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Manganese, copper, and iron are essential trace metals necessary for proper functioning of the human body, and play important roles in cellular responses, signaling, regulation, and overall cell function and metabolism, making them necessary for normal function of the brain. Inflammation is present in nearly every disease, while systemic inflammation has been linked to alterations in brain trace metal homeostasis, which can cause detrimental effects to the brain, such as in cases of neurodegenerative diseases. This study aimed to determine the effects of inflammation on brain trace metal biology, through the utilization of two routes of inflammation in a mouse model study. We hypothesized that inflammation from TNF-α injections in C57BL/6J and utilization of LDLr-/- male mice would cause alterations of manganese, copper, and iron levels in the brain. In order to determine this, we dissected brain tissue into 3 regions, the midbrain, striatum, and cortex. Protein content was determined for the brain tissues, and metal analysis was performed in order to determine the concentration of each metal per milligram of protein. The findings from this study suggested that in the presence of inflammation, there may be increases of manganese in the cortex, copper in the striatum, and iron in the midbrain and striatum. The increases seen in this study seemed to decrease with the addition of carbon nanodot treatment, which promotes further research. The continuation of work on this subject has the potential to enhance research on inflammation and neurodegeneration, and potentially reduce the progression of neurodegenerative diseases through reduction of inflammation.

THE CONSEQUENCES OF PUTATIVE INFLAMMATION ON BRAIN TRACE METAL BIOLOGY

by

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APPROVAL PAGE

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CHAPTER I: INTRODUCTION

Introduction

Manganese, copper, and iron are essential trace metals necessary for proper functioning of the human body and must remain in the optimal physiological range for health (1). These metals play important roles in cellular responses, signaling, regulation, and overall cell function and metabolism, making them necessary for normal function of the brain (1). Inflammation is present in many common diseases, and systemic inflammation has been linked to alterations in trace metal homeostasis (2). When these metals become altered or disrupted there can be detrimental effects to the brain, such as in cases of neurodegenerative diseases (2). The blood brain barrier is a functional and structural barrier to the brain that plays an important role in maintaining an optimal environment for the brain, but there can be disruptive changes in the blood brain barrier when systemic inflammation is present (3). Inflammation is prevalent worldwide, as it is common in many of the diseases that contribute to the top causes of death, and the inflammation present within these common disease states has been linked to neurological disruptions, such as altered trace metal homeostasis (3,4). Since inflammation is omnipresent in many conditions, it is important to understand the effects it could have on brain trace metal homeostasis, as even small changes in homeostasis can cause detrimental effects on the brain and body. This study aimed to determine the effects of systemic inflammation on brain trace metal biology, through the utilization of two routes of inflammation in a mouse model study. The two routes of inflammation used were TNF-α injections in C57BL/6J mice, and application of LDL receptor knockout (LDLr-/-) mice, a mouse model known to display inflammation. We hypothesized that inflammation in C57BL/6J and LDLr-/- male mice would cause alterations of manganese, copper, and iron levels in the brain.

Study Objective and Design

The objective of this study was to assess the effects of inflammation on brain levels of trace metals manganese, copper, and iron in C57BL/6J and LDLr-/- male mice. This study employed the use of two different groups of mice in order to determine changes in brain trace metal biology in two separate states of inflammation. The two groups of mice (two experiments) and two routes of inflammation that were utilized were as follows:

1. **Experiment 1: Systemic acute inflammation via tumor necrosis factor alpha (TNF-α) injections**

Sample size= 20

The control mice group received 100mL of sterilized saline via intraperitoneal (IP) injection once a day for 7 consecutive days. TNF-α mice received an IP injection of 25 ug/kg of TNF-α once a day for 7 consecutive days to induce systemic inflammation. The co-treatment mice groups received both the TNF-α (25ug/kg), along with either carbon nanodots (CND) at 2.5mg/kg daily or resveratrol (RES) at 10mg/kg daily for 7 consecutive days.

Table 1: Experiment 1 Study Design

C57BL/6J: Saline Control (n=5)	Saline control via IP injection to mice
C57BL/6J: TNF-α (n=5)	TNF-α 25ug/kg of body weight via IP injection to mice
C57BL/6J: Co-treatment: TNF-α + Carbon Nanodots (CDN) (n=5)	TNF-α 25ug/kg body weight + CND at 2.5mg/kg of body weight via IP injection to mice
C57BL/6J: Co-treatment: TNF-α + Resveratrol (RES) (n=5)	TNF-α 25ug/kg of body weight + RES at 10mg/kg of body weight via IP injection to mice

2. **Experiment 2: Systemic chronic inflammation via utilization of LDL receptor knockout mice (LDLr-/-), a known animal model displaying severe inflammation**

Sample size= 36

The saline control C57BL/6J mice group received 100mL of sterilized saline via IP injection 2 times per week, 3 days apart each. The CND control C57BL/6J mice group received 2.5mg/kg body weight of CND via IP injection 2 times per week, 3 days apart each. Each of the LDLr-/- mice received their specified treatments of CND via IP injection 2 times per week, 3 days apart each.

Table 2: Experiment 2 Study Design

C57BL/6J: Saline Control (n=6)	Saline control via IP injection to C57BL/6J mice
C57BL/6J: CND 2.5mg/kg (n=6)	CND 2.5mg/kg body weight via IP injection to C57BL/6J mice
LDLr-/-: CND 0mg/kg (n=6)	CND 0mg/kg body weight via IP injection to LDLr-/- mice
LDLr-/-: CND .1mg/kg (n=6)	CND .1mg/kg body weight via IP injection to LDLr-/- mice
LDLr-/-: CND .5mg/kg (n=6)	CND .5mg/kg body weight via IP injection to LDLr-/- mice
LDLr-/-: CND 2.5mg/kg (n=6)	CND 2.5mg/kg body weight via IP injection to LDLr-/- mice

CHAPTER II: LITERATURE REVIEW

Introduction

Inflammation is prevalent worldwide, as it is present within many of the top causes of death including heart disease, cancer, chronic lower respiratory diseases, stroke, neurodegenerative diseases, and diabetes (4). Manganese, copper, and iron are three trace metals that each play an important role in many physiological processes throughout the body including cellular response, signaling, regulation, and overall cell function and metabolism (1). These trace metals have specific uses within the body and contribute to healthy functioning of the brain, but when inflammation is present there may be changes that occur which affect trace metal homeostasis in the brain (3). In the presence of inflammation, changes to the blood brain barrier occur, which can lead to disruptions in the central nervous system and progression of disease, making the blood brain barrier a crucial member in maintaining a healthy brain (3). Because trace metal homeostasis is crucial to health, it is important to understand the significance of these metals, and also the effects that inflammation could have on the blood brain barrier, causing disruption of trace metal homeostasis. The blood brain barrier plays a crucial role in the protection of the central nervous system, but if damage or dysfunction occurs, it can also lead to induction of disease (3). In the presence of inflammation, there may be changes that occur to the blood brain barrier, allowing for potential infiltration of metals and dysregulation. Therefore, it is critical to understand how inflammation could affect the blood brain barrier, in turn leading to brain trace metal dysregulation. This review will summarize the functions of manganese, copper, and iron in the brain and body, while also discussing inflammation, the blood brain barrier, and disturbances in the homeostasis of these trace metals.

Manganese

Manganese is an essential trace metal that plays an important role for normal development of organs as well as a common cofactor in several enzymes (5). Arginase, glutamine synthetase, and manganese superoxide dismutase are each manganese-dependent enzymes that cannot be replaced by another metal, therefore adequate manganese in the diet is crucial for the functioning of these enzymes (5). Manganese deficiency is rare, but has been shown to impair growth, reduce fertility, and cause skeletal and birth defects (6). It is essential that manganese remains in homeostasis, because if homeostasis is altered it can lead to neurological dysfunction and other neurotoxic conditions (5). The regulation of absorption and secretion of manganese is impervious, so the balance of manganese within the body usually is stable but in cases of overexposure manganese toxicity can occur, in which manganese will accumulate in certain brain regions, causing detrimental effects (6). It is necessary to understand the effects of manganese toxicity on the brain and body, along with how inflammation could alter brain manganese levels.

