F(1,23)=3.888$, $p=0.061$). Females in the Mn DW group gained about 17% and females in the saline DW group gained about 19% BW by the end of the 8-week exposure period and there were not any significant differences in percent BW change among the females. Water intake by both male and female Mn-exposed mice, throughout the entire experimental period, was similar to their respective controls (Figure 4.2).

Mn consumption did not affect the estrus cycle ($F(1,22)<0.600$, $p>0.1$) Figures 3 and 4); both control and Mn-exposed females continued to cycle throughout the study. After 8 weeks of Mn exposure, the relative weights of brain, kidney, liver, spleen, brown, subcutaneous, epididymal/ovarian, or retroperitoneal adipose tissues were not affected by Mn in either sex (Table 4.1; $F(3,20)<1.032$, $p>0.1$). Females exposed to Mn did have heavier thymus than controls ($F(3,20)=8.726$, $p=0.008$). In terms of absolute organ and adipose tissue weights, mice were unaffected by Mn exposed in either sex (Table 4.2; $F(3,20)<0.978$, $p>0.1$). Males challenged with LPS had heavier spleens (absolute; Table 4.2; $F(3,20)=14.942$, $p<0.001$) and relative weights than their respective controls ($F(3,20)=14.948$, $p<0.001$). Females challenged with LPS had heavier kidney and spleens (both absolute and relative) and brain (absolute weight only) than their respective controls.

3.2 Behavioral Analysis

3.2.1 OFT

After six weeks of Mn DW exposure, a two-way ANOVA revealed an overall main effect of Mn on locomotor activity with Mn-exposed KO mice being less active, with respect to total distance traveled (Figure 4.5A; $F(3,44)=4.134$, $p=0.048$). Females were less active than males in both distance traveled (Figure 4.5A; $F(3,44)=10.306$, $p=0.002$) and in the number of rearings (Figure 4.5B; $F(3,44)=7.021$, $p=0.011$) and this decreased activity was driven mainly by Mn-exposed females being generally less active ($p=0.015$, $q=2.534$). Over the 30-minute test duration, locomotor activity decreased ($F(3,44)=8.136$, $p=0.007$) irrespective of Mn and sex, as all mice habituated to the arena (Figure 6). Mn-exposure was not associated with any alterations in anxiety-like or fearlessness-like behaviors in either sex. However, females spent significantly less time in the periphery (Figure 4.5C; $F(3,44)=4.061$, $p=0.050$) and more time in the center (Figure 4.5D; $F(3,44)=6.776$, $p=0.013$) of the arena during the first 5-minute exploration phase of the test. This effect was more prominent in the Mn-exposed females but was not statistically significant.

3.2.2 GT

Mn-exposed KO mice, specifically males, had a shorter overlap distance (Figure 4.7D; $F(3,44)=7.133$, $p=0.011$) and a longer stride length (Figure 4.7B; $F(3,44)=26.158$, $p<0.001$) than controls. Female mice took more steps to complete the test than males (Figure 7A; $F(3,44)=19.491$, $p<0.001$). Additionally, female mice had a shorter interstep distance (Figure 4.7C; $F(3,44)=5.366$, $p=0.025$) and stride length (Figure 4.7B; $F(3,44)=26.158$, $p<0.001$).

3.2.3 EZM

Neither Mn DW exposure nor sex had any statistically significant effect on the different EZM parameters that were evaluated. There were a few trending effects of sex, where females spent more time in the open arms and had a greater number of stretch attend posture attempts in the open arm than males, and males spent more time in the closed arm than females.

3.2.4 Post-LPS OFT

After 8 weeks of Mn DW exposure, a three-way ANOVA revealed main effects of sex and LPS on locomotor activity. Males were more active than females with respect to the total distance traveled (Figure 4.8; $F(3,20)=11.117$, $p=0.002$). LPS challenged mice, regardless of Mn treatment or sex, were less active in terms of distance traveled (Figure 4.8A; $F(3,20)=13.460$, $p<0.001$) and rearing activity (Figure 4.8B; $F(3,20)=4.085$, $p=0.050$) during the first 5-minute interval. Over the 15-minute test duration, all mice habituated, and locomotor activity decreased regardless of Mn, sex, and LPS ($F(3,20)=14.042$, $p<0.001$). There were overall main effects of Mn on the times spent in the periphery and center of the open field arena with Mn-exposed mice spending more time in the periphery and less time in the center (Figure 4.8C; $F(3,20)=6.261$, $p=0.017$, and Figure 4.8D; $F(3,20)=6.222$, $p=0.017$, respectively). This Mn effect was predominantly driven by Mn-exposed, vehicle-challenged (Mn+saline) males.

3.3 Plasma Cytokine and Acute Phase Protein ELISAs

In the absence of LPS, plasma IL-6 and IL-10 were undetectable regardless of Mn-exposure and sex (Figure 4.9A, C respectively). Six hours after the LPS administration, there

was a significant increase in plasma IL-6, TNFα, and IL-10 in both Mn and control mice of both sexes. Within LPS challenged mice, a two-way ANOVA revealed an overall main effect of sex, with females' plasma TNFα and IL-10 levels being greater than in males (Figure 4.9B; $F(3,20)=14.251$, $p<0.001$, and Figure 4.9C; $F(3,20)=21.416$, $p<0.001$, respectively). Notably, within LPS, female mice treated with Mn had diminished plasma TNFα levels compared with saline+LPS females (Figure 4.9B; $p=0.003$, $q=4.489$). In both sexes, both plasma SAA (Figure 4.9D; $F(3,20)=397.673$, $p<0.001$) and CRP (Figure 4.9E; $F(3,20)=41.047$, $p<0.001$) increased after LPS administration, and this increase was unaffected by Mn ($F(3,20)<0.284$, $p>0.1$).

4 CX_3CR1^{GFP} (Wild type; WT) and miR15KO (KO) Comparisons

4.1 Male WT and male KO Comparisons

4.1.1 Body Weight, Water Intake, Organ Weight

When comparing male WT and KO mice, the average body weights of either strain were unaffected by Mn consumption, except within WT males there was an end-of-study Mn-induce weight increase (Figure 4.10A; $F(1,23)=5.498$, $p=0.028$). WT males in the control group gained about 20%, WT males in the Mn group gained about 17%, KO males in the Mn group gained about 26% and KO males in the saline group gained about 23% by the end of the 8-week period. Mn-exposed males had an increased percent BW change during weeks 1, 6, and 7 and this Mn effect was driven more by the Mn-exposed KO males (Figure 4.10B; $F(3,44)=15.368$, $p<0.001$). Every week, both the average BW and percent change in BW, the KO males regardless of DW treatment weighed more than the WT males ($F(3,44)=40.831$, $p<0.001$). Throughout the study, water intake by both Mn-exposed WT and KO males was similar to their respective controls (Figure 4.11). There was a week-dependent strain effect, such that during weeks 6, 7, and 8 ($F(3,44)=6.218$, $p=0.030$) KO males consumed more water than WT males. Eight weeks of Mn exposure did not affect relative brain, kidney, liver, spleen, thymus, brown adipose, subcutaneous adipose, retroperitoneal adipose, or epididymal adipose tissues in either strain (Table 4.4; $F(3,44)<2.908$, $p>0.1$). WT males, but not KO males, challenged with LPS had

heavier livers (absolute; Table 4.5, and relative) than controls. KO males challenged with LPS, but not WT males, had heavier spleens than vehicle controls (Table 4.4; $F(3,20)=14.948$, $p<0.001$, and Table 5; $F(3,20)=14.942$, $p<0.001$). Mn-exposed WT males, both with and without LPS, had heavier livers than controls (Table 4.4; $F(3,20)=7.096$, $p=0.015$). WT males regardless of DW treatment or LPS challenge had heavier brain and liver than KO males (Table 4.4; $F(3,44)>4.683$, $p<0.001$). Interestingly, KO males, regardless of DW treatment or LPS challenge, had heavier adipose tissues (BAT, SQ, ET, and RT), both relative and absolute, than WT males ($F(3,44)>14.567$, $p<0.001$) as well as heavier thymus (absolute; $F(3,44)=13.625$, $p<0.001$).

4.1.2 OFT

After 6 weeks of Mn DW exposure, Mn did not have an effect on horizontal locomotor activity, both WT and KO males had similar travel distance (Figure 4.12A; $F(3,44)=0.549$, $p=0.463$) and number of grid crossings (stats; data not shown). Mn did have an effect on vertical activity (rearings), Mn-exposed WT males reared less than controls (Figure 4.12B; $F(3,44)=5.631$, $p=0.022$). A two-way ANOVA revealed an overall main effect of strain on rearing activity, with KO males rearing more than WT males and within Mn, Mn-exposed KO males reared more than Mn-exposed WT males ($p=0.026$, $q=2.312$). All mice habituated to the arena over time (Figure 13). There was not an overall Mn effect on anxiety-like or fearlessness-like behaviors between WT and KO males. Mn-exposed WT males spent less time in the periphery (Figure 4.12C; $F(1,23)=4.129$, $p=0.048$) and more time in the center (Figure 4.12D; $F(1,23)=5.431$, $p=0.024$) than controls. KO males, regardless of DW treatment spent similar times in the periphery and center of the arena as saline treated WT males ($F(3,44)=2.041$, $p=0.160$).

4.1.3 GT

Mn-exposed WT males took more steps to complete the test than controls (Figure 4.14A; $F(1,23)=4.120$, $p=0.048$), while KO males regardless of DW treatment were not

significantly different in the number of steps taken than WT control males. Mn-exposed KO males had a longer stride length than their saline treated KO males (Figure 4.14B; p=0.022, q=2.308), this Mn-induced increase in stride length was not different from WT controls (F(3,44)=2.377, p=0.125). There was a main effect of strain, where saline treated WT males had a longer stride length (p=0.009, q=2.635) and hindpaw interstep distance than saline treated KO males (Figure 4.14C; p=0.012, q=2.627), while Mn-exposed KO males were not significantly different from WT controls. A two-way ANOVA did reveal a main effect of Mn on fore/hindpaw overlap. Mn-exposed mice had a smaller overlap than controls (Figure 4.14D; F(3,44)=8.223, p=0.006) and this was driven by saline treated KO males having a wider overlap distance than either Mn-exposed KO males and saline treated WT males (F(3,44)=11.421, p=0.002).

4.1.4 EZM

In the EZM, Mn-exposed WT males spent more time in the open arms (Figure 4.15A; p=0.014, q=2.553) and less time in the closed arms (Figure 4.15B; p=0.017, q=2.469) than their controls. Mn-exposed WT males also displayed an increase in the number of closed and open arm entries, which was male driven (q=2.145, p<0.001; data not shown). Additionally, Mn-exposed WT males had fewer stretch attempts in the closed arm than controls (Figure 4.15C; p=0.002, q=3.236). These Mn effects were not present in the KO males.

4.1.5 Post-LPS OFT

After 8 weeks of Mn DW exposure, a three-way ANOVA revealed the main effect of LPS on locomotor activity. The LPS challenged males, regardless of strain or Mn treatment, traveled less distance (Figure 4.16A, F(3,20)=5.750, p=0.021) and had decreased vertical activity (Figure 4.16B; F(3,20)=4.590, p=0.038) during the first 5-minute interval. Over the 15-minute test duration, all mice habituated to the arena and locomotor activity decreased irrespective of Mn, LPS, and strain (F(3,20)=40.829, p<0.001). Within saline challenged males, Mn-exposed WT males spent less time in the periphery (Figure 4.16C; p=0.025, q=2.325) while the opposite was observed in the KO males (p=0.002, q=3.312). Additionally, saline treated WT males spent

more time in the periphery than saline treated KO males (p<0.001, q=4.089). Saline challenged and Mn-exposed (Mn+saline) WT males spent more time in the center than their controls (Figure 4.16D; *p*=0.029, *q*=2.259) and the opposite was seen in the saline challenged Mn-exposed KO males (*p*=0.002, *q*=3.291). Furthermore, saline treated KO males spent significantly more time in the center than WT males (*p*<0.001, *q*=4.123). Within LPS challenged males, saline treated WT males spent more time in the periphery than saline treated KO males (*p*=0.001, *q*=3.524). Saline treated KO males spent more time in the center than WT males (*p*=0.001, *q*=3.525). Mn-exposed WT males spent more time in the center than controls (*F*(3,20)=9.838, *p*=0.003), and while not significant, Mn-exposed KO males did numerically spend less time in the center than saline treated KO males.

4.1.6 Plasma Cytokine and Acute Phase Protein ELISAs

In the absence of LPS, plasma IL-6, TNFα, and SAA were undetectable regardless of Mn treatment and strain (Figure 4.17A, B, and D, respectively). Six hours after a single administration of LPS, increased plasma levels of IL-6, TNFα, SAA, and CRP were observed in Mn and control mice of both strains (Figure 4.17A, B, D, and E respectively; *F*(3,44)>8.071, *p*<0.001). A two-way ANOVA, within LPS challenged mice, revealed a main effect of strain, with WT males having lower plasma IL-10 (Figure 4.17C; *F*(3,20)=69.887, *p*<0.001) and SAA (Figure 17D; *F*(3,20)=10.687, *p*=0.002) than KO males. Baseline IL-10 (without LPS) was higher in WT males than in KO males (Figure 4.17C; *F*(3,20)=17.816, *p*<0.001) and decreased after LPS administration, whereas the reverse was seen in KO males. Baseline CRP was not significantly different between WT and KO males, and it increased in both strains after LPS challenge.

4.2 Female WT vs Female KO Comparisons

4.2.1 Body Weight, Water Intake, Organ Weight, and Estrus Cycle

When comparing female WT and KO mice, the body weights were unaffected by Mn (Figure 4.18A; *F*(3,44)=, *p*=0.930). During weeks 1, 3, 4, 5, and 6 KO females, regardless of DW treatment, weighed more than WT females (*F*(3,44)>5.311, *p*<0.001). By the end of the

study, WT females gained about 20% for both treatment groups (Figure 4.18B), KO females in the Mn DW group gained about 17% and KO females in the saline DW group gained about 19% BW by the end of the 8-week exposure period. During weeks 2 and 6 KO females did have a higher percent BW change than WT females ($F(3,44)>11.049$, $p=0.002$), and during week 4 Mn-exposed females regardless of strain had a higher percent BW change than controls ($F(3,44)=4.807$, $p=0.034$). Throughout the entire experimental period, water intake of both WT and KO Mn-exposed females were similar to their respective controls (Figure 4.19). There was a week-dependent strain effect, where during weeks 3, 4, 6, 7, and 8 ($F(3,44)>5.665$, $p=0.013$), KO females consumed more water than WT females, this was only a trending strain effect during weeks 1 and 2. Mn consumption did not affect estrus cycle of either strain ($F(3,44)<0.600$, $p>0.1$; Figures 4.20 and 4.21); Mn-exposed and control females of both strain continued to cycle throughout the study. However, numerically the KO females were in a not receptive stage more frequently than WT females. Eight weeks of Mn exposure did not affect relative organ or adipose tissue weights of WT females (Table 4.5; $F(3,20)<2.908$, $p>0.1$). it did cause an increase in thymus weight of KO females ($F(3,20)=8.726$, $p=0.008$) without affecting the other organs weights. Females, regardless of strain or LPS challenge, that were exposed to Mn had lighter ovarian adipose tissue than females that were treated with saline (Table 4.5; $F(3,44)=4.735$, $p=0.036$). LPS challenged KO females had heavier kidney and spleens (Table 4.5, 4.6; relative and absolute weight respectively) than their respective controls ($F(3,20)>14.942$, $p<0.001$). WT females, regardless of DW treatment or LPS challenge, had heavier brain and liver weights, in terms of relative weight ($F(3,44)>4.875$, $p<0.001$) and lighter ovarian and retroperitoneal adipose tissue weights (both relative and absolute; $F(3,44)>8.344$, $p<0.001$) than KO females.

4.2.2 OFT

After 6 weeks of Mn DW exposure, Mn did not have an effect on horizontal locomotor activity as both WT and KO females traveled similar distance ($F(3,44)=0.682$, $p=0.413$; data not

shown) and similar number of grid crossings ($F(3,44)=2.508$, $p=0.12$; data not shown). A two-way ANOVA revealed main effects of Mn and strain on vertical locomotor activity (number of rearings). Mn-exposed females made fewer rearing attempts than their respective controls (Figure 4.23A; $F(3,44)=9.027$, $p=0.004$). Both saline treated and Mn-exposed KO females made more rearing attempts than their WT counterparts ($p=0.031$, $q= 2.230$). All mice habituated to the arena during the 30-minute test duration and locomotor activity decreased regardless of Mn and strain (Figure 4.23). WT females, regardless of DW treatment, spent more time in the center of the arena (Figure 4.22B; $F(3,44)=6.302$, $p=0.016$) than KO females, and this was driven by the Mn-exposed WT females spending more time in the center.

4.2.3 GT

A two-way ANOVA revealed a main effect of strain on total steps, stride length, interstep distance, and fore/hindpaw overlap distance. WT females took fewer steps to complete the test than KO females (Figure 4.24A; $F(3,44)=4.390$, $p=0.042$), which was predominantly driven by saline treated WT females taking fewer steps than either Mn-exposed WT females ($p=0.042$, $q=2.095$) or saline treated KO females ($p=0.036$, $q=2.165$). KO females had a shorter interstep distance than WT females (Figure 4.24C; $F(3,44)=4.734$, $p=0.035$) and WT females had a smaller fore/hindpaw overlap than KO females (Figure 4.24D; $F(3,44)=5.934$, $p=0.019$). WT females had a longer stride length than KO females (Figure 4.24B; $F(3,44)=39.54$, $p<0.001$) and this was driven by Mn-exposed females having a shorter stride length than controls ($F(3,44)=7.004$, $p=0.009$).

4.2.4 EZM

In the EZM, Mn did not have an effect between WT and KO females. WT females spent less time in the open arm (Figure 4.25A; $F(3,44)=18.847$, $p<0.001$) and more time in the closed arms (Figure 4.25B; $F(3,44)=15.584$, $p<0.001$) than KO females. Additionally, WT females made more closed arm stretch attempts than KO females (Figure 4.25C; $F(3,44)=25.305$, $p<0.001$).

4.2.5 Post-LPS OFT

After 8 weeks of Mn DW exposure, a three-way ANOVA revealed a main effect of LPS on locomotor activity. The LPS challenged females, regardless of DW or strain, traveled less (Figure 4.26A; $F(3,20)=5.336$, $p=0.026$) and made fewer rearings (Figure 4.26B; $F(3,20)=6.062$, $p=0.018$) than their respective controls during the first 5-minute interval. Neither Mn nor strain had any significant effects on times spent in the periphery or center of the arena ($F(3,20)<0.0942$, $p>0.1$; data not shown). Over the 15-minute test duration, mice habituated and locomotor activity decreased regardless of Mn, LPS, and strain ($F(3,20)=22.204$, $p<0.001$).

4.2.6 Plasma Cytokine Acute Phase Protein ELISAs

In the absence of LPS, plasma IL-6, TNFα, IL-10 and SAA were undetectable regardless of Mn treatment or strain (Figure 4.27A, B, C, and D respectively). Six hours after a single LPS administration, increased plasma levels of IL-6, TNFα, IL-10, SAA, and CRP were detected in Mn and control mice of both strains (Figure 4.27A, B, C, D, and E respectively; $F(3,44)>51.829$, $p<0.001$). A two-way ANOVA, within LPS, revealed main effects of Mn and strain. Saline treated females, of both strains, had higher plasma IL-6 than Mn treated females (Figure 4.27A; $F(3,20)=4.036$, $p=0.048$). This Mn effect was more prominent in Mn-exposed WT females having lower plasma IL-6 levels than their controls. Mn-exposed females, of both strains, had lower levels of TNFα, IL-10, SAA, and CRP than control females (Figure 4.27B, C, D, and E respectively; $F(3,20)>22.184$, $p<0.001$). Baseline CRP (without LPS) was not significantly different between DW treatments or strain, and it increased in both strains and DW treatments after LPS administration.

5 Discussion

In this exploratory study we investigated the effects of Mn exposure through drinking was on motor and mood-related behaviors of both male and female mice lacking miRNA155, with estrus cycle monitored throughout the study. Additionally, we examined sex and genetic differences (miRNA155 deficiency) in the effects of DW Mn exposure and the response to an

LPS challenge. Mn in the DW did not impact water intake in either sex or strain. While Mn did not affect body weight of either sex in the KO mice, it is interesting to note that both KO males and females consistently weighed more than WT males and females while on a standard, low-fat rodent chow (4.5%). In Gaudet et al. (2016) female miR-155 knockout mice did not gain weight, compared to wild-type controls, on the control diet (10%) and did significantly gain weight on the high fat diet (45%).

Previous rodent studies that investigated neurobehavioral outcomes of Mn exposure have had varying results reported. These varying reports could be due to study variations including Mn dose/concentration/administration/duration of exposure, strain, mouse age, and behavior tests utilized, however they all have a common behavioral domain that is affected by Mn and that is locomotor alterations (Avila et al., 2010; Fleming et al., 2018; Krishna et al., 2014; Liu et al., 2019; Ludwig et al., 2024; Moreno et al., 2009; Sepúlveda et al., 2012). In the current study, Mn-exposed KO mice, particularly KO females, showed reduced horizontal locomotor activity, as evidenced by a decreased travel distance in the open field test (OFT). However, Mn exposure did not affect vertical locomotor activity in KO mice, unlike the decreased vertical activity observed in Mn-exposed WT mice. While Mn-exposed KO females reared less than their saline-treated counterparts, they still reared more than Mn-exposed WT females. Gait testing revealed that Mn exposure led to a shorter fore/hindpaw overlap in KO mice, but it did not impact stride length, interstep distance, or the total number of steps—three parameters that showed deficits in Mn-exposed WT mice. Additionally, Mn exposure did not affect activity levels in the KO mice during the elevated zero maze (EZM), whereas WT males exhibited increased general activity, as shown by more open/closed arm entries.

In the context of mood-related behaviors, miR155 deficiency alleviated the anxiety/fearlessness-like behaviors that were observed in the WT mice. Thus, during the OFT, rearing activity is a type of risk assessment behavior (Choleris et al., 2001), in addition to being a type of locomotion activity. While there was a decrease in rearing attempts within the WT

mice, rearing during the OFT was unaffected by Mn in the KO mice. Moreover, Mn exposure did not affect times spent in the center/periphery of the OFT nor did it affect the times spent in the open/closed arms of the EZM in the KO mice, but it did so in the WT mice. Additionally, in the EZM, Mn exposure did not affect the number of stretch attend postures (SAP) attempts made by the KO mice, but it did reduce the number of SAP attempts made by the WT mice. SAPs are another type of risk assessment activity when the mouse will elongate its body toward potential treatment sources (Grewal et al., 1997).

After LPS exposure, both Mn-exposed and control mice of both strains and sexes showed reduced horizontal and vertical locomotor activities, i.e., exhibited sickness behavior (Godbout et al., 2005; Krishna et al., 2016). Both saline and LPS challenged WT males spent more time in the center of the arena than their controls. Interestingly, the control, saline-challenged KO males spent more time in the center of the arena than their WT counterparts and the Mn-exposed KO males, an effect not observed within the KO females. To further the role of MiR-155 and to determine if inflammatory alterations are influenced similarly to the behavioral endpoints we evaluated, we assessed selected plasma cytokine and acute phase proteins (APP) levels. Mn alone did not influence plasma cytokine or APP levels of either sex or strain. Within KO mice, Mn exposure only affected female TNFα levels. Mn exposure increased IL-6 and TNFα levels in LPS challenged WT males, but did not affect either cytokine levels in the KO males. While LPS challenged KO mice all had an increase in TNFα, saline+LPS KO females had higher TNFα levels than Mn+LPS KO females, which was a similar effect seen in the WT females. KO males had similar circulating levels of TNFα as the WT males post LPS and KO females had higher levels than WT females. LPS challenged KO mice, both with and without Mn, had a significant increase in plasma IL-6, IL-10, SAA, and CRP in both sexes. Baseline IL-10 was higher in WT males and decreased after LPS challenge, whereas KO males baseline IL-10 increased post-LPS and there were no differences in baseline IL-10 among WT and KO females. For LPS challenged females, SAA and IL-10 levels were lower in Mn-exposed WT

females but were unaffected by Mn exposure within KO females. While WT females displayed a more robust innate immune response to LPS than WT males, KO males and females had similar levels of proinflammatory cytokines other than TNFα. Plasma cytokine levels of the LPS challenged WT males were opposite the cytokine levels produced in WT females of their respective Mn/saline treatment groups, whereas this sex difference was not observed within the KO mice.

These cytokine and APP profiles suggest that the effects of miR-155 deficiency on behavior are likely influenced by both peripheral and central immune contributions. The differential cytokine responses between KO males and females, particularly in response to LPS, highlight how miR-155 deficiency may alter neuroimmune signaling, potentially through neuroinflammation. For example, although both sexes exhibited increased plasma proinflammatory cytokines after LPS challenge, the absence of miR-155 led to altered cytokine profiles, such as higher TNFα levels in females and distinct IL-10 responses in males, suggesting changes in CNS immune signaling (Nonoguchi et al., 2022; Patel et al., 2023). Moreover, the reduced TNFα levels in Mn-exposed female KO mice compared to their saline-treated counterparts further suggest that miR-155 regulates peripheral immune responses that may influence central immune system, including neuroinflammation, contributing to the observed behavioral changes (Pashangzadeh et al., 2021; Tili et al., 2007). The observed behavioral alterations, such as reduced locomotor activity, likely reflect both peripheral and central contributions, such as activation of microglia or other CNS immune cells (Woodburn et al., 2021; Zhang et al., 2023). Importantly, the lack of prominent sex differences in cytokine responses within KO mice—compared to the marked sex differences in WT mice—suggests that miR-155 plays a more pronounced role in regulating immune responses in the CNS, which may be linked to the behavioral changes observed. Thus, both peripheral and central immune pathways, particularly neuroinflammation, may be key in modulating the sickness behavior associated with miR-155 deficiency.

The current study characterized sex differences in behavioral deficits, or lack thereof, in Mn-exposed KO mice and expanded the existing literature on potential treatment targets for DW Mn overexposure. We have shown that Mn exposure through DW for a subchronic period induces behavioral alterations in WT mice and that these effects are diminished within KO mice. Both sexes of WT mice did demonstrate neurobehavioral dysfunction, and with WT males being more susceptible, when miR-155 is absent in both sexes there is less neurobehavioral dysfunction for both sexes. Immune response data further highlighted the role of miR-155 in modulating neuroimmune signaling. Although Mn exposure did not significantly affect plasma cytokines or APP levels in either strain, WT mice, particularly females, exhibited a stronger proinflammatory cytokine response to LPS, with increased TNFα, IL-6, and IL-10. In contrast, cytokine responses were blunted in KO mice, with KO females showing reduced TNFα levels after Mn exposure. These findings suggest that miR-155 deficiency alters peripheral immune responses, potentially affecting central immune signaling and neuroinflammation, which could contribute to the reduced behavioral impairments in KO mice. The absence of sex differences in cytokine responses in KO mice compared to WT further supports the idea that miR-155 regulates CNS immune responses, influencing behavior. Thus, targeting miR-155 may offer a potential strategy for mitigating neuroinflammation and associated behavioral dysfunction in response to Mn exposure. Additional behavioral testing would be required to confirm that locomotor, mood, and cognitive functions are impacted more in the presence of miR-155.

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Figures and Tables

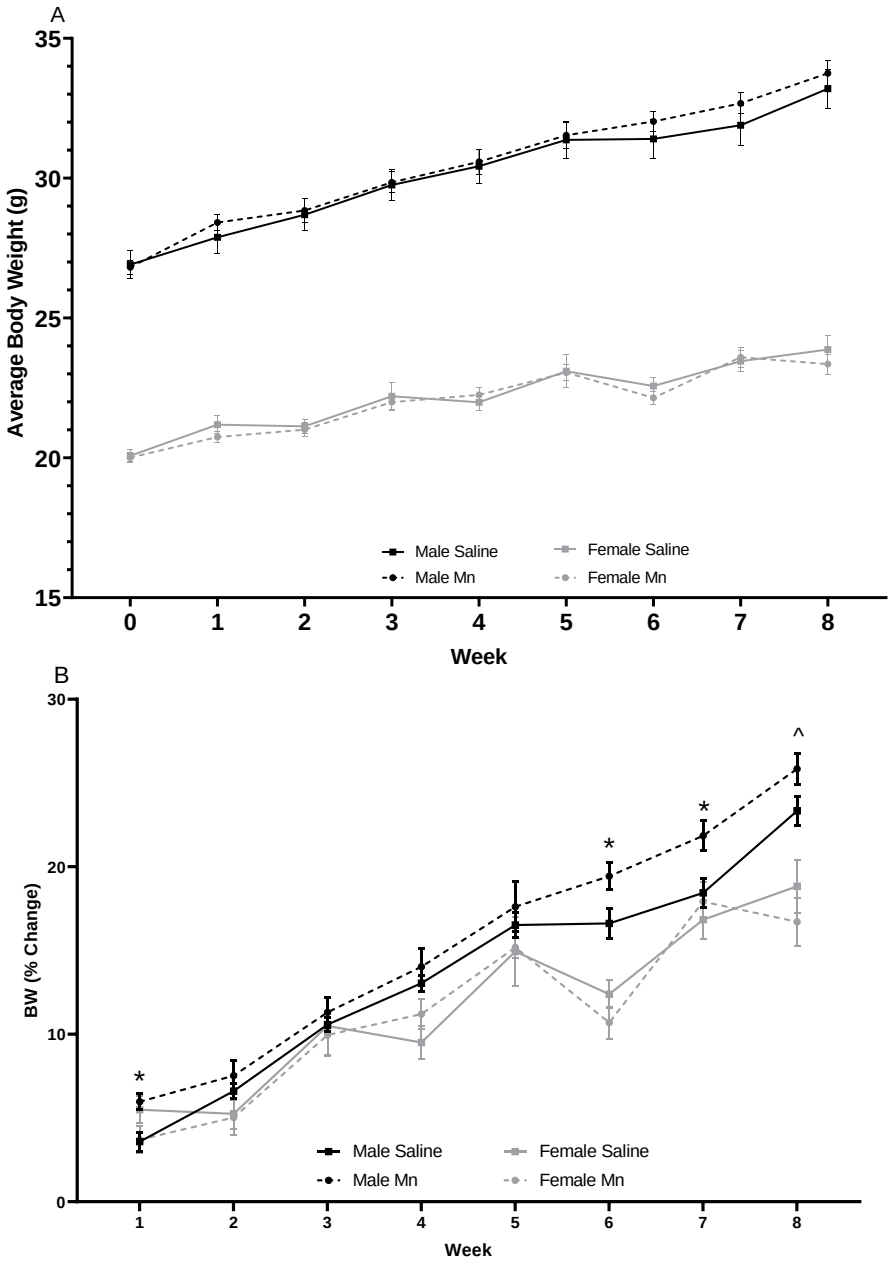

Figure 4.1. Effect of Mn DW (0.4g/L) exposure on body weight (BW) and on percent change in body weight (BW) during the 8-week treatment duration within miR-155 KO male and female mice. A) average weekly BW; B) weekly percent change in BW. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$ and ^indicates a trend $0.05<p<0.10$; n=12/group.

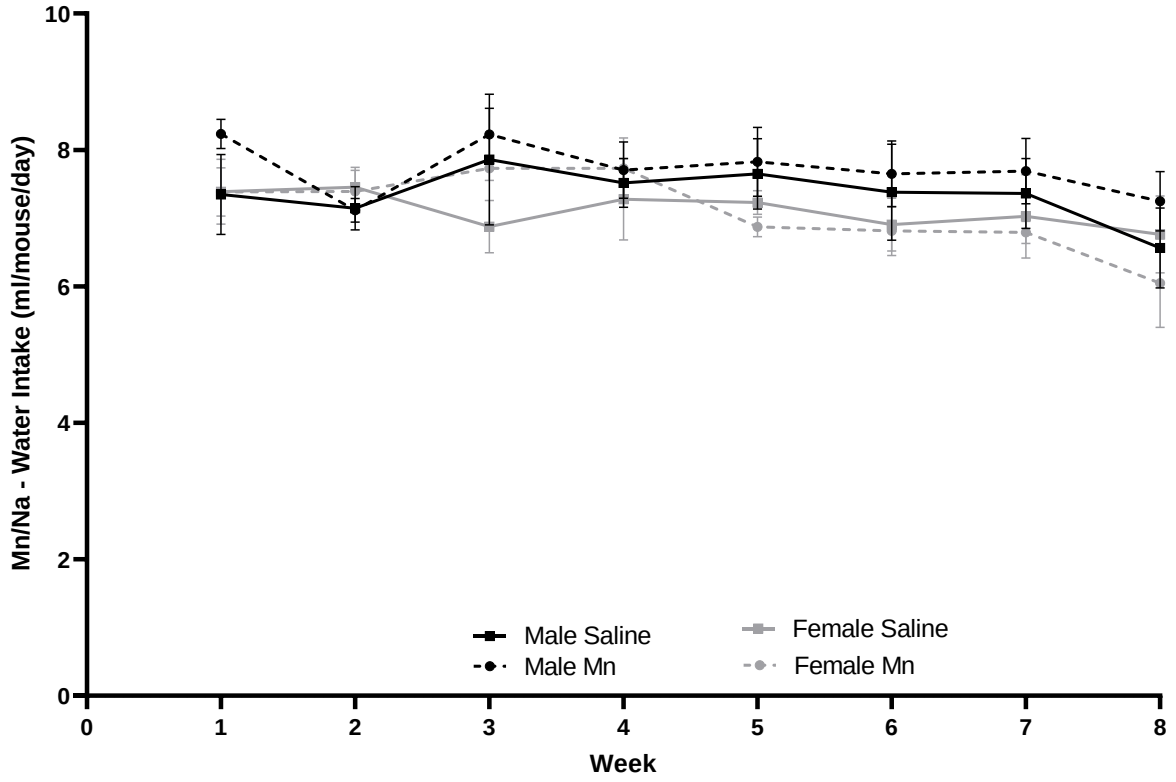

Figure 4.2. Effect of Mn DW (0.4g/L) on water intake (ml/mouse/day) during the 8-week treatment duration within miR-155 KO male and female mice. Water intake is presented as mean ± SEM; *n*=12/group.

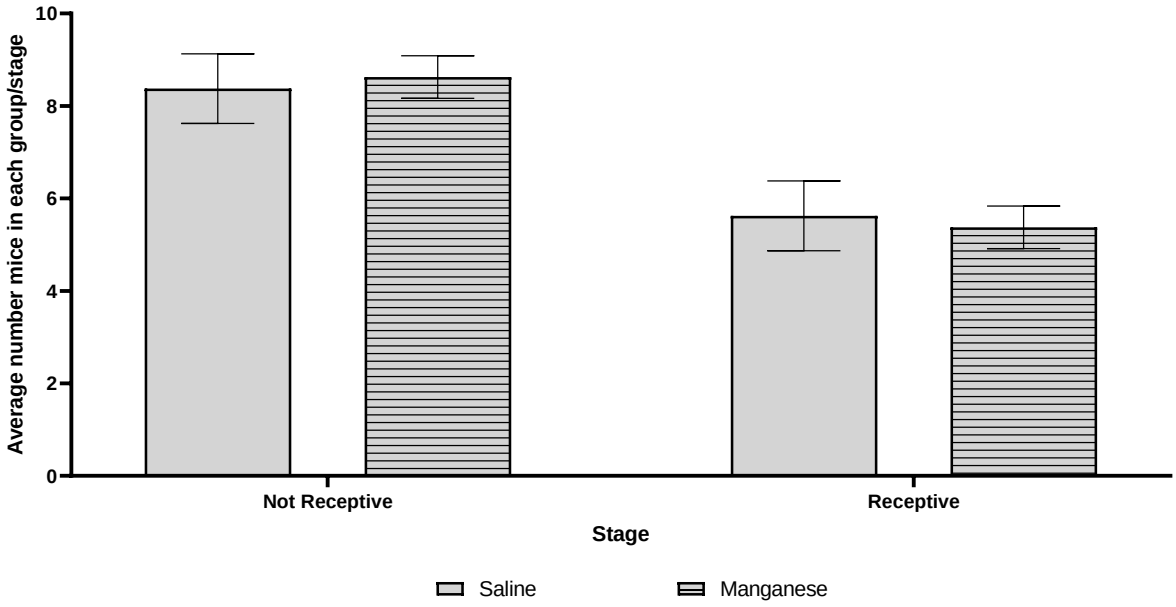

Figure 4.3. Average number of miR-155 KO female mice that are in a receptive stage (estrus and metestrus) or not receptive stage (diestrus and proestrus) of the estrous cycle.