Homeostasis of manganese in the brain is tightly regulated, but when inflammation is present it could result in altered brain manganese levels. Manganese insufficiency is a very rare condition in humans, but toxicity is common and can be inherited due to a gene mutation or acquired due to environmental settings, such as certain occupations where inhalation of manganese-containing dust occurs (6). It is suggested that manganese enters the brain at the blood brain barrier through entry across the cerebral capillaries, but the exact mechanisms of manganese entry into the brain are not fully understood (7). Manganese neurotoxicity can result in neuroinflammation, oxidative stress, mitochondrial dysfunction, and extrapyramidal motor dysfunction, resembling conditions that come with Parkinson's disease (7). Although it has been

shown that manganese toxicity can occur due to environmental exposure or a gene mutation, the literature remains unclear on how inflammation could affect brain manganese levels.

Copper

Copper is critical for many essential enzymes, normal development of the brain, and as a structural cofactor for certain proteins and nucleic acids (8,9). In the brain, copper is also important in energy metabolism, antioxidant defense, iron metabolism and neurotransmitter synthesis (8). In addition, copper also acts as a redox agent, so if in excess it can lead to the generation of toxic reactive oxygen species, thus copper homeostasis in the brain is crucial to health and proper functioning of the brain (8). Because of this, the uptake, storage, and export of copper in the brain is regulated by suitable mechanisms. Both copper toxicity and deficiency have been suggested to result in dysregulated brain function, leading to neurodegeneration, and are associated with conditions such as Menkes disease, Wilson's disease, Alzheimer's disease, Huntington's disease, and Parkinson's disease (8). Consequently, it is important to understand how inflammation could affect copper homeostasis in the brain.

When copper accumulates in the cell, it stimulates the production of proinflammatory cytokines, leading to the development of an inflammatory response and promotes further accumulation of copper due to the proinflammatory cytokines (9). In an inflammatory state, the production of proinflammatory cytokines increases which is suggested to advocate the accumulation of copper in cells, causing oxidative damage (9). In a mouse-model study that focused on how inflammation affects copper uptake in the lung, it was found that in patients with pulmonary diseases the sputum copper increased (10). This study confirmed that due to copper overload in the lung, mice with pulmonary diseases were susceptible to copper-mediated oxidative damage (10). The researchers also demonstrated that tumor necrosis factor (TNF-α), an

inflammatory cytokine, upregulated the expression of a metallic reductase that is involved in facilitating copper uptake (10). If these results are similar to what would happen to the brain, then we could expect that in the presence of inflammation copper levels would be higher in the brain.

Iron

Iron is an additional essential trace metal that is also the most abundant trace metal in the human body (11). Because iron is the amplest trace metal in the body, it is important to understand its mechanisms and involvement in the body. Iron acts as a component of many enzymes, while playing a role in cell proliferation, cellular respiration, DNA synthesis, and energy metabolism (11), It also is involved with hemoglobin, the protein involved in carrying oxygen from the lungs to the cells and tissue throughout the body (11). In the central nervous system, iron plays a significant role in the metabolism of the neurotransmitters catecholamines, which are a group of substances released in response to physical or emotional stress (11,12). The accumulation of iron in the brain has been shown to lead to certain neurological disorders, such as Alzheimer's and Parkinson's disease (13). Because of irons' crucial role in many physiological processes throughout the body, it is important to understand how disruptions to brain iron homeostasis can affect the body.

Iron homeostasis is extremely complex and not fully understood yet. However, it is known that both iron deficiency and overexposure can result in serious complications in vital organs such as the brain (13). Iron deficiency is the leading cause of anemia and has been shown to have adverse effects on early development, and throughout the lifespan, and is associated with poor prognosis in chronic disease patients (13,14). Overexposure of iron, or iron toxicity in the brain has been suggested to play a role in the pathogenesis of neurodevelopmental disorders and

age-related diseases of the brain (13). When iron accumulates in the brain, the mitochondrial respiratory chain becomes impaired, which can lead to a large increase in levels of reactive oxygen species, which can damage proteins and result in cell death (13). There are mechanisms in place to help regulate iron homeostasis in the case of an offense such as inflammation, and as with other metals, this homeostatic regulation occurs mostly at the blood brain barrier (13). Chronic inflammation has been suggested to limit iron availability, while tissue-specific brain iron toxicity can be seen without an increase in systemic iron (15). This suggests that the processes that regulate iron homeostasis could be overwhelmed by a condition that involves chronic inflammation, and it is not yet known whether inflammation is specifically correlated to increases in brain iron levels.

Inflammation

Inflammation is a common condition and adaptive response that underlies many physiological processes in response to conditions such as infections and injuries, which is usually divided into two groups: acute inflammation and chronic inflammation (16). Acute inflammation is a short-term response to infection or injury, in which the initial stages are mediated by mast cells and macrophages, which then trigger the production of other inflammatory mediators (16). This then stimulates recruitment of plasma proteins and leukocytes, and to eliminate the infectious agents, neutrophils are then recruited to release their toxic contents such as reactive oxygen species (16). However, much less is known about the mechanisms of chronic inflammation, which has been suggested to not only be present in, but also contribute to many diseases such as autoimmune diseases, atherosclerosis, cardiovascular disease type 2 diabetes, and cancer (16,17).

Chronic diseases remain at the root of the leading causes of death worldwide, and in the shadow of chronic diseases is chronic inflammation, which upregulates pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1, interleukin 6 (IL-6), and cyclooxygenase-2 (18). Chronic inflammation is prolonged and systemic, and rather than the inflammation being caused by infection or injury, it is associated with malfunction of the tissue such as an imbalance in a physiological system due to a chronic disease state (16). During inflammation, there is tight control over the inflammatory pathways in order to limit tissue damage, but in chronic illness this regulation is less understood (19). In the case of chronic diseases, the inflammatory response persists and there tends to be more tissue and organ damage, along with abnormal repair of tissue (18). This information could suggest that chronic systemic inflammation could have adverse effects on the blood brain barrier, in turn causing neuroinflammation and dysregulation of brain trace metals.

The Blood Brain Barrier

The blood brain barrier is a necessary feature of the central nervous system that acts as a functional and structural barrier to the brain from toxins and pathogens in the bloodstream, while also acting to regulate the movement of molecules, cells, and metals between the blood and the brain (20). Because the blood brain barrier plays such a crucial role in the homeostatic mechanisms of the brain, it is important to understand some of the mechanisms behind this control. There is critical restraint within the blood brain barrier in order to help control homeostasis of the central nervous system and allow proper function and protection of neural tissue (20). The physiological barrier is composed of several barriers such as the choroid plexus and the capillary bed of the central nervous system, and consists of the metabolic, physical and transport properties of the endothelial cells (20,21). To understand how inflammation could

affect the blood brain barrier, it is beneficial to have modest knowledge on how this barrier functions.

The blood brain barrier consists of blood vessels that help deliver oxygen and nutrients to the brain, while removing harmful products such as metabolic waste and carbon dioxide (20). In order for this barrier to function properly, there must be interactions between the neurons and microglia, astrocytes, and pericytes, which are each important cells of the brain (22). The barrier is composed of three main cellular elements, which are the endothelial cells, astrocytes, and pericytes (20). In between the endothelial cells, a tight junction forms to act as a diffusion barrier, which is how the barrier prevents pathogens from entering the brain (20,22). If the tight junction becomes impaired there can be dysfunction in the blood brain barrier, which in turn would complicate neurological diseases or conditions such as stroke (23). Astrocytes are a type of glial cell, which are able to induce barrier properties and contain proteins that are needed in water regulation and homeostasis of the brain (20). On the other hand, pericytes are a type of cell that are embedded in the vascular basement membrane, which are important in blood vessel formation, blood flow control to the brain, and maintenance of the blood brain barrier (20). A properly functioning blood brain barrier is necessary for the human body to remain healthy, as without its homeostatic mechanisms' disease could quickly occur. In neurological disorders such as Parkinson's and Alzheimer's disease, there is a loss of some or all the barrier's functions, which can lead to neuronal dysregulation and dysfunction (20). Because of this, it is important to better understand the impacts that inflammation has on the blood brain barrier.

Inflammation and the Blood Brain Barrier

Within the blood brain barrier, every single cell plays an important role in the protection and homeostatic regulation of the brain, but if one constituent fails to function properly, the

barrier can break down, resulting in neurodegeneration, neuroinflammation, and impaired brain homeostasis (24). Because of this, it is important to understand the effects that inflammation has on the barrier function, in order to understand how it could contribute to changes in trace metal homeostasis in the brain. When oxidative stress is present, such as in neurological disorders, blood brain barrier breakdown is seen, but it is difficult to determine if the breakdown is due to oxidative stress itself (24). The oxidative stress is suggested to contribute to breakdown of the blood brain barrier through damage of proteins and upregulation of inflammatory mediators, which are understood to compromise the barrier (24). This breakdown is seen in many neurological diseases, but it is difficult to determine whether inflammation itself leads to the barrier disruption, rather than the disease itself, although it is known that inflammation can induce barrier dysfunction (24,25,26). With this, it is necessary to understand the impact that inflammation has on the barrier.

In many *in vitro* and *in vivo* studies, a component of gram-negative bacteria called lipopolysaccharide (LPS) is used to model systemic inflammation (25). In *in vitro* brain microvascular endothelial cell studies, LPS was shown to have disruptive effects on ions and solutes such as albumin at the blood brain barrier (25). In *in vivo* studies, LPS also has shown disruptive changes to the barrier 60% of the time, while increasing barrier permeability, but the effects of LPS on the barrier were not the same among all studies (25,26). Because of this, it is agreeable to suggest that systemic inflammation in LPS studies can cause disruption changes to the blood brain barrier, although the mechanisms behind this are not fully understood. Some of the proposed mechanisms are due to nitric oxide and prostanoids, which are mediators of the inflammatory pathways and are synthesized by the cerebrovascular endothelial cells (25). There is often endothelial damage and changes to astrocytes that occur with systemic inflammation,

along with increased permeability of the barrier, which are each proposed mechanisms that could lead to further dysfunction of the barrier (25).

In an Alzheimer's disease mouse model of amyloid precursor protein transgenic mice, the injection of LPS increased permeability of the blood brain barrier, which allowed for the entrance of proinflammatory cytokines TNF-α and IL-6 into the brain and further promoted neuroinflammation (26). With other neurodegenerative diseases, it is also suggested that inflammation could be a potential risk factor for blood brain barrier dysfunction, and it is suggested that this disruption is an early event that occurs before the diffusion of inflammatory cytokines into the brain (26,27). After the inflammatory molecules infiltrate the brain, they damage the microglia, astrocytes, and pericytes, resulting in neuronal damage and continued barrier dysfunction (27). The infiltration of inflammatory cytokines has also been shown to reduce tight junction expression, which immediately affects the integrity of the barrier, allowing for more permeability of unwanted molecules or metals (25). Therefore, it can be suggested that inflammation could directly cause barrier dysfunction, allowing for the penetration of undesirable molecules into the brain, which could lead to dysregulated brain homeostasis. Further research is needed in order to fully understand the role of systemic inflammation on blood brain barrier function.

Brain Trace Metal Dysregulation

Since we have discussed how inflammation can cause changes to the blood brain barrier such as increased permeability, endothelial damage, and reduced tight junction expression, it is necessary to discuss how inflammation could lead to brain trace metal dysregulation. Disruption of trace metal homeostasis has been seen in many conditions such as in Parkinson's disease, there has been an increase of iron levels in the substantia nigra, along with decreased copper in

this same region (28). Iron levels in the striatum and cortex have shown to be comparable to the controls, while some studies have shown no overall increase in iron levels in the brain in Parkinson's disease (28). Whether the dysregulation seen in the substantia nigra is due to inflammation itself or because of the neurodegeneration present in the disease state is inconclusive. In Wilson's disease, copper levels have been shown to be increased, while in post-mortem studies there has been increased iron seen in the striatum (28). As discussed, manganese dysregulation is often seen in manganese over-exposure, but whether inflammation could lead to manganese dysregulation in the brain is unknown (6).

Obesity is prevalent worldwide and has been considered a global epidemic that affects over 2 billion people (29). Within obesity, it is known that inflammation is present due to the activation of inflammatory pathways (29). In mouse model studies focusing on diet induced obesity and trace metal dysregulation, there have been changes in trace metal homeostasis seen within the brain (30,31,32). In a study that looked at the effects of diet induced obesity on trace metal homeostasis and gene expression, it was found that in male mice, diet induced obesity led to changes in iron and manganese in the brain (30). This suggested that diet induced obesity could potentially contribute to the development of a neurodegenerative disease due to the alterations in brain trace metal homeostasis (30). In another mouse model study that explored the impact of obesity on brain iron levels, it was discovered that in obese mice there were significant changes in iron levels when compared to the control mice (31). This study focused on the midbrain, thalamus, hippocampus, and striatum, but the differences in iron were only seen in the midbrain and thalamus (31). Because of the altered trace metal homeostasis seen within this study, the results from this study could suggest a potential link between obesity and neurodegeneration.

According to another study that looked at trace metal dysregulation due to diet induced obesity, obesity has also been shown to decrease copper levels in the midbrain of male and female mice, while iron was shown to increase in males only (32). With obesity, not only is inflammation a common consequence, but also oxidative stress and mitochondrial dysfunction (32). Each of these consequences could result in disruptive changes to the blood brain barrier, thus giving the expected results of altered trace metal homeostasis. Another study looked at the effects of heavy alcohol consumption in mice on brain iron, copper and zinc levels (33). The results found that heavy alcohol consumption increased iron levels, and decreased copper and zinc, each in the hippocampus (33). In humans, high alcohol consumption has been associated with neurodegeneration possibly due to the disruption in iron levels, and can lead to alcoholic liver disease, the most common type of chronic liver disease worldwide, in which hepatic inflammation is present (33,34). The results from each of these studies focusing on obesity suggest that in the presence of environmental stress such as obesity, brain trace metal metabolism is altered. Since inflammation is present within diet induced obesity and heavy alcohol consumption the question on whether inflammation causes changes in brain trace metal biology stands.

Conclusion

The blood brain barrier is a complex structure needed to regulate the transport of molecules and cells in and out of the brain, while maintaining a normal physiological environment to protect the brain from damage (3). Inflammation is a condition involved in many disease states, which through complicated mechanisms has been suggested to enhance breakdown and dysfunction of the blood brain barrier (19). This breakdown of the barrier allows for enhanced permeability into and out of the brain, leaving the brain susceptible to altered trace

metal homeostasis, which can be detrimental on the brain (27). Because of this, the question of whether or not inflammation can directly cause changes in brain trace metal homeostasis has not been determined. There is plenty of research that focuses on the effects of neurodegenerative diseases, chronic diseases, and obesity on brain trace metal homeostasis, but there are few studies that focus on neuroinflammation and brain trace metal biology. Additional research is needed to determine how inflammation can affect trace metal biology in the brain, including trace metals manganese, copper, and iron.

Hence, the objective of the proposed research is to assess the effects of acute and chronic inflammation on brain levels of trace metals manganese, copper, and iron in C57BL/6J and LDLr-/- male mice via two routes of inflammation:

1. Systemic inflammation in C57BL/6J mice via TNF-α injections, an inflammatory cytokine that causes inflammation.
2. Systemic inflammation via utilization of LDLr-/- mice, a known animal model displaying severe inflammation.

CHAPTER III: METHODOLOGY

Animals and Diet

The tissues that were used in this study were received from the Jia lab in the Biology department at the University of North Carolina at Greensboro, with approval for animal care and procedures by the Animal Care and Use Committee at the University of North Carolina at Greensboro. Two separate groups of male mice brain tissue were received from their lab, as mentioned before in the study design, with Experiment 1 being C57BL/6J male mice and having been injected with TNF-α to induce inflammation and treated with resveratrol and carbon nanodots (CND). Experiment 2 consisted of male mice who were LDL-receptor knockout mice (LDLr-/-), as this would induce obesity and atherosclerosis, suggesting inflammation, and the treatment was CND in varying amounts, described below. The Experiment 1 mice were fed a regular diet provided by the UNCG Animal Research Facility, while the Experiment 2 mice were fed an atherogenic diet (TD:88137), which is used to accelerate atherosclerosis and closely resembles the Western Diet, to help induce obesity. The number of animals required for this experiment was determined through preliminary studies and was based on a power analysis using estimated variance.

Carbon Nanodot Treatment

Recently, research on the potential antioxidant capacities of carbon nanodots has been emerging, and the mice for this project were received from a lab that looks at the use of carbon nanodots as a nanomaterial for biomedical research such as a drug delivery, an anti-inflammatory, a bioimaging tool, and a potential antioxidant (35). Because of this, it is important to briefly understand the current research on carbon nanodots as a treatment for inflammation, as it was used in the present project to treat inflammation in TNF-α and LDLr-/- mice in order to

look at potential changes in brain trace metals. Not only have carbon nanodots been studied for their antioxidant and anti-inflammatory properties, but they also have become popular in research due to their excellent photoluminescence, biocompatibility, and stability (36). Current research looking at the effects of carbon nanodots on inflammation has found that carbon nanodots reduce the expression of proinflammatory genes, act as an electron donor to support free radical scavenging, promote the phagocytic activity of macrophages, and can protect against oxidative damage from reactive oxygen species (35,36,37). Specifically, one study looked at the effects of carbon nanodots in atherosclerosis, which corresponds with the physical state of the LDLr-/- mice used in the present study (37). Each of these findings support the hypothesis that carbon nanodots may be a potential treatment for chronic conditions in which an inflammatory state is present, which is why we also wanted to look at the differences in brain trace metals based on varying levels of carbon nanodot treatment in LDLr-/- mice.