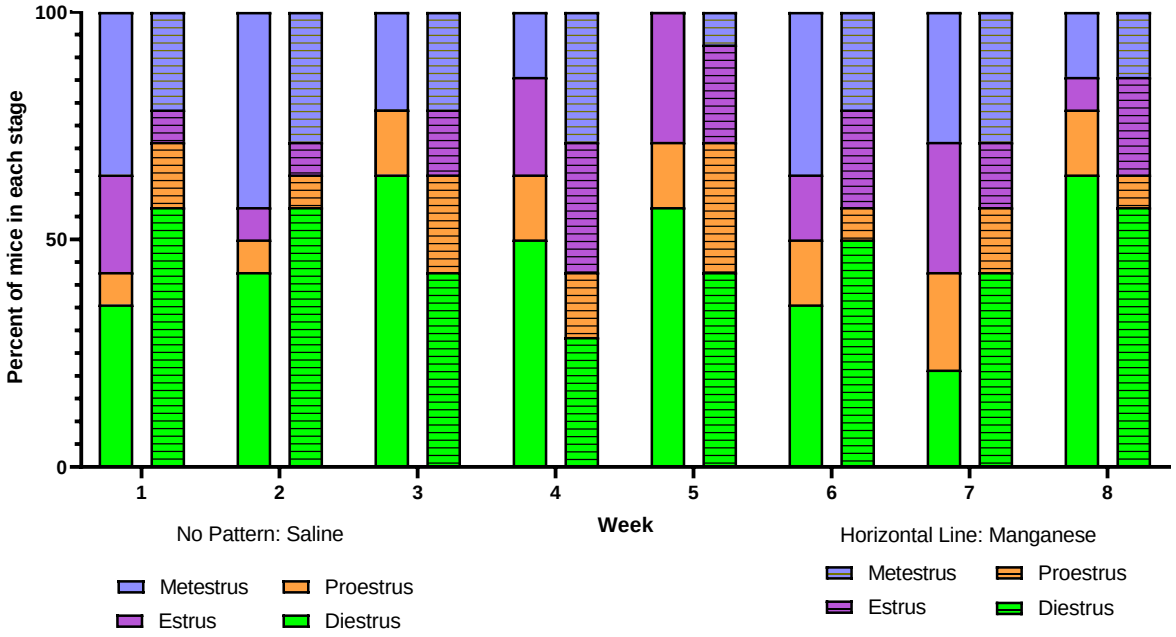

Figure 4.4. Percent of miR-155 KO female mice that are in each stage of the estrous cycle throughout the 8-week treatment duration (*n*=12/group).

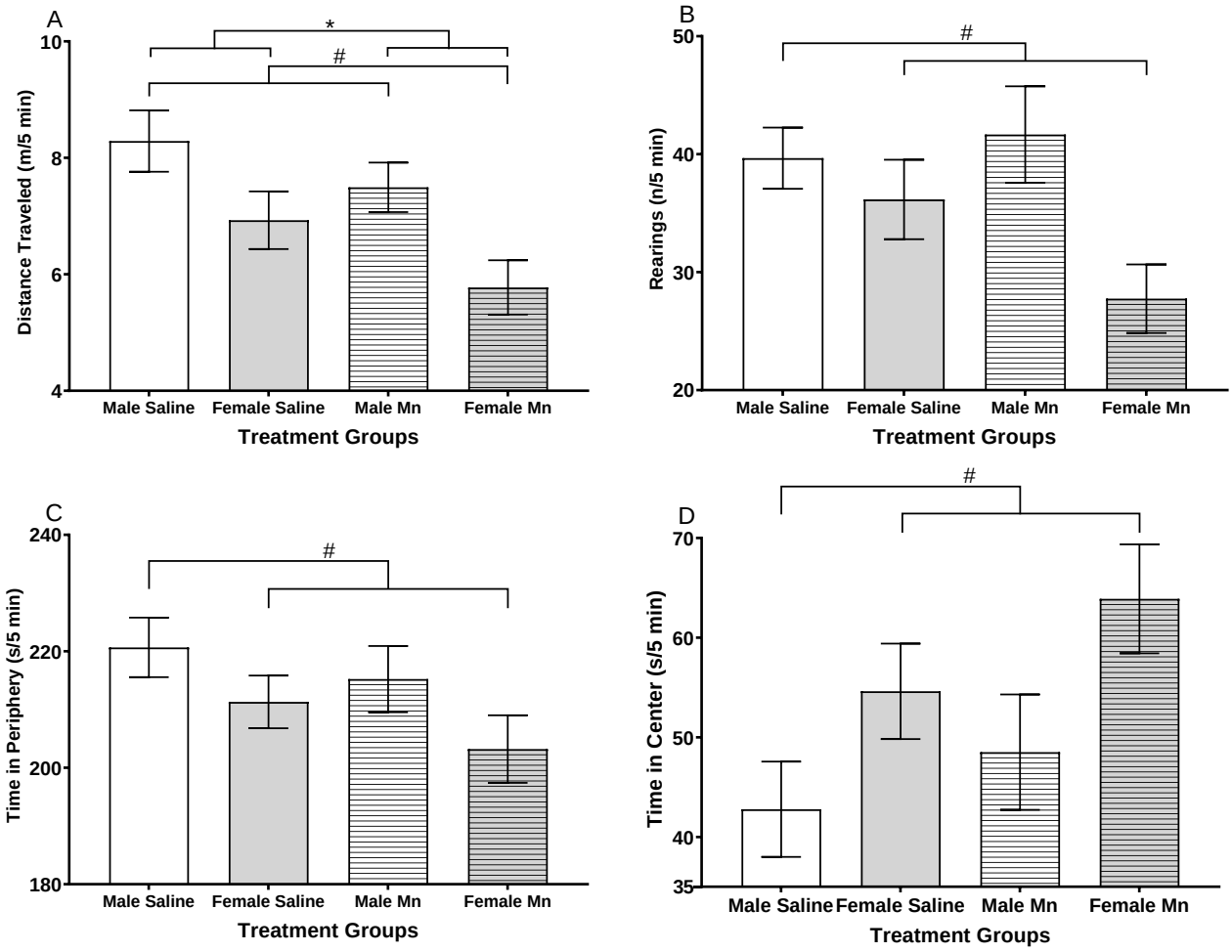

Figure 4.5. Open Field Test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or sex differences during the open field test within miR-155 KO male and female mice. A) distance traveled, B) rearing activity, C) time spent in the periphery, or D) time spent in the center of the open field arena (first 5 minutes). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; # indicates a significant effect of sex $p<0.05$; $n=12$/group.

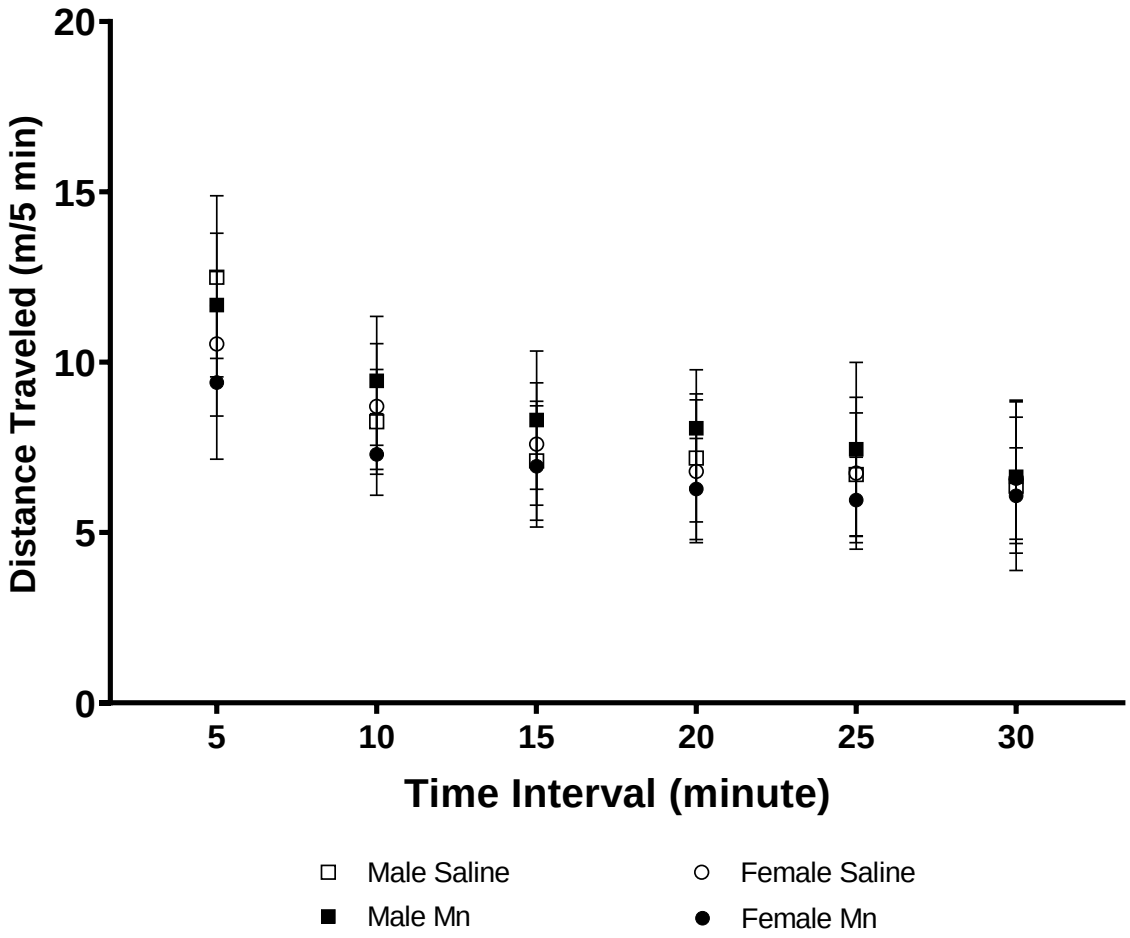

Figure 4.6. Open Field Test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure on the total distance traveled per 5-minute interval in the open field test arena within miR-155 KO male and female mice. Data are presented as mean ± SEM. *n*=12/group.

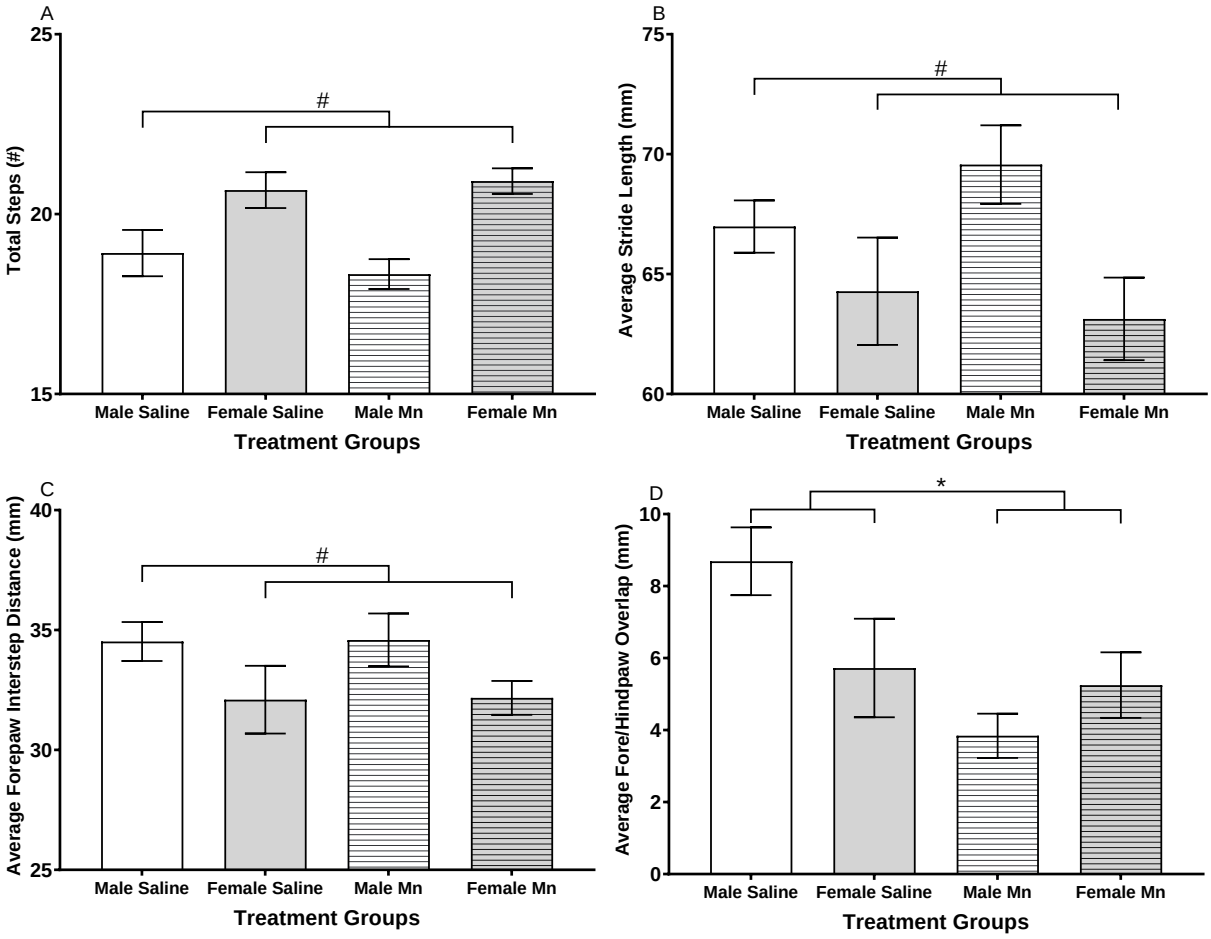

Figure 4.7. Gait Test. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or sex differences on parameters of gait within miR-155 KO male and female mice. A) total number of steps taken, B) average stride length, C) forepaw interstep distance, D) average fore/hindpaw overlap distance. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; $^{\#}$indicates a significant effect of sex $p<0.05$; $n=12$/group.

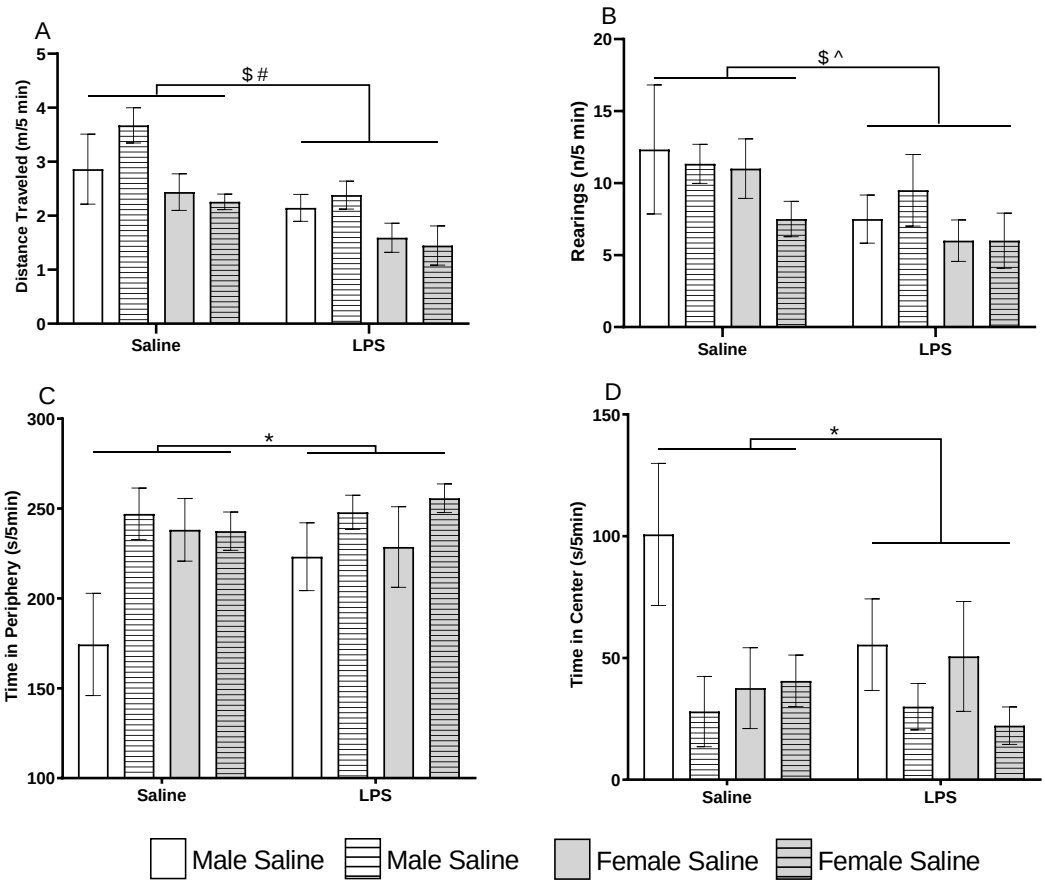

Figure 4.8. Post LPS Open Field Test. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or sickness behavior, four hours after a single dose of either saline or LPS (0.3 mg/kg BW) during post LPS open field test (first 5 minutes) within miR-155 KO male and female mice. A) distance traveled, B) rearing activity, C) time spent in the periphery, and D) time spent in the center. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; [#]indicates a significant effect of sex $p<0.05$; [$]indicates a significant effect of LPS $p<0.05$; ^indicates a trend $0.05<p<0.10$; $n=12$/group.