Brain Tissue Collection

Mice were humanely anesthetized, and the mice heads were removed from the body and placed into test tubes to be kept in the -80□ freezer in the Jia lab. The mice tissues were sent to the Erikson lab, and kept in the -80□ freezer. Upon time for dissection, the test tubes were removed from the -80°C freezer and placed over ice. The mice brains were removed from the skull and dissected on an iced metal tray sagittally into left and right hemispheres. Next, the hemispheres were dissected into 4 regions: midbrain, striatum, cortex, and the "rest" of the brain. The "rest" was not used for this study but saved in the case of needing it for future research. The midbrain, striatum, and cortex were divided into pre-labeled, RNAse free test tubes to be kept in the -20□ freezer until time for analysis. The bodies of the mice were used by the Jia lab for other research purposes.

Protein Analysis

For total protein analysis, the brain tissue samples were first sonicated using the sonic dismembrator (Model 50, Fisherbrand, Fisher Scientific, USA) into a cold radioimmunoprecipitation-assay (RIPA) buffer, until smooth. Once sonicated, this homogenate was then used to determine protein concentration by using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc., USA). This protein assay gives a high-precision determination of protein concentration, read as microliters per milliliter, with a linear working range of 20 to 2000µl/mL.

Trace Metal Analysis

50µl aliquots of the homogenate of brain tissue were digested into pre-labeled test tubes with 50µl ultra-pure nitric acid for trace metal analysis for 4 days at room temperature (21□). After the first 12 hours and every 24 hours following, the sample tubes were opened to release trapped gas, closed, vortexed for 15 seconds, and placed back under the hood. At the end of the 4 days, 50µl of the digested homogenate was then diluted based on the metal to be analyzed into pre-labeled test tubes with 2% nitric acid for trace metal analysis. Concentrations of manganese, copper, and iron were determined using a graphite furnace atomic absorption spectroscopy (GFAAS), (Model AA240, Agilent Technologies Inc., USA). All samples were run in duplicates, as the Varian AA240 gives accurate determination of metal concentrations. Bovine liver (NBS standard reference material, USDC, Washington DC, USA) was digested in ultrapure nitric acid and used as an internal standard during analyses.

Statistical Analyses

Using both the protein concentrations (µl/mL) and trace metal concentrations (µg/mg), data were entered into a Microsoft Excel template that calculated micrograms of metal per

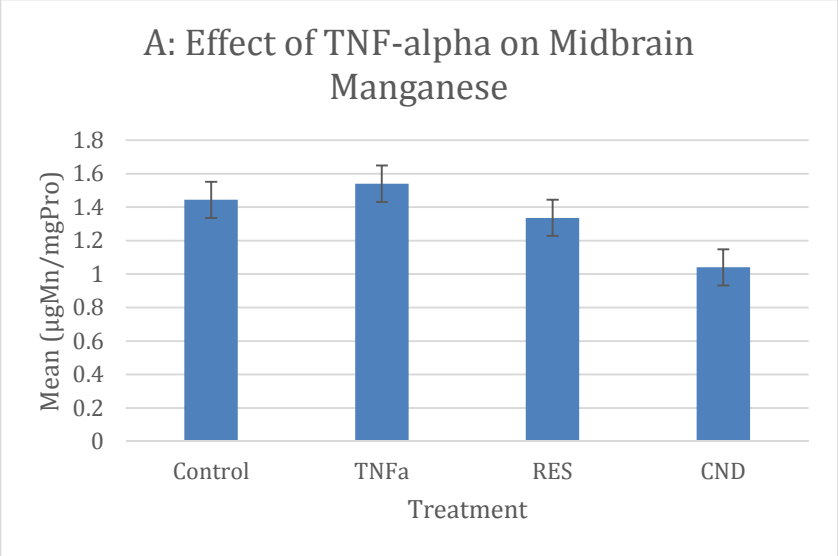
milligram of protein (μg/mg). Using these data on trace metal concentrations, the following analyses were conducted in SPSS (version 28.0.0; IBM SPSS; Armonk, NY) using a general linear model with repeated measures (brain regions) and treatment as the between subjects' effects. Significance level for these analyses was set at $p<0.05$, and "approaching significance at $p>0.05/<0.10$. A Tukey's HSD test was used for post hoc analyses. In order to determine if there were any differences in brain trace metals between the C57BL/6J control and LDLr-/- control, an independent t-test was conducted before the general linear model with repeated measures.

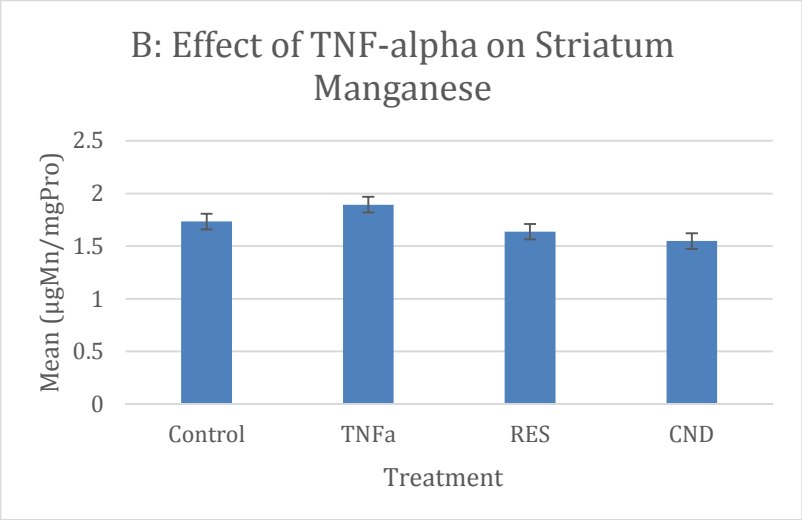
CHAPTER IV: RESULTS

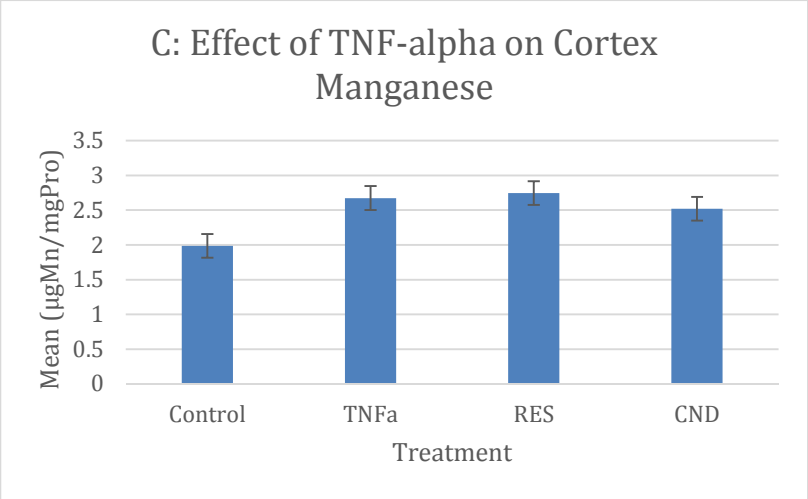
Experiment 1: Manganese

For all metal analyses in Experiment 1, an independent t-test was performed in order to assess if there were any significant differences between the saline control and TNF-α injected mice in order to determine whether the injections independently caused changes in trace metal, in which no significant differences were found. Manganese concentrations were significantly different across the brain regions ($p<.001$) but there was no significant treatment effect. It is apparent that manganese increased in each brain region in the TNF-α injected mice, with the highest increases seen in the cortex (Figure 1C). In the resveratrol and CND treatment groups for the midbrain and striatum, manganese also slightly decreased (Figure 1A & 1B).

Figure 1: Mean Manganese Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).





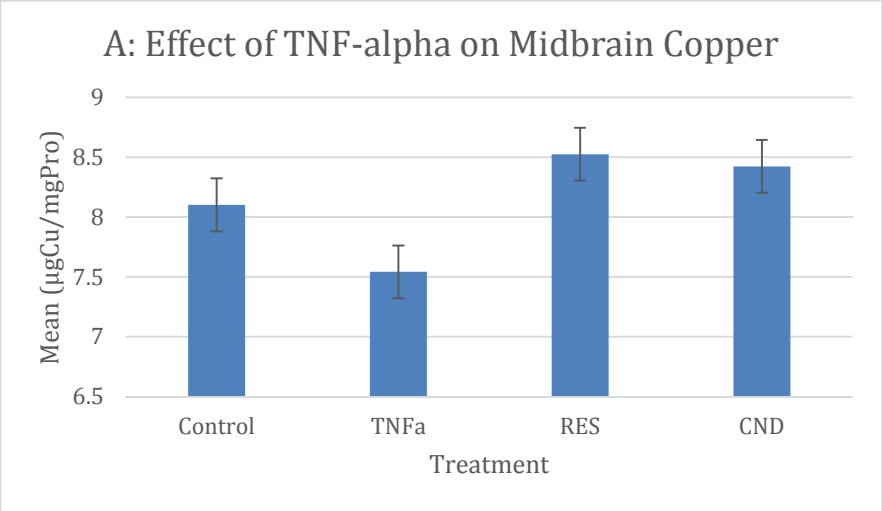


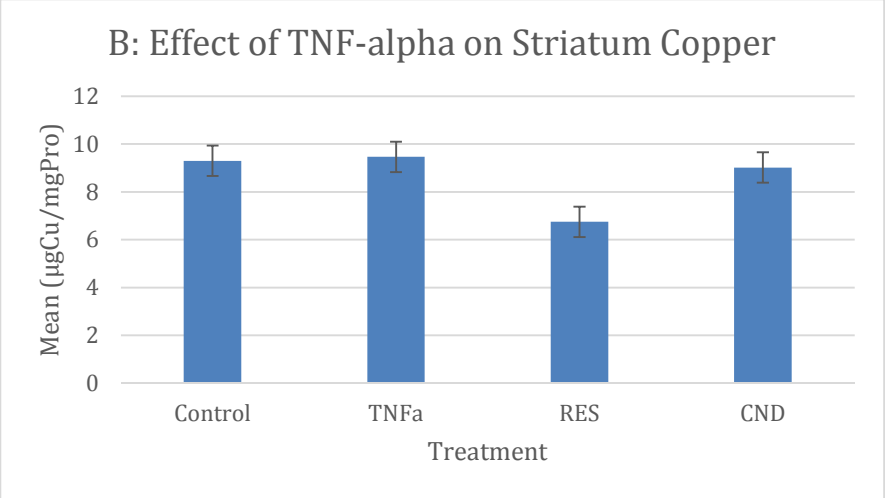
Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. Brain manganese showed statistically significant regional effects (p=<.001) with the cortex having the highest concentrations.

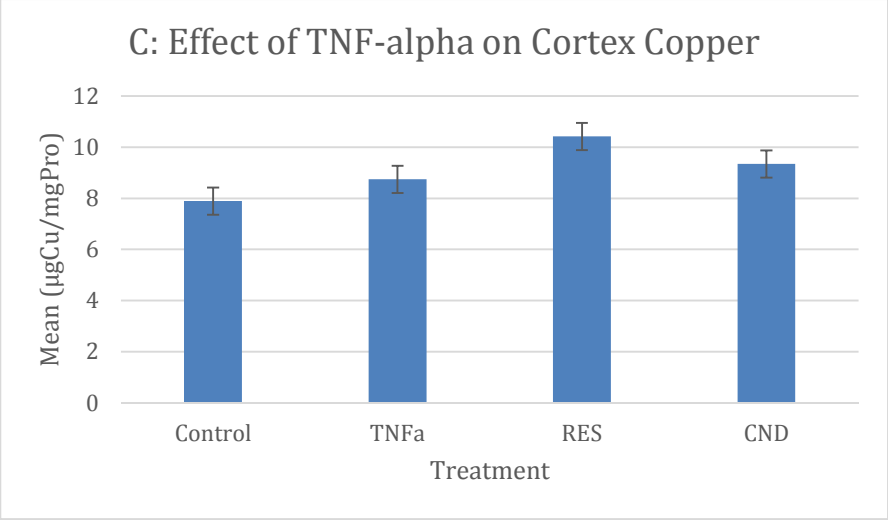
Experiment 1: Copper

Copper concentrations were not significantly different across the brain regions, but they were approaching significance between brain regions (p=.057), while there was no treatment effect seen. The figure revealed mixed results when looking at the changes in copper among brain regions and treatment groups.

Figure 2: Mean Copper Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).





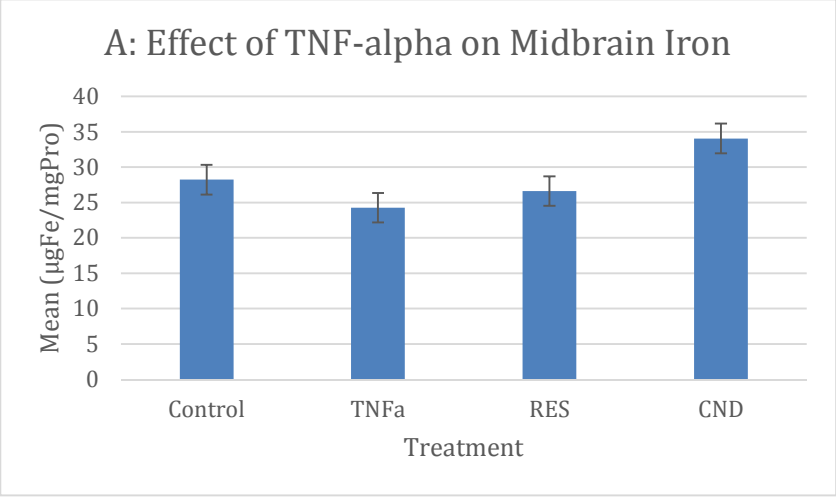


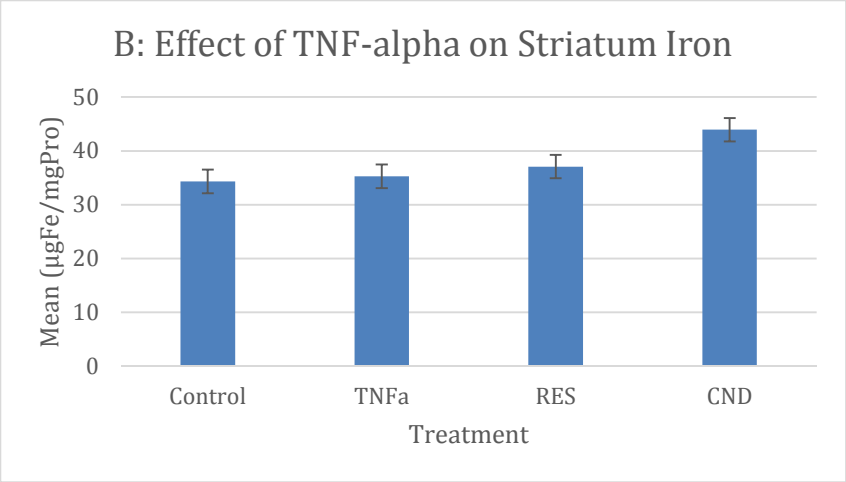
Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. The test of within subjects' contrasts was approaching significance at p=.057, with the highest concentrations of copper being seen in the resveratrol group in the cortex.

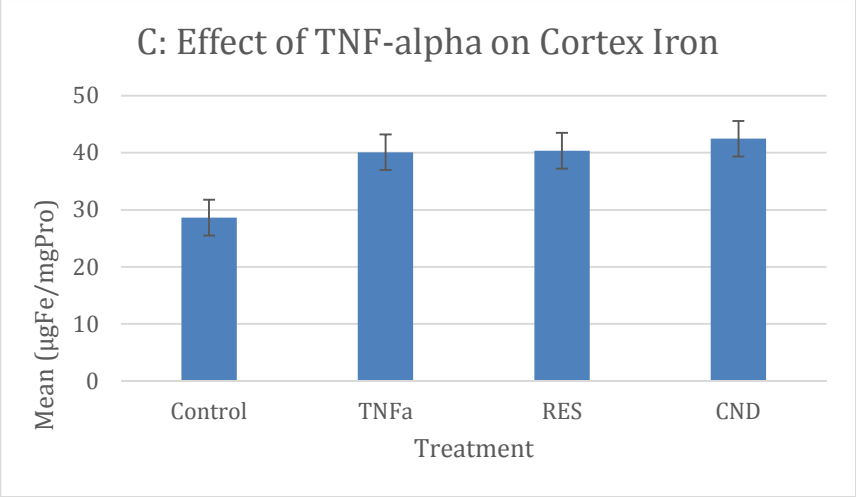
Experiment 1: Iron

Of the 20 samples within the dataset, three outliers were removed when analyzing iron, two from the resveratrol group, and one from the CND group, leaving 17 samples for analyses. The outliers were assessed using a box and whiskers plot, and if indicated as an outlier by an asterisk we would remove it. Iron concentrations were significantly different across the brain regions (p<.001), while there was no iron by treatment effect. Iron seemed to increase in both the striatum and cortex in the TNF-α injected mice, but no decreases were seen in the treatment groups (Figure 3B & 3C).