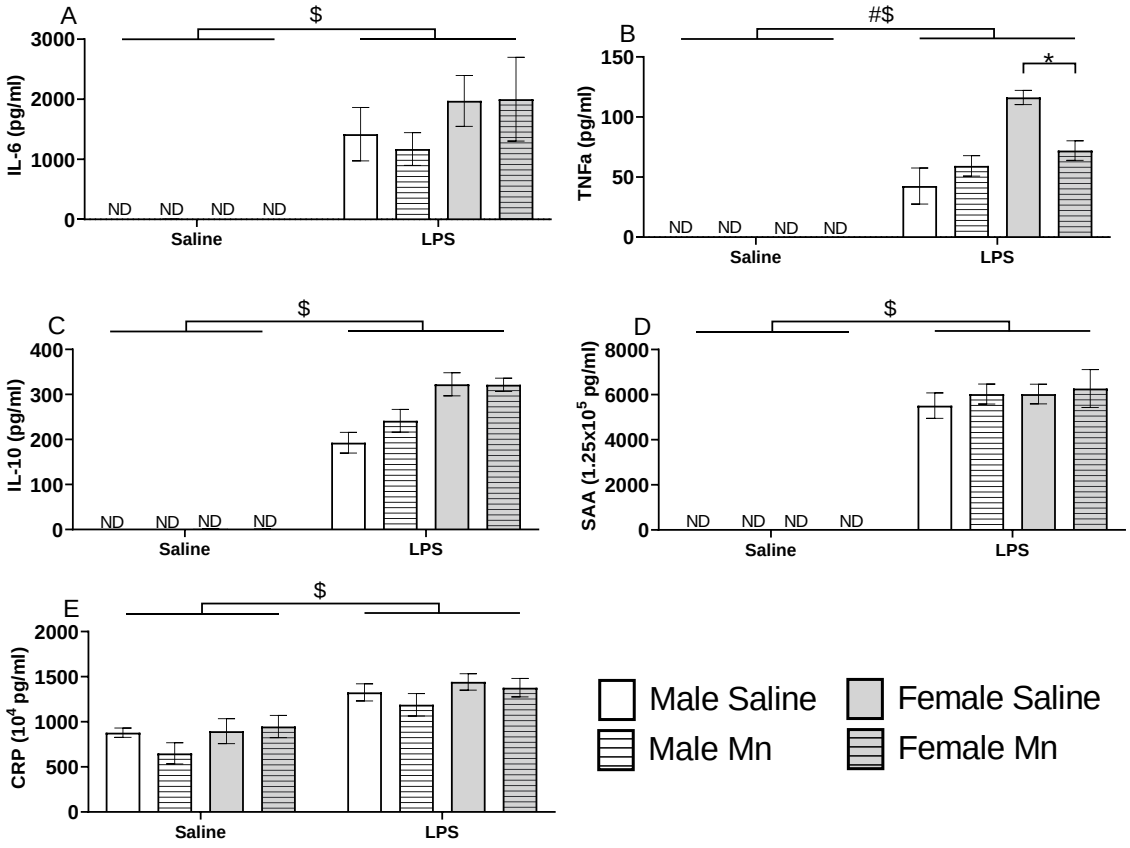

Figure 4.9. Plasma cytokine and acute phase protein (APP) levels. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, sex differences, and/or effect of acute LPS administration (0.3 mg/kg BW) on plasma cytokine levels 6 hours after LPS administration within miR-155 KO male and female mice. A) IL-6, B) TNFα, C) IL-10 D) SAA, and E) CRP. Data are presented as mean ± SEM. $^{\#}$ indicates a significant effect of sex $p<0.05$; * Indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS $p<0.05$; not detected (ND); $n=6$/group.

Table 4.1. Relative tissue weight (mg/kg BW) for each treatment group after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge within miR-155 KO male and female mice. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge			
Sex	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
Female	Brain	19.5 ± 0.88	20.6 ± 0.58	20.5 ± 0.32	21.4 ± 0.60
	Kidney	11.0 ± 0.29	**12.9 ± 0.48**	12.3 ± 0.41	**13.8 ± 0.54**
	Liver	45.0 ± 1.03	44.1 ± 2.60	39.9 ± 1.48	43.0 ± 2.25
	Spleen	3.5 ± 0.30	**4.6 ± 0.21**	3.3 ± 0.15	**4.3 ± 0.18**
	Thymus	**1.8 ± 0.11**	2.1 ± 0.18	**2.2 ± 0.15**	2.4 ± 0.08
	BAT	3.4 ± 0.43	8.4 ± 4.85	3.2 ± 0.54	3.2 ± 0.24
	SQ	4.9 ± 1.13	3.7 ± 0.18	3.3 ± 0.56	3.7 ± 0.48
	OT	22.7 ± 4.49	16.4 ± 3.17	12.2 ± 2.21	14.6 ± 2.39
	RT	6.2 ± 1.81	4.9 ± 1.18	4.0 ± 0.97	4.1 ± 0.91
Male	Brain	14.7 ± 0.43	14.8 ± 0.45	14.9 ± 0.42	14.9 ± 0.34
	Kidney	12.1 ± 0.41	12.7 ± 0.79	12.4 ± 0.41	12.6 ± 0.39
	Liver	43.4 ± 1.75	42.3 ± 1.96	41.3 ± 2.45	41.3 ± 1.89
	Spleen	2.2 ± 0.09	**2.9 ± 0.17**	2.5 ± 0.20	**3.0 ± 0.19**
	Thymus	1.6 ± 0.30	1.2 ± 0.08	1.2 ± 0.09	1.3 ± 0.08
	BAT	7.1 ± 1.23	5.9 ± 0.92	6.3 ± 0.83	5.7 ± 0.57
	SQ	5.8 ± 1.35	5.4 ± 0.60	4.7 ± 0.81	5.6 ± 0.46
	ET	28.2 ± 5.05	30.3 ± 3.84	25.2 ± 3.56	26.2 ± 2.24
	RT	10.7 ± 1.77	11.8 ± 1.62	8.4 ± 1.27	9.1 ± 0.66

Tissue abbreviations: BAT, brown adipose tissue; SQ, subcutaneous adipose tissue; ET, epididymal adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue.

Table 4.2. Absolute tissue weight (g) for each treatment group after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge within miR-155 KO male and female mice. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge											
Sex	Organ/Fat	**Saline-Saline**			**Saline-LPS**			**Mn-Saline**			**Mn-LPS**		
Female	Brain	0.475	±	0.00523	**0.492**	**±**	**0.00407**	0.483	±	0.00372	0.487	±	0.00324
	Kidney	0.271	±	0.00833	**0.309**	**±**	**0.00998**	0.29	±	0.00926	0.314	±	0.00725
	Liver	1.112	±	0.0728	1.055	±	0.0682	0.943	±	0.041	0.986	±	0.0689
	Spleen	0.0852	±	0.00844	**0.11**	**±**	**0.00736**	0.0786	±	0.00351	**0.0969**	**±**	**0.0022**
	Thymus	0.0431	±	0.0018	0.05	±	0.00479	0.052	±	0.00274	0.055	±	0.00207
	BAT	0.0706	±	0.00907	0.176	±	0.102	0.0667	±	0.0114	0.0665	±	0.005
	SQ	0.102	±	0.0238	0.0779	±	0.00382	0.0689	±	0.0117	0.0775	±	0.0101
	OT	0.477	±	0.0944	0.345	±	0.0665	0.257	±	0.0465	0.306	±	0.0503
	RT	0.131	±	0.038	0.104	±	0.0248	0.0836	±	0.0205	0.0853	±	0.0192
Male	Brain	0.478	±	0.00537	0.485	±	0.00621	0.481	±	0.00384	0.484	±	0.00553
	Kidney	0.393	±	0.0193	0.42	±	0.0287	0.404	±	0.0159	0.408	±	0.0131
	Liver	1.41	±	0.0649	1.393	±	0.0648	1.343	±	0.0827	1.344	±	0.0768
	Spleen	0.0709	±	0.00299	**0.0969**	**±**	**0.0076**	0.0803	±	0.00565	**0.0987**	**±**	**0.00577**
	Thymus	0.0518	±	0.0104	0.0392	±	0.00173	0.0388	±	0.00251	0.0419	±	0.00323
	BAT	0.148	±	0.0259	0.124	±	0.0193	0.132	±	0.0174	0.119	±	0.012
	SQ	0.122	±	0.0283	0.113	±	0.0125	0.0985	±	0.0171	0.117	±	0.00959
	ET	0.592	±	0.106	0.636	±	0.0807	0.528	±	0.0747	0.55	±	0.047
	RT	0.224	±	0.0373	0.248	±	0.0341	0.176	±	0.0266	0.192	±	0.0138

Tissue abbreviations: BAT, brown adipose tissue; SQ, subcutaneous adipose tissue; ET, epididymal adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue.

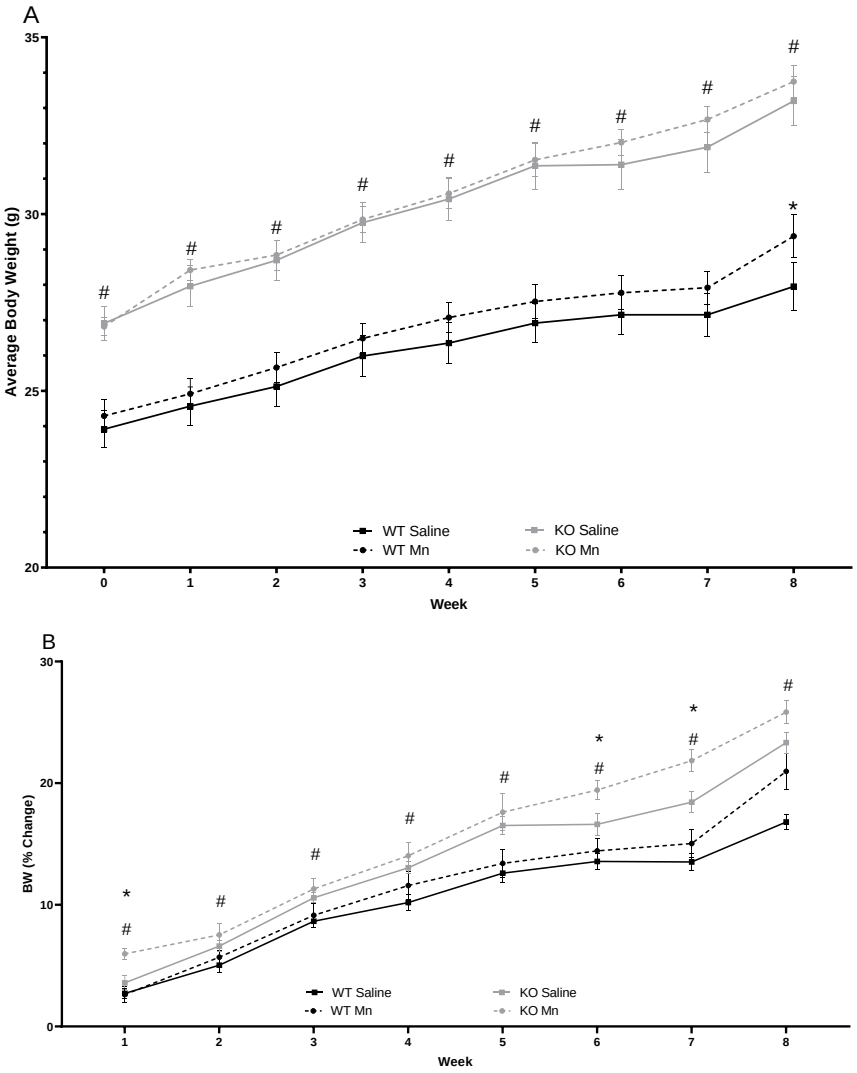

Figure 4.10. Effect of Mn DW (0.4g/L) exposure on body weight (BW) and on percent change in body weight (BW) during the 8-week treatment duration within male WT and miR-155 KO mice. A) average weekly BW; B) weekly percent change in BW. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$ and ^indicates a trend $0.05<p<0.10$; $n=12$/group.

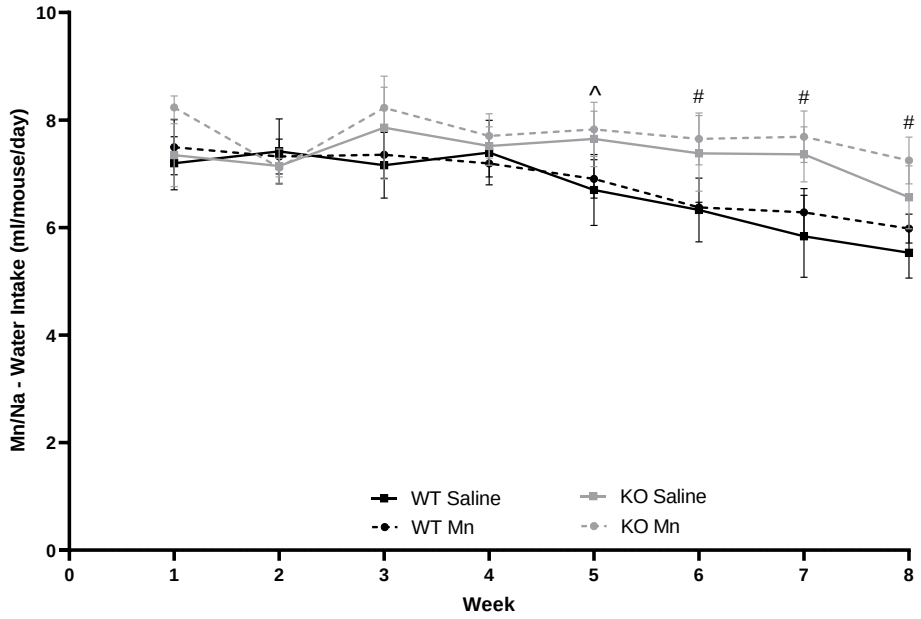

Figure 4.11. Effect of Mn DW (0.4g/L) on water intake (ml/mouse/day) during the 8-week treatment duration within male WT and miR-155 KO mice. Water intake is presented as mean ± SEM. #Indicates a significant strain difference $p<0.05$ and ^indicates a trending strain difference $0.05<p<0.1$; $n=12$/group.

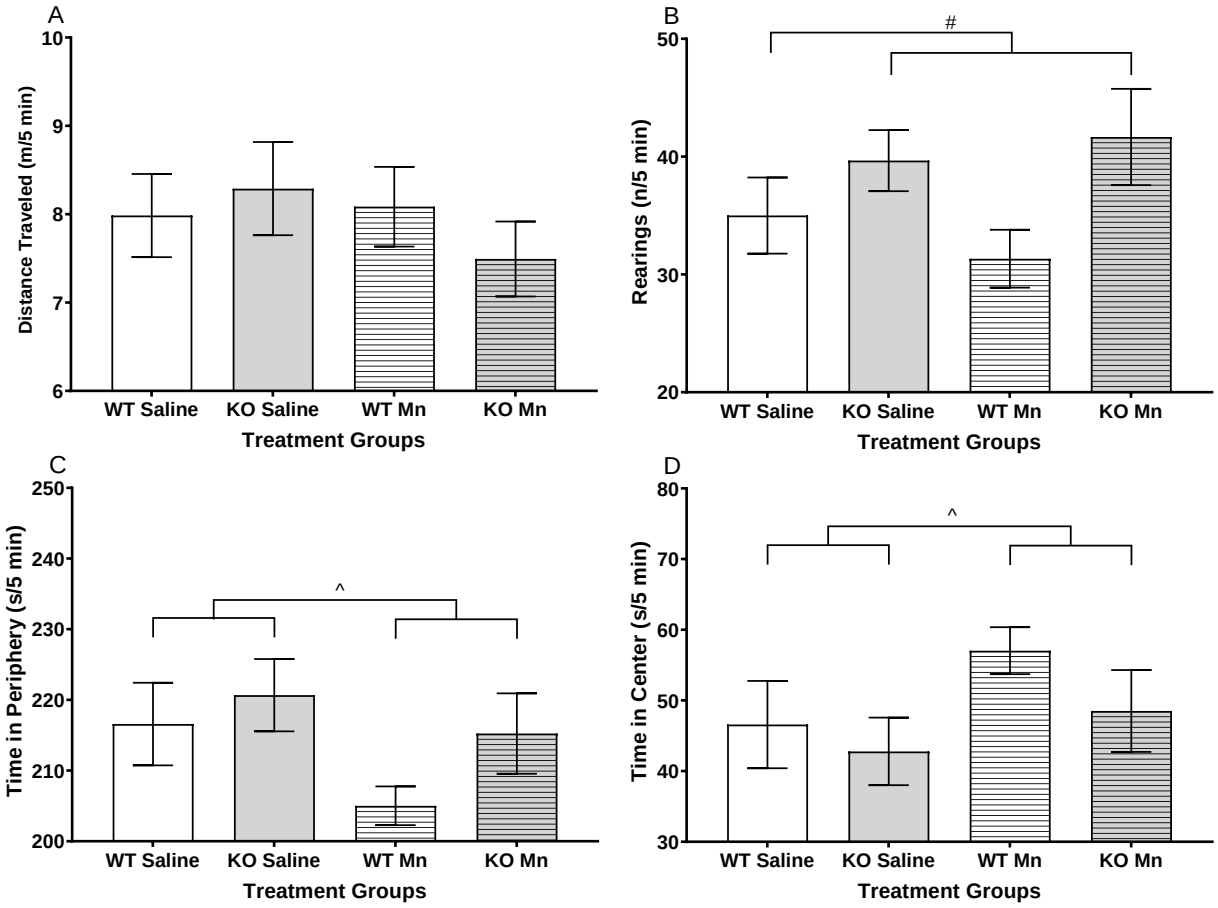

Figure 4.12. Open Field Test within male WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences during the open field test within male WT and KO mice. A) distance traveled, B) rearing activity, C) time spent in the periphery, or D) time spent in the center of the open field arena (first 5 minutes). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p < 0.05$; # indicates a significant effect of strain $p<0.05$; n=12/group.

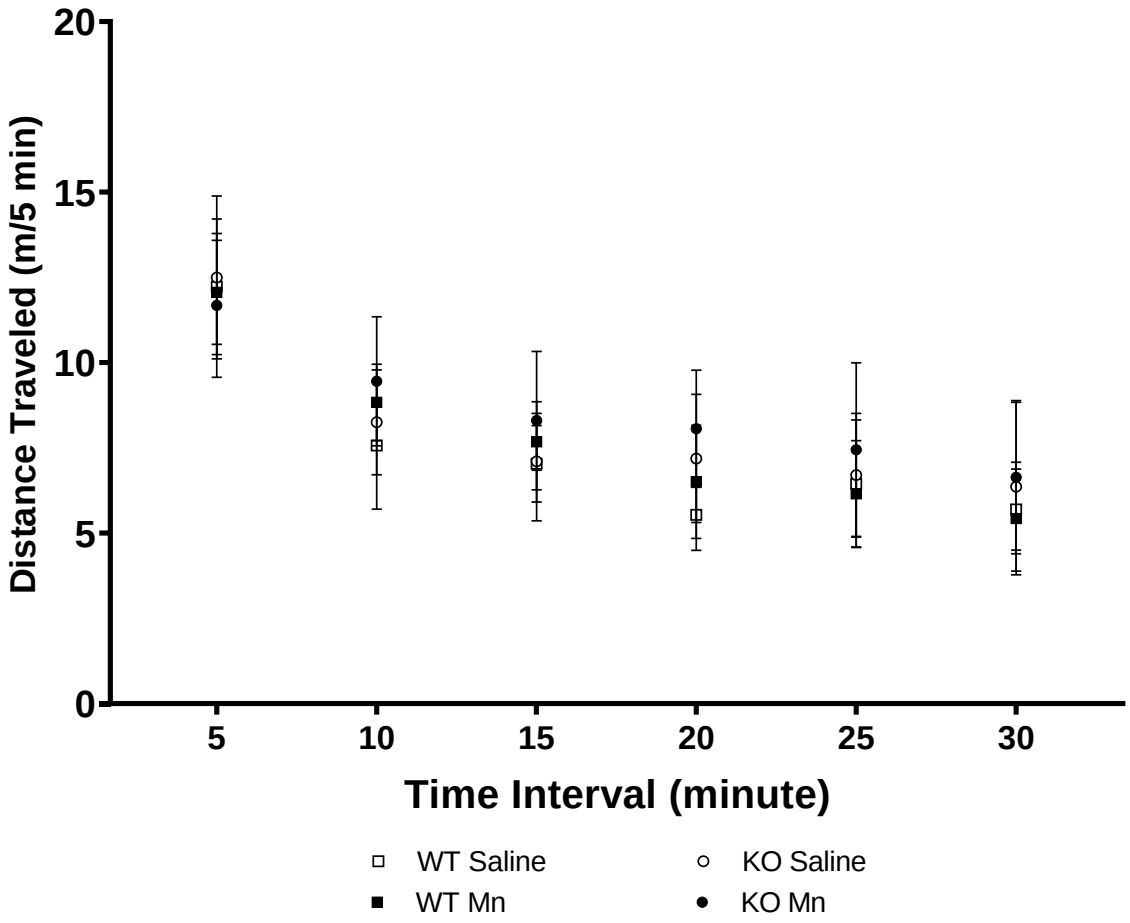

Figure 4.13. Open Field Test Habituation within male WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure on the total distance traveled per 5-minute interval in the open field test arena within male WT and KO mice. Data are presented as mean ± SEM. *n*=12/group.

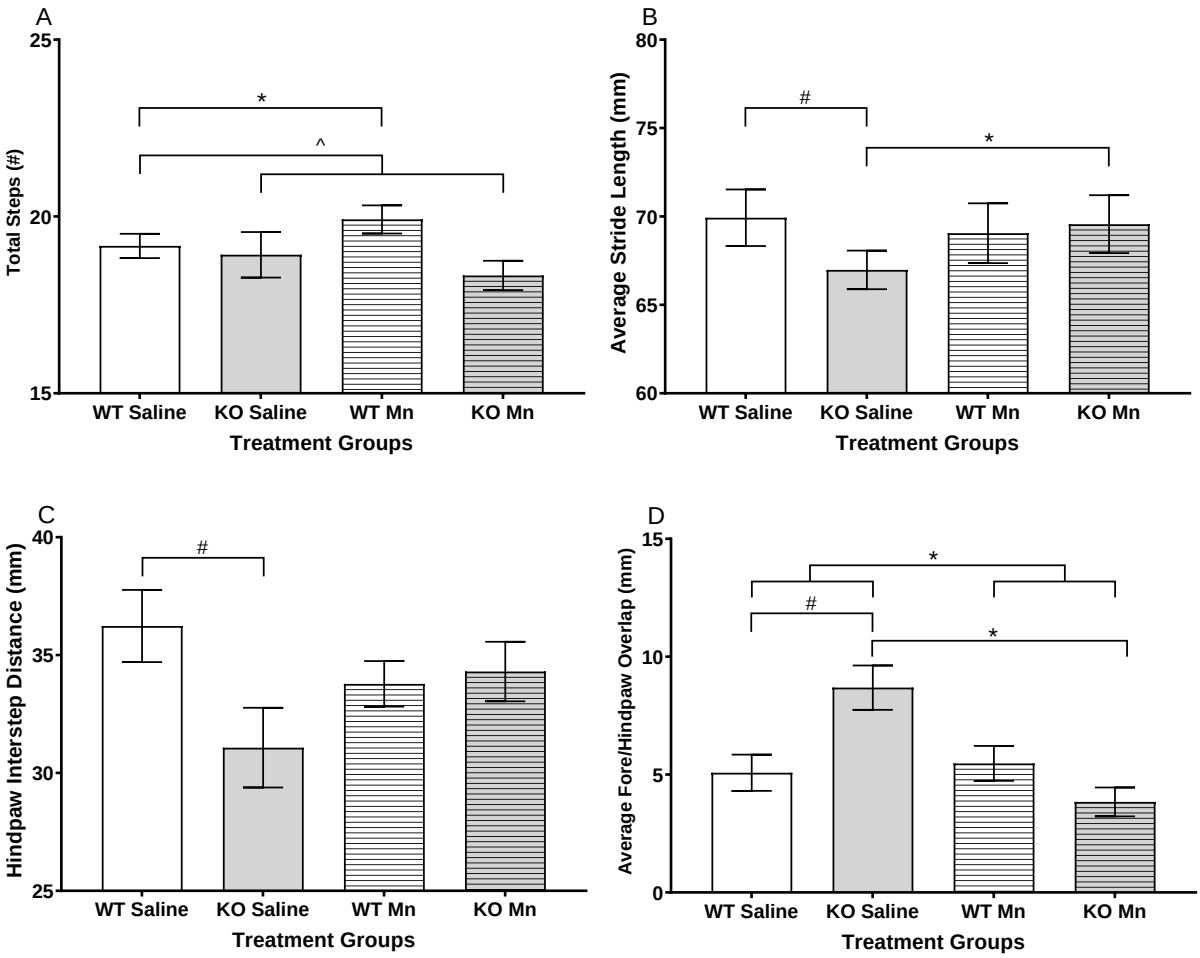

Figure 4.14. Gait Test within male WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences on Gait within male WT and KO mice. A) total number of steps taken, B) average stride length, C) forepaw interstep distance, D) average fore/hindpaw overlap distance. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; $^{\#}$indicates a significant effect of strain $p<0.05$; $n=12$/group.

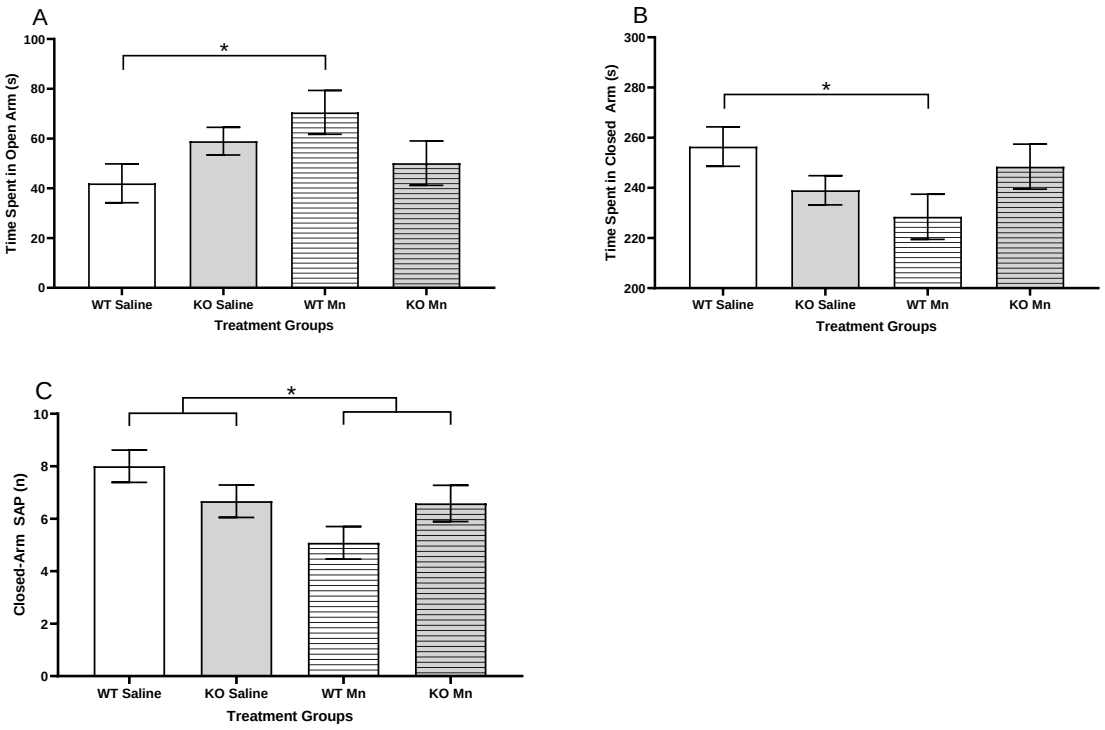

Figure 4.15. Elevated Zero Maze within male WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences during the elevated zero maze within male WT and KO mice. A) time spent in the open arm(s), B) time spent in the close arm(s), C) number of stretch attend posture attempts in the closed arm(s). Data are presented as mean ± SEM. ^Indicates 0.05<*p*<0.10; *n*=12/group.

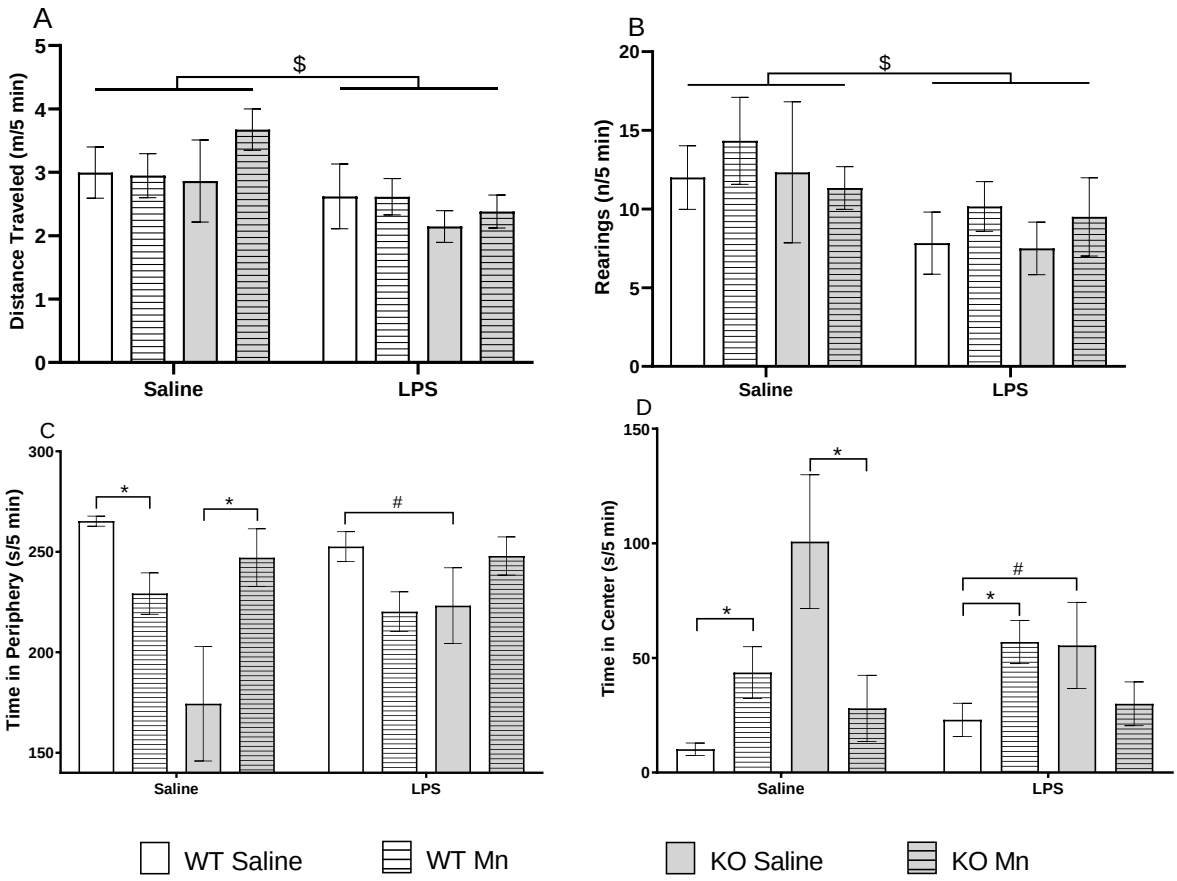

Figure 4.16. Post LPS Open Field Test within male WT and miR-155 KO mice. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, strain differences, and/or sickness behavior, four hours after a single dose of either saline or LPS (0.3 mg/kg BW) during post LPS open field test (first 5 minutes) within male WT and KO mice. A) distance traveled, B) rearing activity, C) time spent in the periphery, and D) time spent in the center. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; #indicates a significant effect of strain $p<0.05$; $indicates a significant effect of LPS $p<0.05$; ^indicates a trend $0.05<p<0.10$; $n=12$/group.

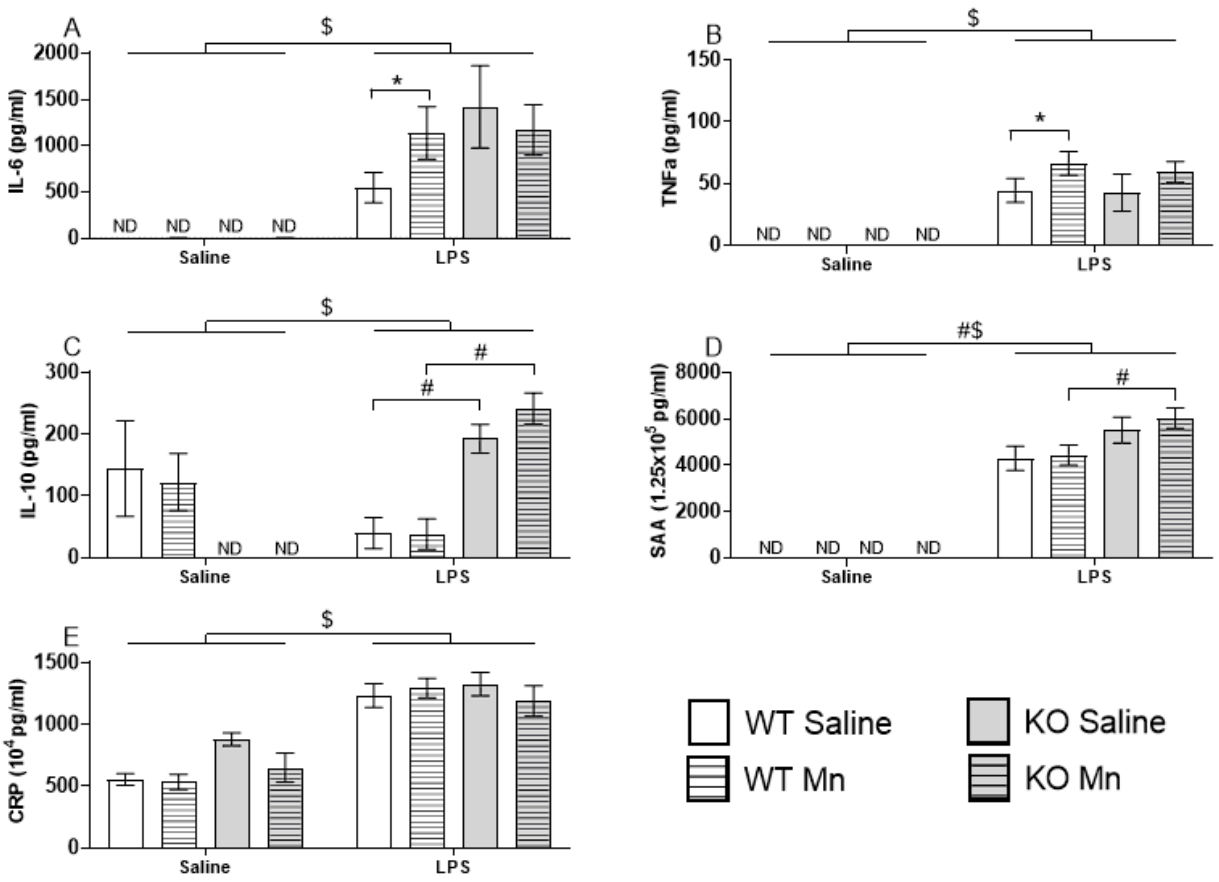

Figure 4.17. Plasma cytokine and acute phase protein (APP) levels within male WT and miR-155 KO mice. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, strain differences, and/or effect of acute LPS administration (0.3 mg/kg BW) on plasma cytokine levels 6 hours after LPS administration within male WT and KO mice. A) IL-6, B) TNFα, C) IL-10 D) SAA, and E) CRP. Data are presented as mean ± SEM. $^{\#}$ indicates a significant effect of strain $p<0.05$; * Indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS $p<0.05$; not detected (ND); n=6/group.

Table 4.3. Relative tissue weights (mg/kg BW) for each treatment group within male WT and miR-155 KO mice after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge. Mean ± SEM. Bold indicates $p<0.05$, * indicates a significant effect of Mn, $^{\$}$ indicates a significant effect of LPS, $^{\#}$ indicates a significant effect of strain; $n=6$/group.