Figure 3: Mean Iron Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).







Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. Brain iron showed statistically significant within subjects' effects (p=<.001), with the cortex having the highest concentrations.

Experiment 2: Body Weights

Body weights for the mice in Experiment 2 have been included, as the LDLr-/- mouse model typically results in overweight or obesity. For the mice in Experiment 2, the LDLr-/- mice were all significantly heavier than the C57BL/6J mice (Table 3). It is known that within obesity there is low-grade inflammation present due to the activation of inflammatory pathways (29). With this, it may be suggested that not only do the LDLr-/- mice have inflammation present due to their atherogenic state, but also their obese state. Body weights for Experiment 1 were

excluded as there were no significant differences seen in their weights when given TNF-α injections.

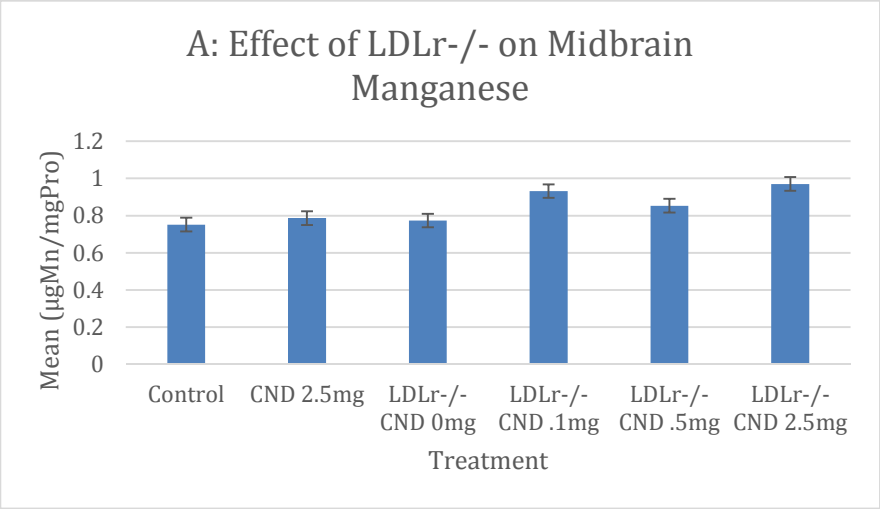
Table 3: Experiment 2 Body Weights. Body weights for mice used in Experiment 2 are expressed in grams. LDLr-/- mice were significantly heavier than C57BL/6J mice ($p<0.001$) as indicated by superscript letters.

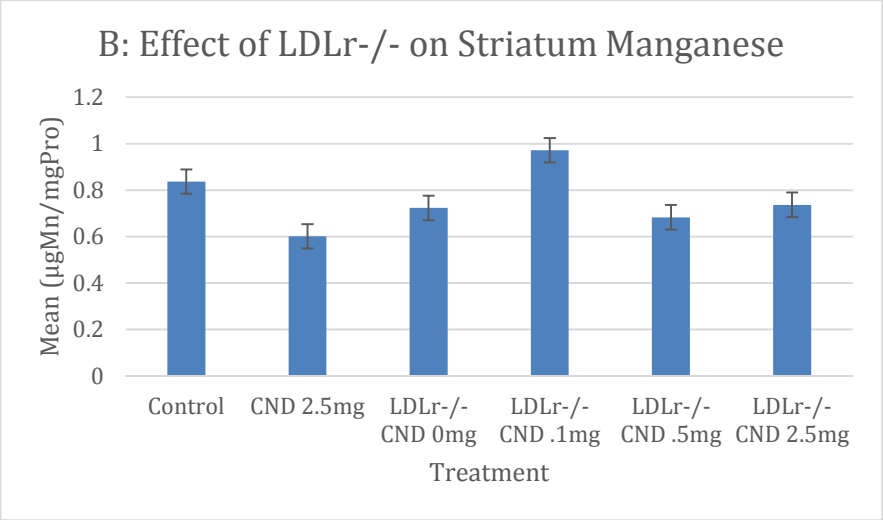
	Mean Body Weight (g)	SEM
C57BL/6J Saline Control	33.92[a]	1.03
C57BL/6J CND 2.5mg/kg	30.71[a]	0.81
LDLr-/- CND 0mg/kg	42.79[b]	1.05
LDLr-/- CND .1mg/kg	43.74 [b]	2.49
LDLr-/- CND .5mg/kg	42.48 [b]	1.64
LDLr-/- CND 2.5mg/kg	44.50 [b]	1.27

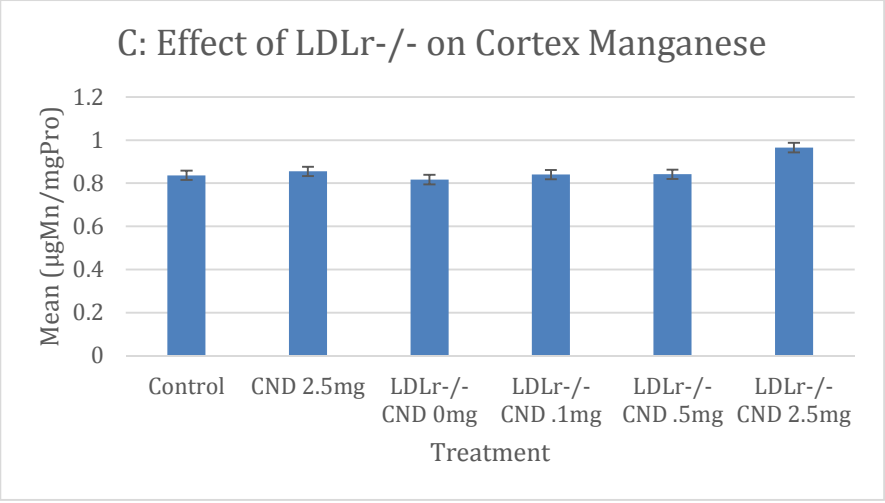
Experiment 2: Manganese

For all metal analyses in Experiment 2, an independent t-test was performed in order to assess if there were any significant differences between the C57BL/6J saline control and LDLr-/- 0mg CND group, in order to see if there was a difference in mouse type, in which no significant differences were found. There were no brain regional manganese differences due to treatment, but there was a treatment effect ($p=.034$). The figure shows mixed results when looking at changes in manganese levels between groups and with the varying levels of CND treatment.

Figure 4: Mean Manganese Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).