		DW Treatment - LPS Challenge			
Strain	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
WT Male	Brain	18 ± 0.33	17.5 ± 0.52	16.7 ± 0.47	17 ± 0.41
	Kidney	13.6 ± 0.32	13.6 ± 0.44	13 ± 0.52	13.3 ± 0.31
	Liver	41.3 ± 2.37	**$^{\$}$45.5 ± 0.72**	***44 ± 1.15**	**$^{\$}$48.3 ± 0.86**
	Spleen	2.6 ± 0.11	2.6 ± 0.54	2.4 ± 0.18	3.1 ± 0.26
	Thymus	1.1 ± 0.15	1.1 ± 0.16	1.2 ± 0.07	1.1 ± 0.05
	BAT	3.5 ± 0.27	2.9 ± 0.13	2.3 ± 0.22	3.5 ± 0.27
	SQ	2.5 ± 0.29	2.5 ± 0.29	3.2 ± 0.23	2.6 ± 0.39
	ET	10 ± 0.55	10.5 ± 1.22	13.1 ± 2.23	13.1 ± 2.13
	RT	3.3 ± 0.48	3.3 ± 0.36	4.9 ± 1.11	5.3 ± 1.29
KO Male	$^{\#}$Brain	14.7 ± 0.43	14.8 ± 0.45	14.9 ± 0.42	14.9 ± 0.34
	Kidney	12.1 ± 0.41	12.7 ± 0.79	12.4 ± 0.41	12.6 ± 0.39
	$^{\#}$Liver	43.4 ± 1.75	42.3 ± 1.96	41.3 ± 2.45	41.3 ± 1.89
	Spleen	2.2 ± 0.09	**$^{\$}$2.9 ± 0.17**	2.5 ± 0.20	**$^{\$}$3.0 ± 0.19**
	Thymus	1.6 ± 0.30	1.2 ± 0.08	1.2 ± 0.09	1.3 ± 0.08
	$^{\#}$BAT	7.1 ± 1.23	5.9 ± 0.92	6.3 ± 0.83	5.7 ± 0.57
	$^{\#}$SQ	5.8 ± 1.35	5.4 ± 0.60	4.7 ± 0.81	5.6 ± 0.46
	$^{\#}$ET	28.2 ± 5.05	30.3 ± 3.84	25.2 ± 3.56	26.2 ± 2.24
	$^{\#}$RT	10.7 ± 1.77	11.8 ± 1.62	8.4 ± 1.27	9.1 ± 0.66

Tissue abbreviations: BAT, brown adipose tissue; ET, epididymal adipose tissue; RT, retroperitoneal adipose tissue; SQ, subcutaneous adipose tissue.

Table 4.4. Absolute tissue weight (g) for each treatment group within male WT and miR-155 KO mice after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge. Mean ± SEM. Bold indicates $p<0.05$, * indicates a significant effect of Mn, $ indicates a significant effect of LPS, # indicates a significant effect of strain; n=6/group.

		DW Treatment - LPS Challenge											
Strain	Organ/Fat	**Saline-Saline**			**Saline-LPS**			**Mn-Saline**			**Mn-LPS**		
WT Male	Brain	0.474	±	0.0056	0.472	±	0.0059	0.474	±	0.006	0.479	±	0.0052
	Kidney	0.357	±	0.007	0.376	±	0.0159	0.372	±	0.0208	0.376	±	0.0159
	Liver	1.086	±	0.0609	**$1.238**	**±**	**0.0639**	***1.251**	**±**	**0.05**	**$1.366**	**±**	**0.0426**
	Spleen	0.069	±	0.0026	0.086	±	0.0071	0.067	±	0.0052	0.086	±	0.0071
	Thymus	0.029	±	0.0041	0.028	±	0.0043	0.034	±	0.0017	0.033	±	0.0018
	BAT	0.092	±	0.0069	0.08	±	0.0053	0.092	±	0.0073	0.1	±	0.0109
	SQ	0.067	±	0.0077	0.068	±	0.0074	0.067	±	0.0083	0.075	±	0.0139
	ET	0.264	±	0.0152	0.284	±	0.0302	0.38	±	0.0733	0.377	±	0.0755
	RT	0.088	±	0.0124	0.088	±	0.0101	0.142	±	0.0348	0.153	±	0.0439
KO Male	Brain	0.478	±	0.00537	0.485	±	0.00621	0.481	±	0.00384	0.484	±	0.00553
	Kidney	0.393	±	0.0193	0.42	±	0.0287	0.404	±	0.0159	0.408	±	0.0131
	#Liver	1.41	±	0.0649	1.393	±	0.0648	1.343	±	0.0827	1.344	±	0.0768
	Spleen	0.0709	±	0.00299	**$0.0969**	**±**	**0.0076**	0.0803	±	0.00565	**$0.0987**	**±**	**0.00577**
	#Thymus	0.0518	±	0.0104	0.0392	±	0.00173	0.0388	±	0.00251	0.0419	±	0.00323
	#BAT	0.148	±	0.0259	0.124	±	0.0193	0.132	±	0.0174	0.119	±	0.012
	#SQ	0.122	±	0.0283	0.113	±	0.0125	0.0985	±	0.0171	0.117	±	0.00959
	#ET	0.592	±	0.106	0.636	±	0.0807	0.528	±	0.0747	0.55	±	0.047
	#RT	0.224	±	0.0373	0.248	±	0.0341	0.176	±	0.0266	0.192	±	0.0138

Tissue abbreviations: BAT, brown adipose tissue; ET, epididymal adipose tissue; RT, retroperitoneal adipose tissue; SQ, subcutaneous adipose tissue

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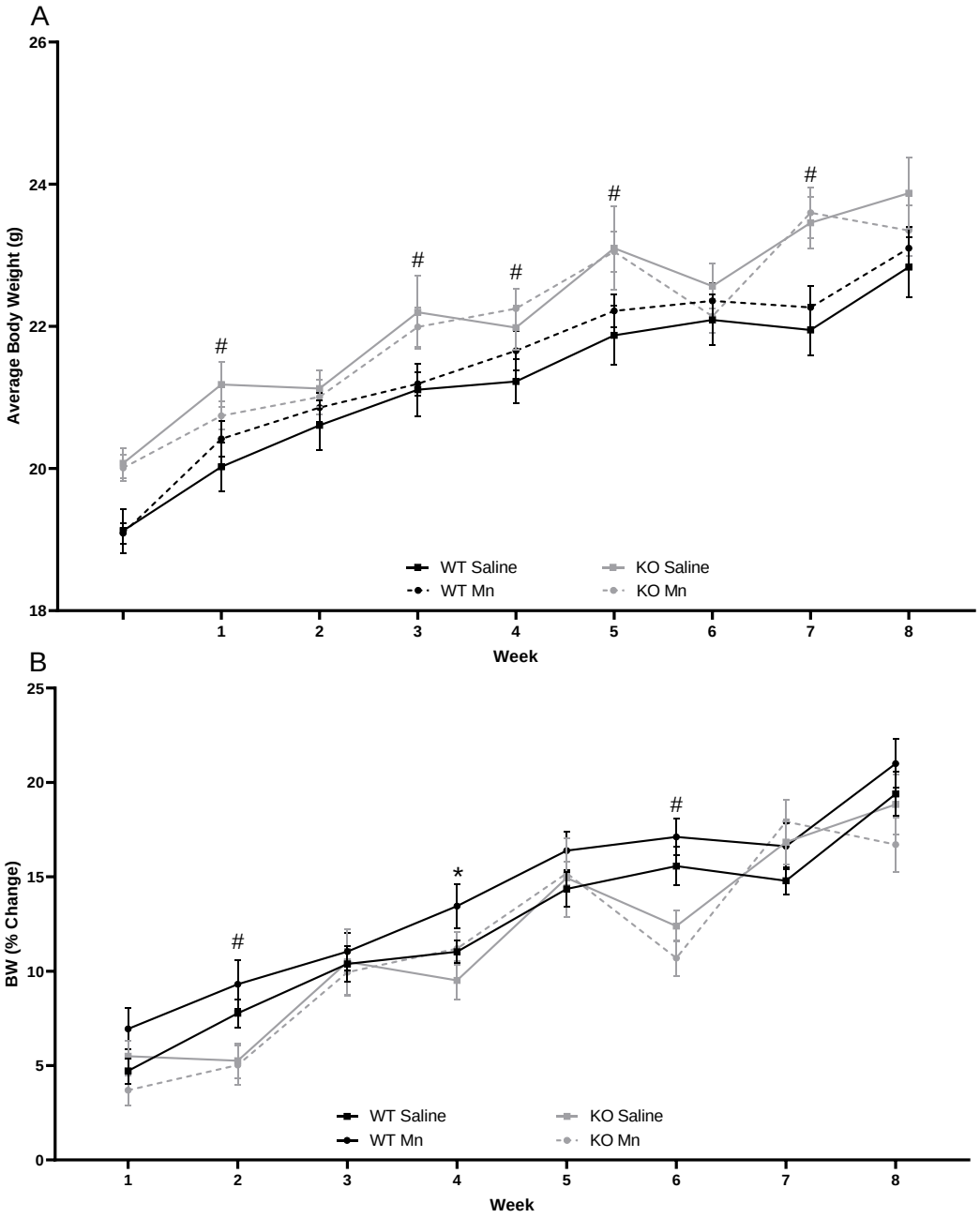

Figure 4.18. Effect of Mn DW (0.4g/L) exposure on body weight (BW) and on percent change in body weight (BW) during the 8-week treatment duration within female WT and miR-155 KO mice. A) average weekly BW; B) weekly percent change in BW. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$ and ^indicates a trend $0.05 < p < 0.10$; n=12/group.

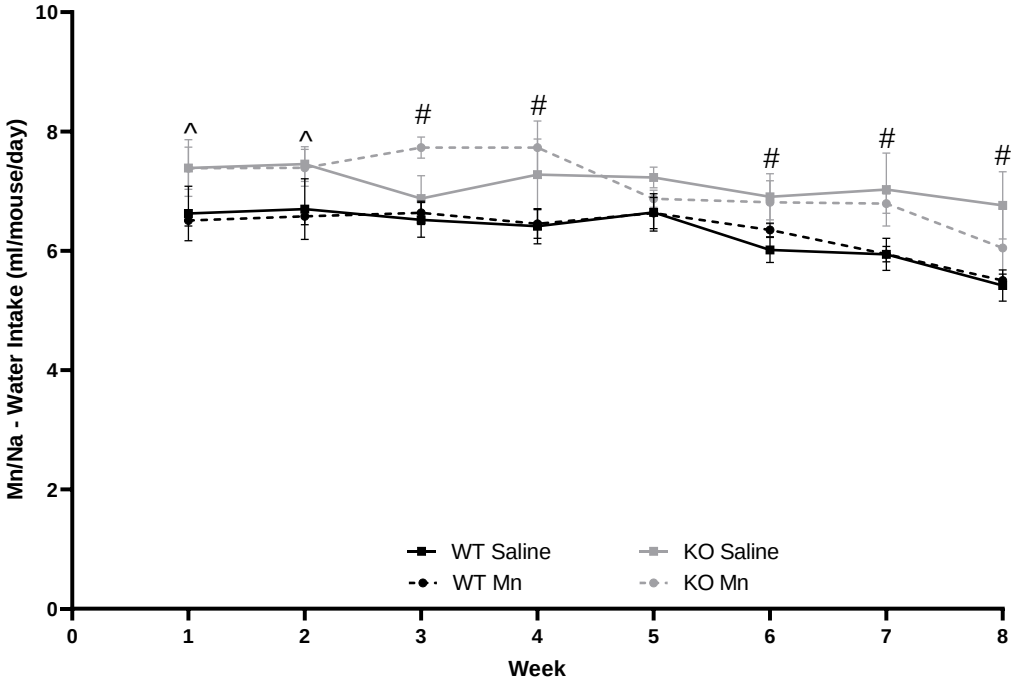

Figure 4.19. Effect of Mn DW (0.4g/L) on water intake (ml/mouse/day) during the 8-week treatment duration within female WT and miR-155 KO mice. Water intake is presented as mean ± SEM; *n*=12/group.

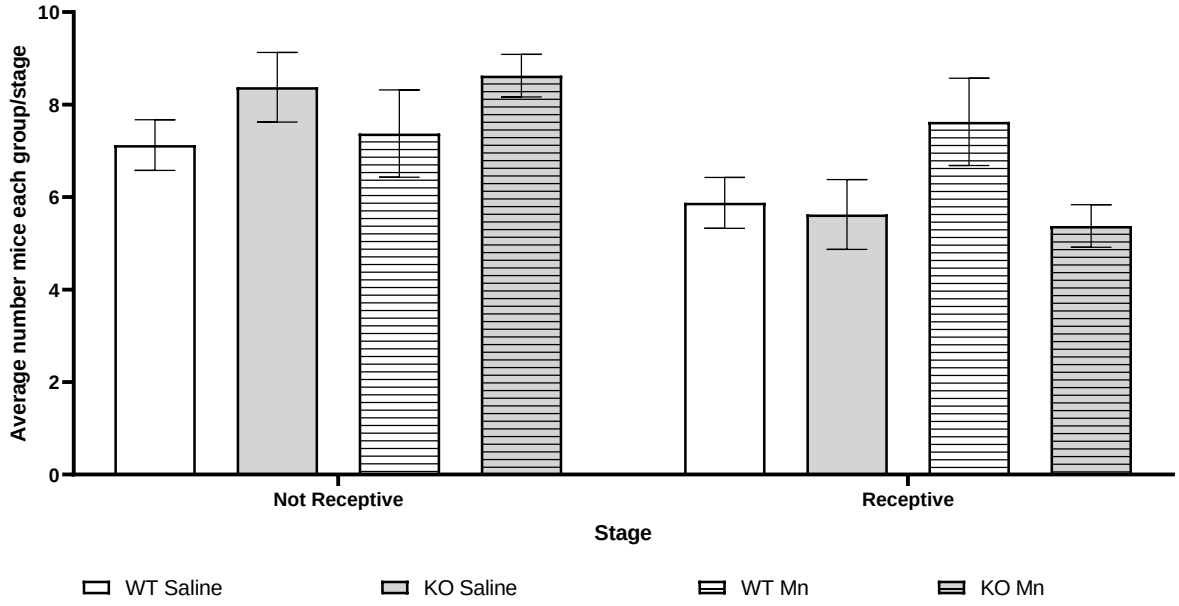

Figure 4.20. Average number of female WT and miR-155 KO mice that are in a receptive stage (estrus and metestrus) or not receptive stage (diestrus and proestrus) of the estrous cycle.

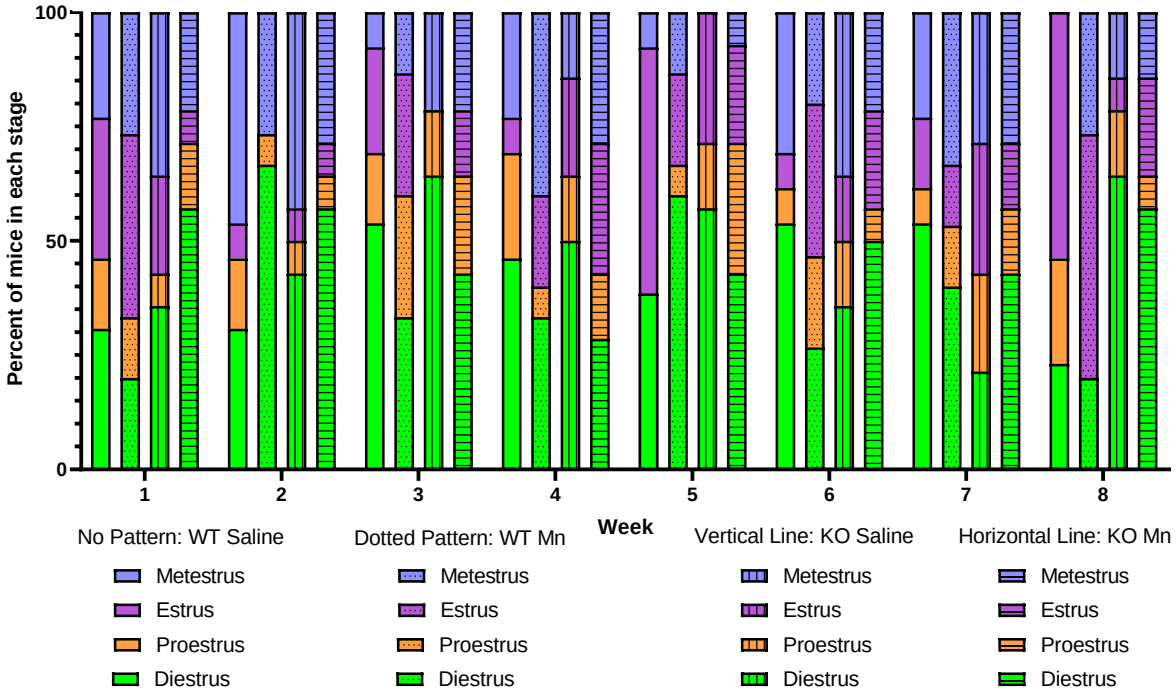

Figure 4.21. Percent of female WT and miR-155 KO mice that are in each stage of the estrous cycle throughout the 8-week treatment duration (*n*=12/group).

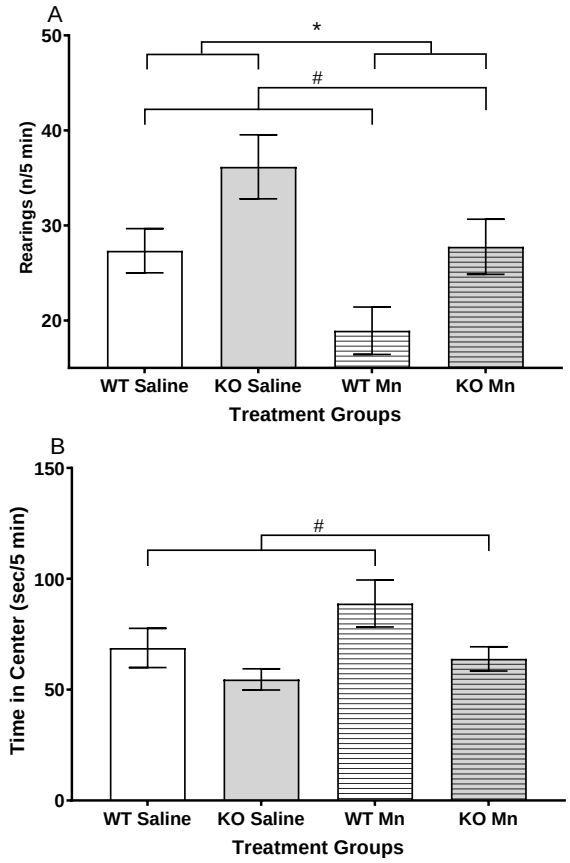

Figure 4.22. Open Field Test within female WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences during the open field test within female WT and KO mice. A) rearing activity, B) time spent in the center of the open field arena (first 5 minutes). Data are presented as mean ± SEM. * Indicates a significant effect of Mn $p<0.05$; $^{\#}$ indicates a significant effect of strain $p<0.05$; $n=12$/group.

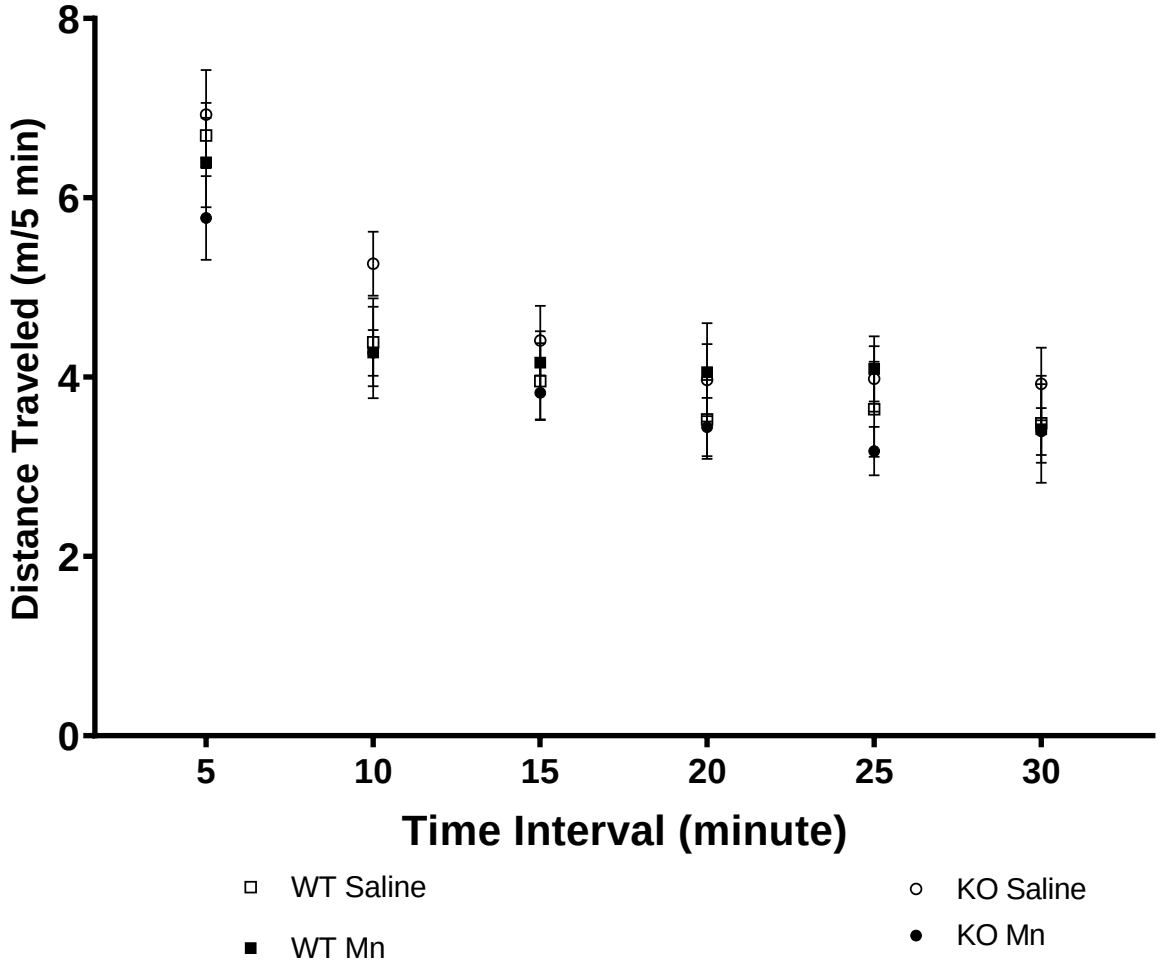

Figure 4.23. Open Field Test Habituation within female WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure on the total distance traveled per 5-minute interval in the open field test arena within female WT and KO mice. Data are presented as mean ± SEM. *n*=12/group.

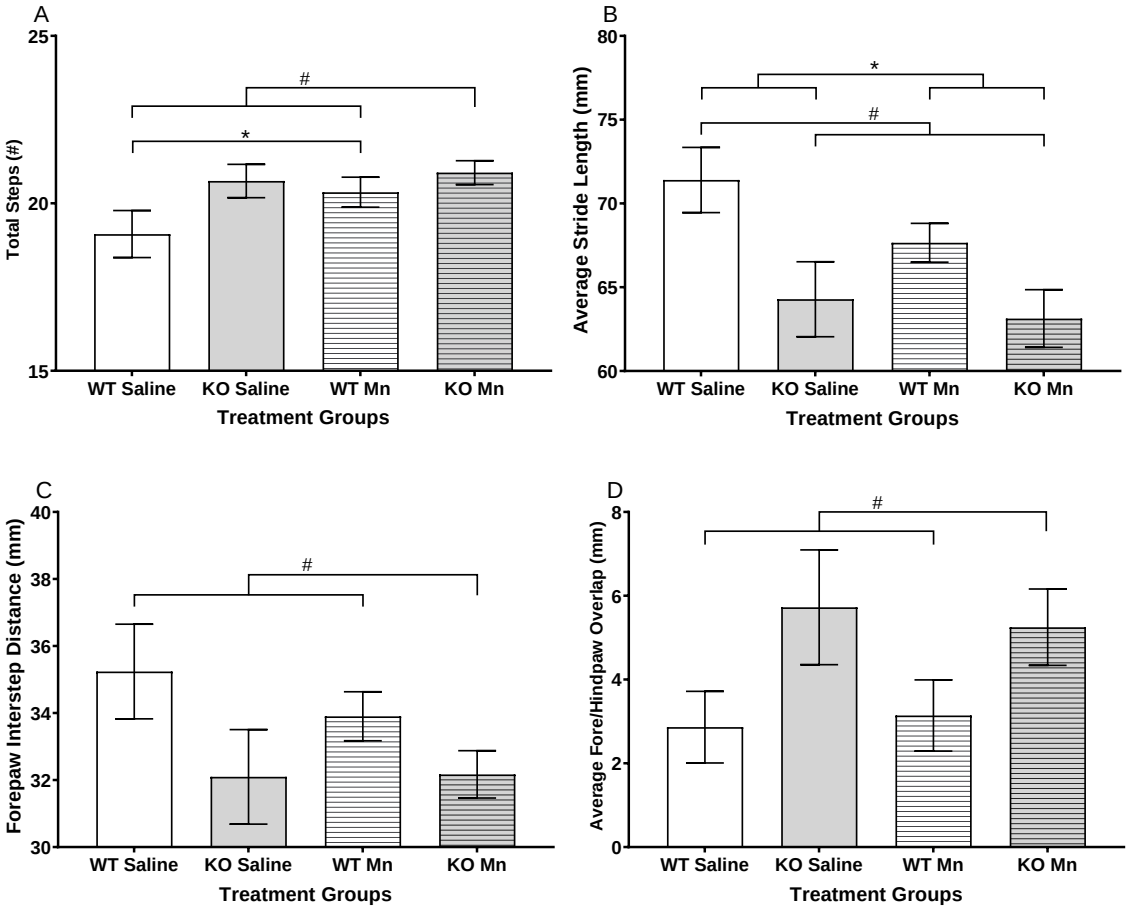

Figure 4.24. Gait Test within female WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences on Gait within female WT and KO mice. A) total number of steps taken, B) average stride length, C) forepaw interstep distance, D) average fore/hindpaw overlap distance. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; $^{\#}$indicates a significant effect of strain $p<0.05$; $n=12$/group.

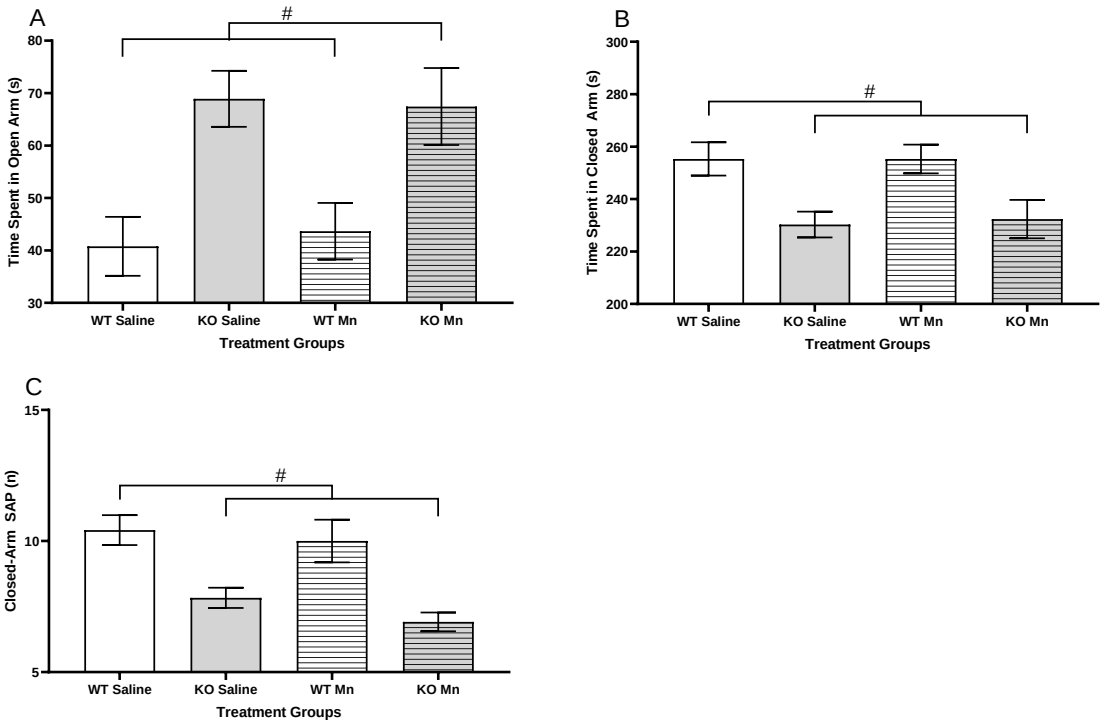

Figure 4.25. Elevated Zero Maze within female WT and miR-155 KO mice. Effect of Mn after 6 weeks of DW (0.4 g/L) exposure and/or strain differences during the elevated zero maze within female WT and KO mice. A) time spent in the open arm(s), B) time spent in the close arm(s), C) number of stretch attend posture attempts in the closed arm(s). Data are presented as mean ± SEM. ^Indicates 0.05<*p*<0.10; *n*=12/group.

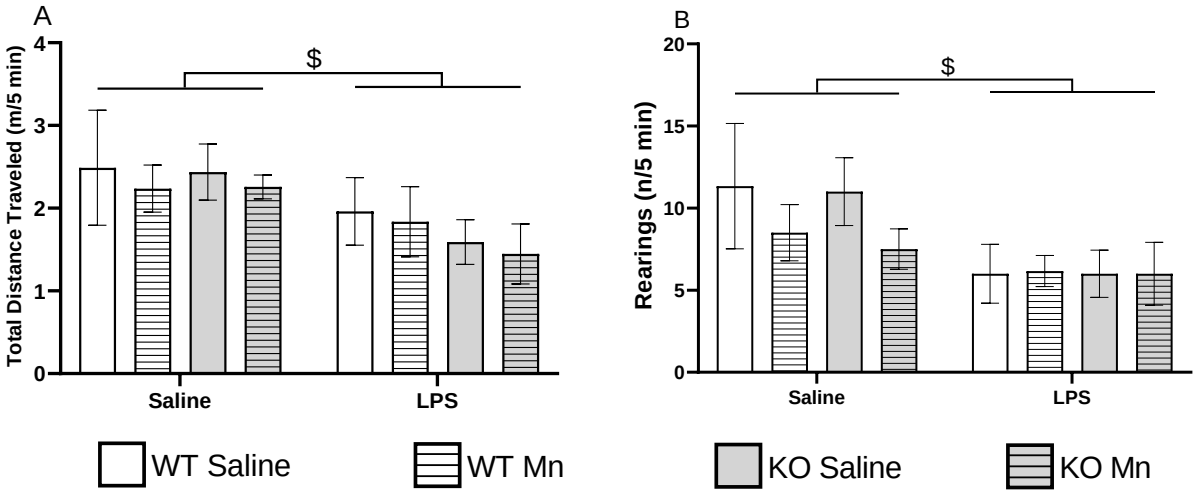

Figure 4.26. Post LPS Open Field Test within female WT and miR-155 KO mice. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, strain differences, and/or sickness behavior, four hours after a single dose of either saline or LPS (0.3 mg/kg BW) during post LPS open field test (first 5 minutes) within female WT and KO mice. A) distance traveled and B) rearing activity. Data are presented as mean ± SEM. *Indicates a significant effect of Mn $p<0.05$; $^{\#}$indicates a significant effect of strain $p<0.05$; $^{\$}$indicates a significant effect of LPS $p<0.05$; ^indicates a trend $0.05<p<0.10$; $n=12$/group.

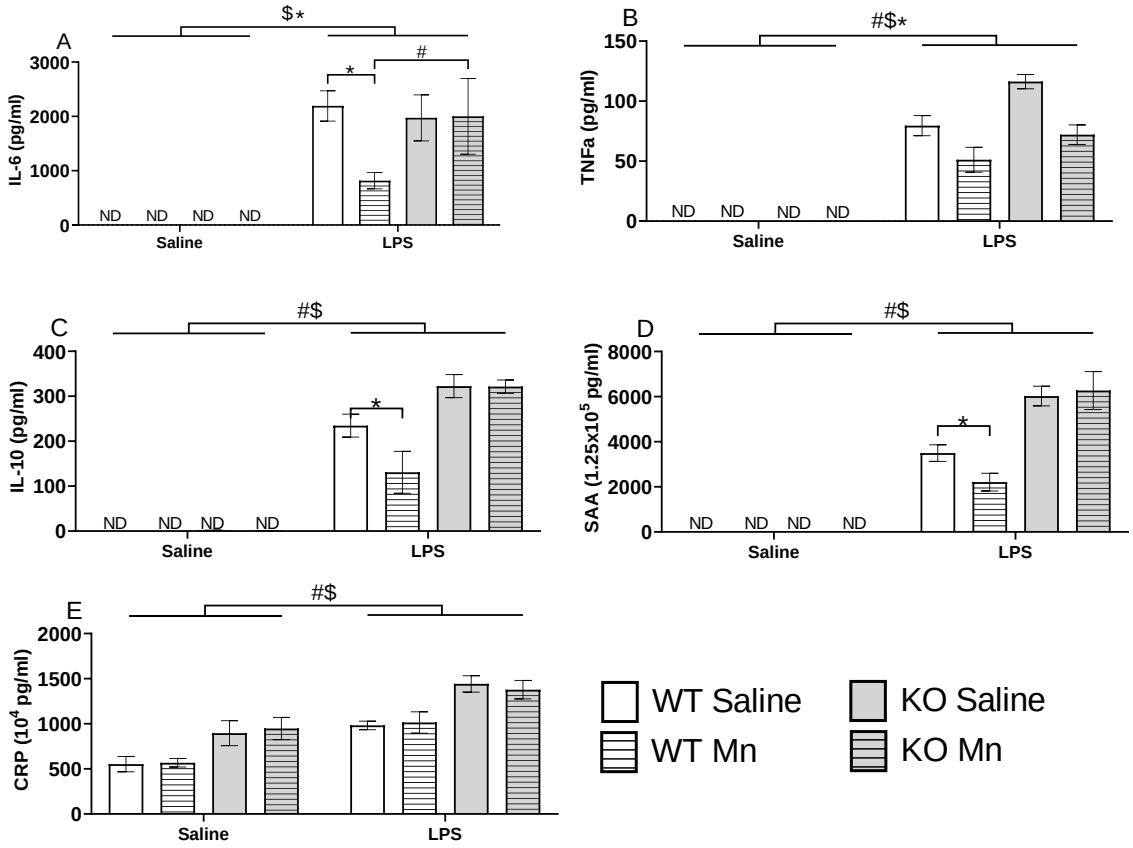

Figure 4.27. Plasma cytokine and acute phase protein (APP) levels within female WT and miR-155 KO mice. Effect of Mn after 8 weeks of DW (0.4 g/L) exposure, strain differences, and/or effect of acute LPS administration (0.3 mg/kg BW) on plasma cytokine levels 6 hours after LPS administration within female WT and KO mice. A) IL-6, B) TNFα, C) IL-10 D) SAA, and E) CRP. Data are presented as mean ± SEM. $^{\#}$ indicates a significant effect of strain $p<0.05$; * Indicates a significant effect of Mn $p<0.05$; $^{\$}$ indicates a significant effect of LPS $p<0.05$; not detected (ND); n=6/group.