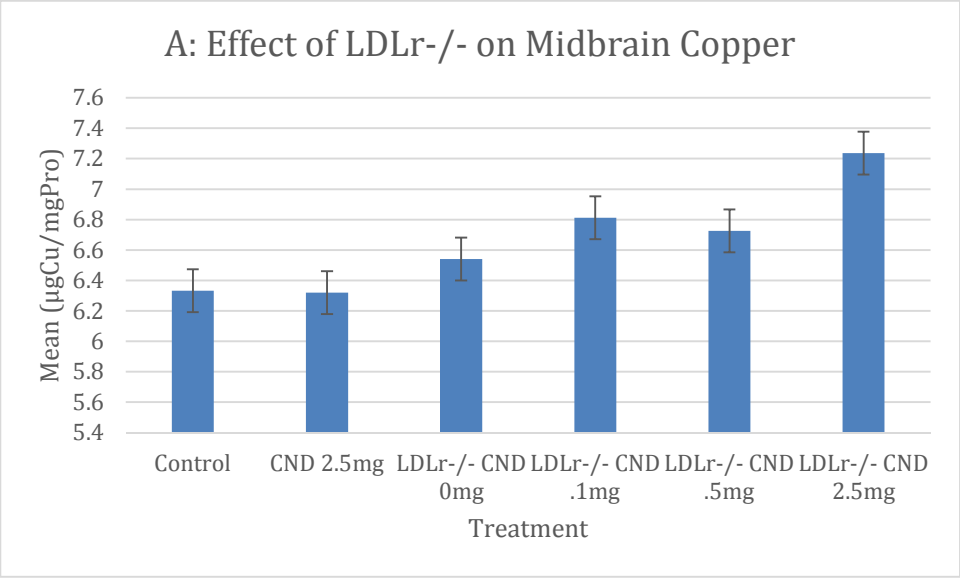


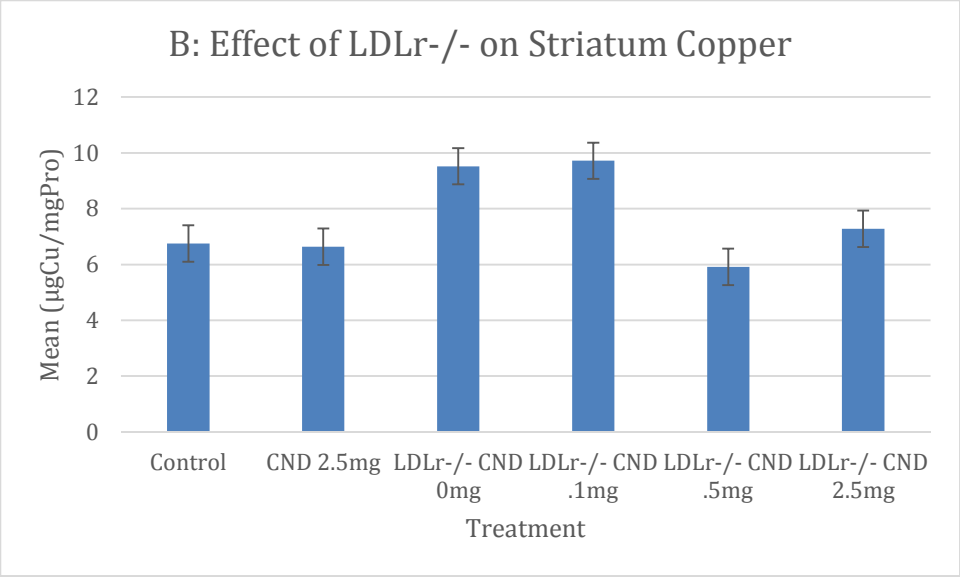
Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. The test of between subjects' effects revealed a statistically significant effect at p=.034, with the highest increase of manganese seen in the LDLr-/- .1mg CND group in the striatum. The post hoc analysis was approaching significance at p=.061.

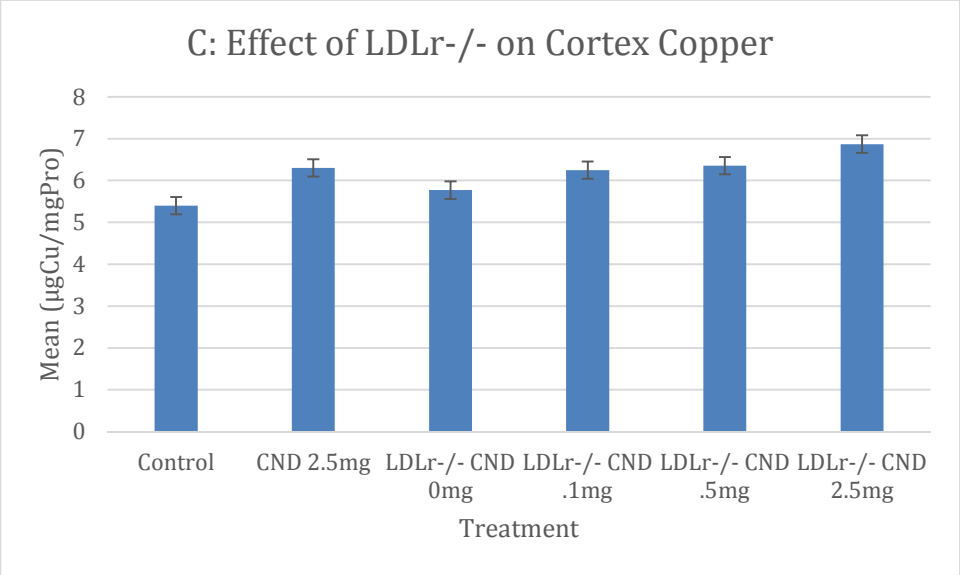
Experiment 2: Copper

Of the 36 samples, two outliers were removed from the analyses of copper, one from the LDLr-/- .1mg CND group, and the other from the LDLr-/- .5mg CND group, leaving 34 samples total. The outliers were assessed using a box and whiskers plot, and if indicated as an outlier by an asterisk we would remove it. Copper concentrations were significantly different across brain regions (p=.003) and there was a significant brain region by treatment effect (p=.050). There was a treatment effect seen (p=.018). There are promising results in the striatum, in which copper was shown to increase in the LDLr-/- mice and decrease with the higher doses of CND treatment (Figure 5B).

Figure 5: Mean Copper Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).







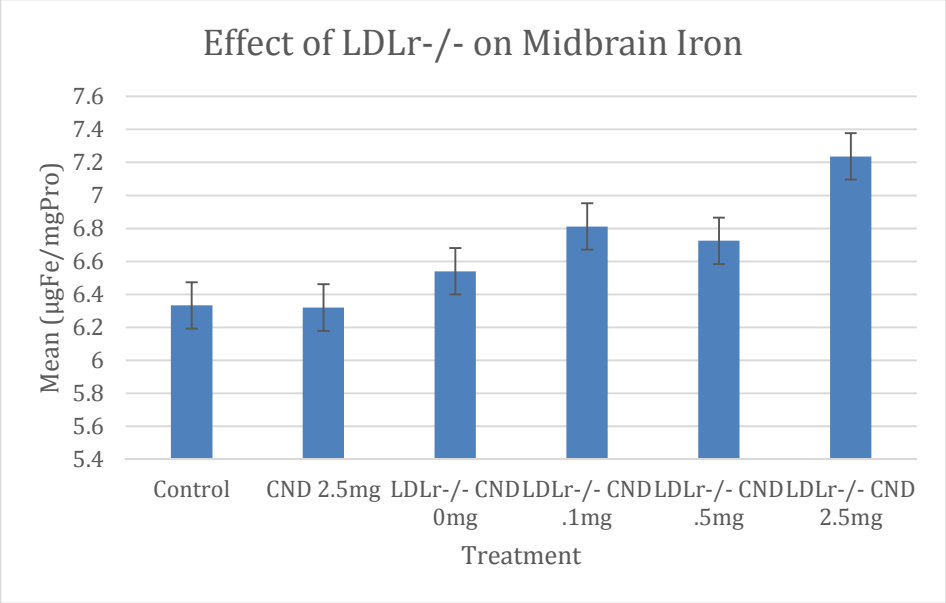
Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. Brain copper was significantly elevated in the striatum with the LDL receptor knockout groups 0 mg and 0.1 mg showing the greatest increase compared to saline control (p=.050). Overall, there was a treatment effect on copper concentration (p=.018).