Table 4.5. Relative tissue weights (mg/kg BW) for each treatment group within female WT and miR-155 KO mice after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge			
Strain	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
WT Female	Brain	21.6 ± 0.4	22.3 ± 0.72	21 ± 0.69	20.8 ± 0.64
	Kidney	12.6 ± 0.12	13.5 ± 0.35	12.5 ± 0.2	13.3 ± 0.25
	Liver	46.8 ± 0.94	46.5 ± 2.43	47.1 ± 1.28	**51.4 ± 0.86**
	Spleen	3.4 ± 0.21	**4.5 ± 0.21**	3.1 ± 0.23	**4.9 ± 0.34**
	Thymus	2.2 ± 0.09	2.2 ± 0.12	1.9 ± 0.24	1.9 ± 0.54
	BAT	3.9 ± 0.26	3.9 ± 0.17	3.8 ± 0.31	4.7 ± 0.6
	SQ	3.2 ± 0.21	3.7 ± 0.6	3.5 ± 0.71	3.1 ± 0.49
	OT	9.3 ± 1.3	9.9 ± 0.98	8 ± 1.49	8.3 ± 0.71
	RT	2.5 ± 0.41	3.2 ± 0.4	2 ± 0.35	2.9 ± 0.48
KO Female	Brain	19.5 ± 0.88	20.6 ± 0.58	20.5 ± 0.32	21.4 ± 0.60
	Kidney	11.0 ± 0.29	**12.9 ± 0.48**	12.3 ± 0.41	**13.8 ± 0.54**
	Liver	45.0 ± 1.03	44.1 ± 2.60	39.9 ± 1.48	43.0 ± 2.25
	Spleen	3.5 ± 0.30	**4.6 ± 0.21**	3.3 ± 0.15	**4.3 ± 0.18**
	Thymus	**1.8 ± 0.11**	2.1 ± 0.18	**2.2 ± 0.15**	2.4 ± 0.08
	BAT	3.4 ± 0.43	8.4 ± 4.85	3.2 ± 0.54	3.2 ± 0.24
	SQ	4.9 ± 1.13	3.7 ± 0.18	3.3 ± 0.56	3.7 ± 0.48
	OT	22.7 ± 4.49	16.4 ± 3.17	12.2 ± 2.21	14.6 ± 2.39
	RT	6.2 ± 1.81	4.9 ± 1.18	4.0 ± 0.97	4.1 ± 0.91

Tissue abbreviations: BAT, brown adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue; SQ, subcutaneous adipose tissue.

Table 4.6. Absolute tissue weight (g) for each treatment group within female WT and miR-155 KO mice after 8 weeks of Mn/saline DW exposure and 6 hours post-LPS challenge. Mean ± SEM. Bold indicates $p<0.05$; $n=6$/group.

		DW Treatment - LPS Challenge			
Strain	Organ/Fat	**Saline-Saline**	**Saline-LPS**	**Mn-Saline**	**Mn-LPS**
WT Female	Brain	0.477 ± 0.0034	0.484 ± 0.0039	0.474 ± 0.0121	0.458 ± 0.0111
	Kidney	0.278 ± 0.005	0.293 ± 0.0063	0.284 ± 0.0076	0.294 ± 0.0075
	Liver	1.04 ± 0.0403	1.012 ± 0.0562	1.067 ± 0.0217	1.131 ± 0.0223
	Spleen	0.075 ± 0.0039	**0.097 ± 0.0044**	0.07 ± 0.0059	**0.107 ± 0.0078**
	Thymus	0.048 ± 0.0021	0.047 ± 0.0032	0.043 ± 0.0062	0.052 ± 0.0106
	BAT	0.087 ± 0.0064	0.085 ± 0.0044	0.086 ± 0.0063	0.104 ± 0.0132
	SQ	0.072 ± 0.0058	0.082 ± 0.0147	0.079 ± 0.0157	0.07 ± 0.011
	OT	0.209 ± 0.0329	0.218 ± 0.024	0.184 ± 0.0373	0.184 ± 0.0172
	RT	0.057 ± 0.0098	0.072 ± 0.0115	0.045 ± 0.0086	0.064 ± 0.0111
KO Female	Brain	0.475 ± 0.00523	**0.492 ± 0.00407**	0.483 ± 0.00372	0.487 ± 0.00324
	Kidney	0.271 ± 0.00833	**0.309 ± 0.00998**	0.29 ± 0.00926	0.314 ± 0.00725
	Liver	1.112 ± 0.0728	1.055 ± 0.0682	0.943 ± 0.041	0.986 ± 0.0689
	Spleen	0.0852 ± 0.00844	**0.11 ± 0.00736**	0.0786 ± 0.00351	**0.0969 ± 0.0022**
	Thymus	0.0431 ± 0.0018	0.05 ± 0.00479	0.052 ± 0.00274	0.055 ± 0.00207
	BAT	0.0706 ± 0.00907	0.176 ± 0.102	0.0667 ± 0.0114	0.0665 ± 0.005
	SQ	0.102 ± 0.0238	0.0779 ± 0.00382	0.0689 ± 0.0117	0.0775 ± 0.0101
	OT	0.477 ± 0.0944	0.345 ± 0.0665	0.257 ± 0.0465	0.306 ± 0.0503
	RT	0.131 ± 0.038	0.104 ± 0.0248	0.0836 ± 0.0205	0.0853 ± 0.0192

Tissue abbreviations: BAT, brown adipose tissue; OT, ovarian adipose tissue; RT, retroperitoneal adipose tissue; SQ, subcutaneous adipose tissue.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

1 Overview

Manganese (Mn) is a naturally occurring transition metal and is found throughout the environment. It is essential to human health, necessary for the synthesis of lipids, amino acids, and carbohydrates as well as an important cofactor for biological processes including bone formation, energy metabolism, blood clotting, digestion, metabolism, and nervous system function. However, overexposure to this metal can be neurotoxic, particularly via inhalation in occupational settings and through Mn contaminated drinking water (DW) for the general public. Adverse neurological outcomes include locomotor, mood, and cognitive alterations. Unfortunately, Mn intoxications, especiallaly chronic ones, lead to lasting deficits that are managed symptomatically and via chelation therapies. Thus, exposure prevention is the best course of action. Furthermore, the majority of neurological effects of excess Mn, especially in the DW, have mainly been studied in males leaving the adverse effects of Mn on females understudied.

The goal of this dissertation work was to investigate sex effects of subchronic exposure to Mn through the DW and evaluate any beneficial or negative effects of miR-155 deficiency, or LPS challenge at the neurochemical, inflammatory, and behavioral levels. Here, we utilized both male and female mice from two strains - CX_3CR1^{GFP} (wildtype, WT) and mi-R155 knockout (KO) - on a C57BL/6 background. The goals were to 1) evaluate behavioral alterations in mice that were exposed to Mn via DW, 2) assess alterations to neurotransmitter systems and glial cell activity caused by subchronic Mn DW exposure, 3) investigate whether the absence of miR-155 influences these behavioral and inflammatory responses to Mn DW exposure, and 4) to

determine if deactivating miR-155 could serve as a potential therapeutic target. The following chapter will summarize the findings and limitations of these studies, as well as provide insight into future research directions.

2 Summaries of Conducted Studies

Given the lack of female data, Chapter 2 concentrated on evaluating the behavioral (mood and locomotor) alterations of male and female WT mice after subchonic Mn expsoure via the drinking water (DW; 8 weeks). This was to determine if the effects of Mn were sex-specific. Additionally, a lipopolysaccharide (LPS) challenge was conducted at the end of the Mn exposure period to assess whether sex played a role in the response of Mn-exposed mice to an inflammagen. After 6 weeks of Mn DW exposure, both sexes of WT mice had gait impairments, i.e., shorter stride length and interstep distance, which caused the Mn-exposed mice to take more steps to complete the test than their saline treated counterparts. Vertical locomotor activity was impacted by Mn exposure, with mice from the Mn DW groups rearing less during the open field test (OFT). General activity was also decreased within the Mn-exposed mice, there was less open/closed arm entries during the elevated zero maze (EZM). In regards to tests of mood, Mn-exposure resulted in a decrease in fear/anxiety-like behaviors. Mn-exposed mice spent more time in the open areas of both the OFT and the EZM. Risk assessment behaviors, including rearing attempts during the OFT and stretch attend posture attempts during the EZM, were reduced among Mn-exposed mice, particularly the males. After 8 weeks of Mn exposure, mice were challenged with LPS and Mn-exposed males, but not females, continued to exhibit increased fearlessness-like behavior during the OFT i.e., by spending more time in the center of the arena, even when they were challenged with LPS. Both liver and brain Mn levels were increased after the subchronic Mn DW exposure and the increase was not sex- or LPS-dependent. Mn alone did not affect circulating cytokines in either sex; however, Mn-exposed/LPS-challenenged males had potentiated plasma cytokine levels, while the reverse was observed in the females. The data from this study demonstrated that subchronic Mn DW

exposure does increase the level of Mn in the brain and that it leads to behavioral changes in both sexes. Though it also indicates that males may have been more susceptible to the Mn-induced effects due to males demonstrating adverse alterations across multiple behavior tests and during multiple time points. The male-specific Mn+LPS augmented cytokine production further indicated that the effects of Mn are sex-biased.

Due to the behavioral and inflammatory alterations in the periphery obserevd in these mice, and because Mn is known to disrupt neurotransmitter homeostatis, the neurochemical characterization and the modulatory effects that inflammation plays on neurotransmitter homeostasis and neuroinflamation were of intereset. In Chapter 3, neurochemical monoamine and amino acid neurotransmitters were measured in multiple brain regions, including the striatum, ventral hippocampus, and the prefrontal cortex. For neuroinflammation, astrocyte and microglia activation was measured, and the number of these cells were counted in various dorsal and ventral hippocampal areas and in the substantia nigra. In the striatum, Mn exposure did not affect monoamine neurotransmitters, including dopamine (DA) in either sex. This is particularly interesting because the striatum is a brain region that highly inervated with dopaminergic projections and is involved with motor, especially fine motor. The Mn-induced behavioral alterations that were summarized in Chapter 2 occurred in the absence of measurable changes in DA homeostasis. While DA is typically considered the neurotransmitter that predominantly controls striatal-based locomotor function, the Mn induced increase in GABA levels in males and the decreased GLU levels in females, which both lead to decreased exitatory tone (GLU/GABA ratio), coould be reponsible for the motor perturbation we observed and taken place before the Mn hallmark alterations of striatal DA occurred. Thus, it is conceivable that the behavioral changes observed in Chapter 2 were not linked to Mn-induced alterations in striatal dopamine (DA) levels. However, when Mn and LPS exposures were combined, striatal DA levels were reduced in females. In females, higher levels of 5-HT and 5-HIAA after LPS exposure suggest increased serotonin activity or turnover in the striatum. In

males exposed to LPS, 5-HIAA levels increased without a change in 5-HT levels, implying enhanced neuronal activity in that region. The hippocampus is a brain region that is associated with learning and memory; Mn toxicity is linked to cognitive impairments and behavioral changes, like reduced fear/increased risky behaviors, as seen in the male mice during Chapter 2. Males exposed to Mn showed increased GABA levels, leading to a lower GLU/GABA ratio, which may contribute to manic-like behaviors, including impulsivity (Peres et al., 2016; Takeda et al., 2002; Tinkov et al., 2021). In LPS-exposed females, elevated 5-HIAA levels in the ventral hippocampus suggest enhanced serotonin turnover, possibly indicating increased anxiety or overactivity in the serotonergic system. Hormones like estrogen offer neuroprotection against Mn-induced neurotoxicity. This protective effect is mediated, partly, by preventing oxidative stress and Mn-induced disruption of glutamate homeostasis (Pajarillo et al., 2018). The prefrontal cortex is a brain region that plays a role in executive functions including planning, decision-making, working memory, attention, focus, learning, impulse control, as well as regulation of stress responsivity. In Chapter 2, Mn exposure led to decreased fear and increased risky behaviors, which were persistent in the males. The Mn-induced rise in GABA suggested greater inhibitory tone in the PFC, possibly contributing to this behavior. The decreased GLU/GABA ratio, especially in males, may explain the observed impulsivity and risky behaviors. Mn also increased GLN and GLU levels in the PFC, which may indicate disruptions in the uptake or recycling mechanisms, potentially contributing to excitotoxicity and dysregulated neurotransmitter homeostasis. Interestingly, the PFC was the only region showing Mn-related changes in monoamines. Mn-exposed males had lower 5-HT levels, which may be linked to their risky behaviors, while Mn-exposed females had higher 5-HIAA levels, indicating potential overactivation of the serotonergic system, which could increase anxiety over time. Since the hippocampus is important for learning and memory, as Mn exposure has been reported to impact these aspects, it was important to investigate microglia and astrocytes in several areas of the ventral and dorsal hippocampus alongside neurotransmitter homeostasis in the

hippocampus. Mn exposure led to sex-dependent changes in glial cell activity in various brain regions. In the dorsal hippocampus, Mn exposure increased microglia activation in females, especially in the Mn+LPS group in the hilar region, while males showed higher microglia presence in the CA3. Astrocyte numbers were unaffected by Mn, but females had greater microglial activation and astrocyte activity in the ventral hippocampus, particularly in the CA1, CA3, and dentate gyrus. In the substantia nigra, Mn-exposed males showed reduced microglial and astrocyte activity, while females had increased activation in the substantia nigra pars compacta. Overall, females exhibited higher levels of glial activation and microglial activity than males, suggesting a more pronounced response to Mn exposure in females. These findings highlight the potential for sex-specific effects of Mn on brain function, particularly in glial cells and neurotransmitter activity. Immunohistochemical analysis of tyrosine hydroxylase (TH), a dopaminergic marker, did/did not reveal any major manganese/LPS induced alterations in the striatum/substantia nigra. However, there were increases/decreases in neuroinflammatory (astrocytes and microglia) cells in the hippocampus (dorsal/ventral/striatum/substantia nigra). The behavioral and biological parameters evaluated in this study provide additional data on Mn overexposure in sexes and how LPS could further antagonize the system.

The work in Chapter 4 begins the exploration of how the different sexes respond behaviorally to subchronic Mn exposure through drinking water when miRNA155 is missing. Mn-exposed KO mice, particularly female KO mice, showed reduced horizontal locomotor activity in the OFT, while Mn-exposed WT mice had reduced vertical activity. KO females exhibited fewer rearings than saline-treated KO females but more than Mn-exposed WT females. Gait analysis revealed reduced fore/hindpaw overlap in Mn-exposed KO mice, whereas stride length, interstep distance, and step count were only affected in Mn-exposed WT mice. In the EZM, Mn had no effect on KO mice but increased activity in WT males. KO mice showed no Mn-induced changes in risk assessment or anxiety-like behaviors, while WT mice spent more time in open areas and had reduced rearing and SAP attempts. LPS challenge reduced locomotor activity

across all treatment groups. Notably, saline-treated KO males spent more time in the center of the OFT than Mn-exposed KO males or WT controls—a pattern not observed in females.

Plasma cytokine and acute phase protein levels were assessed to explore how inflammation differs with and without miR-155. Mn alone did not affect cytokine or APP levels in either sex or strain. However, in KO females, Mn reduced TNFα levels. LPS exposure increased TNFα, IL-6, IL-10, SAA, and CRP in both sexes. IL-10 rose in KO but dropped in WT males post-LPS. Mn reduced IL-10 and SAA only in WT females. WT females mounted a stronger inflammatory response than males, while KO mice showed sex-similar cytokine patterns, suggesting miR-155 deletion modulates both behavioral and immune responses to Mn in a sex-dependent manner.

3 Limitations of Current Studies

While these studies pointed at important new nuances related to sex differences in Mn drinking water exposures, it is imperative to discuss limitations of the current work. While the estrus cycle was staged weekly, it was only staged once a week, and sex hormones were not measured at any point. Estrogen can influence how the females' innate immune system responds to an inflammatory challenge and may play a protective role in Mn exposures.

Other behavior tests were conducted as part of this study; however, they were found to be not significant or had too many confounding variables and thus were excluded from analysis. While the battery of testing encompassed multiple domains, i.e., cognition, mood, and motor, conducting several tests in a row daily could have had carry over effects, such as stress, which may have influenced behavioral results. Even though it was benefitcial to have a strain of mice that already had their microglia tagged with a green flouresenct protein, a technical limitation for this is that during IHC only red could be used to duel tag i.e. astrocytes + microglia or TH+ neurons + microglia – which could be overcome by using a different color and filter combination. A limitation to the study is that cell numbers, i.e., microglia, astrocytes, and TH+ neurons, were not estimated using unbiased stereology, which may impact the accuacy of quantifying cell

populations. To further enhance Chapter 3, Sholl analysis should be considered in future investigaion to analyze the structure of the microglia in order to determine their morphophysiolgocial state. For example, ramiffied microglia would be highly branched and in survelience mode, whereas an amoeboid state would be in response to an inflammatory event. The water intake, in this study, was an estimate for each individual mouse's water intake, since mice were group housed 2-5 per cage. To get a better water intake, mice would have to be single-housed instead. Future investigation of neurochemistry, neuroinflammation, and Mn brain level measurements of the KO mice will need to be conducted to fully explore the impacts of the absence of miR-155 after Mn DW exposure. TNFα production, as stated by Jackson Laboratory, was supposed to be deficient in the KO mice; however, the KO mice produced similar levels of this cytokine as WT mice. In the future, cytokine expression levels (i.e., qPCR of the inflammatory genes) of both strains of mice should be analyzed to determine if cytokine gene expression levels are impacted.

4 Conclusions

Manganese (Mn) is an essential trace metal that is important for several biological processes. Overexposures, particularly via inhalation and consuming Mn contaminated drinking water, of this metal will cause neurological deficits. The dissertation research summarized here investigates the effects of subchronic Mn exposure via drinking water in both male and female mice, focusing on behavioral and neurochemical alterations and the role of central and peripheral inflammation. The findings revealed that Mn exposure led to both behavioral and mood impairments in both sexes, including decreased locomotor activity, gait deficits, and reduced fearless/anxiety-like behaviors. The behavioral effects of Mn exposure seem to be more pronounced within males and more subtler within females. Additionally, Mn exposure in combination with LPS caused sex-biased effects on cytokine production, with males showing increased cytokine levels and females displaying the opposite. Whether this lesser susceptibility to Mn/LPS combination in the females is due to female-specific hormones, such as estrogen,

differential cellular sensitivity/uptake of Mn, or other mechanism, remains to be determined. Neurochemical analyses in the brain showed that Mn exposure did not affect dopamine levels in the striatum, but led to sex-dependent changes in other neurotransmitters in this brain region, such as GABA and serotonin. In particular, males showed increased GABA levels and altered GLU/GABA ratios, while females exhibited changes in serotonin turnover. These neurochemical changes were accompanied by sex-dependent differences in glial cell activity, with females showing more pronounced microglial and astrocyte activation in the hippocampus. The study also assessed how the absence of miR-155, a microRNA involved in immune response, influenced the effects of Mn exposure. The results indicated that mice lacking miR-155 exhibited less pronounced behavioral changes in response to Mn exposure, particularly in females, suggesting that miR-155 may play a role in modulating the effects of Mn on behavior and inflammation. In conclusion, this study highlights sex-dependent differences in the behavioral, neurochemical, and inflammatory effects of Mn exposure, with males being more susceptible to adverse effects. The findings also suggest that inflammation, likely partly dependent on functional miR-155, may contribute to the observed differences in Mn toxicity between sexes.

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