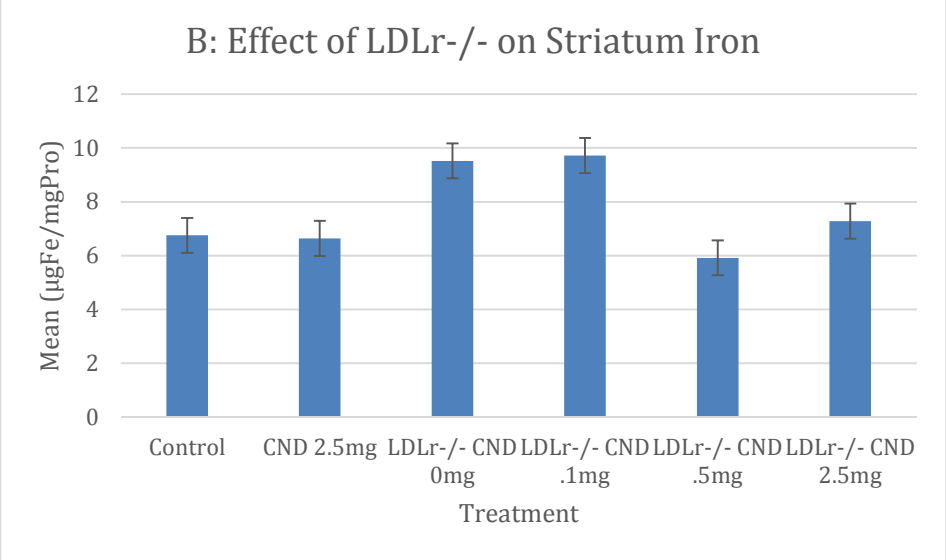
Experiment 2: Iron

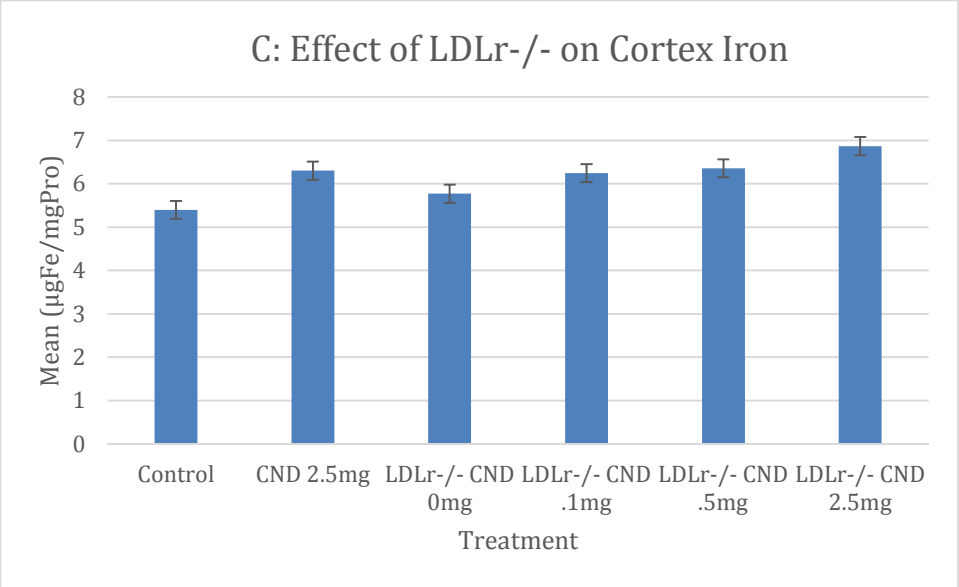
For iron, three outliers were also removed from the dataset, one each from the LDLr-/- 0mg, .1mg, and .5mg CND groups, leaving 33 samples for analyses. The outliers were assessed using a box and whiskers plot, and if indicated as an outlier by an asterisk we would remove it.

The test of within subjects' effects showed that there were significant differences in the means between brain regions (p=<.001), and the test of within subjects' contrasts showed significant results in brain regions by treatment (p=.039). There was no significance seen in the test of between subjects' effects or Tukey's HSD. In both the midbrain and striatum, iron increased in the LDLr-/- mice, and decreased with CND treatment.

Figure 6: Mean Iron Concentration by Brain Region (A=Midbrain, B=Striatum, C=Cortex).







Concentration was determined by Atomic Absorption Spectrometry and expressed in µg Mn/mg protein. Brain iron was significantly elevated in the midbrain, with the LDLr-/- 0mg CND showing the greatest increase compared to the saline control ($p<.001$).

CHAPTER V: DISCUSSION

Inflammation

The effects of inflammation on brain levels of manganese, copper, and iron have not yet fully been understood, but the results from this study give valuable insight to the literature. It is known that the LDLr-/- mouse model would display inflammation, and it is assumed that TNF-α injections would induce inflammation in C57BL/6J mice, but it is indecipherable whether the inflammation in these mice would allow for brain trace metal dysregulation (38,39). This study used both TNF-α injections and LDLr-/- mice which are established models of inducing inflammation. The state of inflammation in Experiment 1 may reflect an acute inflammation, while Experiment 2 could be seen as a chronic state, which could each produce different results in brain trace metal dysregulation.

TNF-α is an inflammatory cytokine that is produced during acute inflammation, while the state of the LDLr-/- mice could be seen as chronic inflammation due to their obese and atherogenic state (40). Since acute inflammation is a short-term response, it may not produce enough neuroinflammation, if any, to inhibit the integrity of the blood brain barrier (16). On the other hand, chronic inflammation may cause more permeability of the blood brain barrier, as this can be seen in certain chronic inflammatory states, which lines up with the utilization of the LDLr-/- mouse model (25). However, much less is known on the mechanisms of chronic inflammation, so it is difficult to determine whether the use of LDLr-/- mouse model would produce different results. Diet induced obesity has been shown to cause changes in brain trace metals in mouse model studies, which is a similar state to the LDLr-/- mice, as their atherogenic diet and genetic modification induced obesity, atherosclerosis, and inflammation (30,31,41).

Because of this, we would expect that these mice would also have dysregulation of brain trace metals, but the present study revealed mixed results.

Manganese

When looking at the analyses for manganese, both experiment groups showed no significant results, but the post hoc Tukey's HSD in Experiment 2 revealed the Saline Control and LDLr-/- .1mg CND groups to be approaching significance (p=.061). It is also notable that manganese increased in the cortex when mice were injected with TNF-α, suggesting that the inflammation present from TNF-α injections in C57BL/6J mice may increase levels of manganese in the cortex (Figure 1). Although manganese dysregulation in the brain is typically seen in over-exposure to the metal, it has also been found in cases of diet-induced obesity in mouse models, which is a similar state to the LDLr-/- mice in the present study (6,30). Despite the fact that this did not produce significant results, there were interactions between the saline control and the LDLr-/- .1mg CND groups. This could suggest that the LDLr-/- mouse may display enough inflammation to alter brain manganese homeostasis, but it is not fully known whether this is the case.

Since the CND treatments showed no alterations in brain manganese levels in the LDLr-/- mice, it may be suggested that the CND treatment may not be reducing inflammation as proposed, but the LDLr-/- mouse model still resulted in minimal dysregulation of manganese in the brain. The lack of changes seen in manganese could also be a promising result that the blood brain barrier may have enough defense that it does not weaken enough in the presence of inflammation to allow for infiltration of manganese, although manganese dysregulation is seen in a variety of neurological disorders, which is the opposite of these results, leaving this unclear (42). This would be especially favorable as manganese toxicity and deficiency can both lead to

negative neurological effects, and recent research is looking at the relationship between manganese and metabolic diseases (43). All things considered, the most notable result when looking at brain manganese levels in response to inflammation is the increase in the cortex among the mice injected with TNF-α, which may suggest manganese may increase in specific regions of the brain in the presence of inflammation, similar to the specific increase seen in the olfactory bulb in diet induced obesity (30).

Copper

When looking at the analyses for copper, there were also some changes seen in trace metal status. For Experiment 1 the within subjects' contrasts test for brain region was approaching significance at p=.057, but there was no treatment effect seen. These results may suggest that short-term TNF-α injections alone, a model of acute inflammation, may not produce enough inflammation to cause changes to the blood brain barrier, and thus changes to brain copper levels. It is known however that in an inflammatory state, the proinflammatory cytokines present promote accumulation of copper, but it is not known whether this accumulation could occur in the brain from TNF-α injections alone (9). Jiang et al demonstrated that in pulmonary disease, which is a chronic inflammatory state, copper in lung sputum increased, and that TNF-α upregulated the expression of a metallic reductase involved in copper uptake (10). These results could suggest that TNF-α would increase copper levels, but it is unclear whether this would affect brain copper levels, as the blood brain barrier defense may remain strong in acute cases of inflammation.

On the other hand, when looking at Experiment 2, the multivariate tests revealed a significant interaction between copper and brain region, and the test of within subjects' effects and within subjects contrasts each gave significant results. The between subjects' effects test

showed a statistically significant interaction between copper and treatment, while the post hoc analysis was approaching significance. The highest levels of copper were seen in the striatum, and other studies have found that in the chronic disease Wilson's disease, copper is also increased (28). However, in diet-induced obesity and heavy alcohol consumption, brain copper levels have been shown to decrease, which raises the question of what exactly causes copper to either increase or decrease in certain inflammatory states (32, 33). The results from the present study suggest that brain copper levels may change in the presence of chronic inflammation such as that seen in LDLr-/- mice, which goes hand-in-hand with other research that has found copper levels to either increase or decrease in certain inflammatory states (28,32,33).

It is apparent that copper increased in the striatum of the LDLr-/- 0mg CND and .1mg CND groups (Figure 5). Another interesting addition to the literature that needs to be further investigated is that those higher levels of copper in the striatum seemed to decrease in the .5mg and 2.5mg CND treated mice, which may suggest potential advantages to the use of CND treatment for inflammation. Shen et al also found CND to be a promising framework for the treatment or diagnosis of inflammation, so future research should look at the effects of CND treatment on diverse inflammatory states (44). There has also been research conducted that showed CND as an effective treatment for cancer similarly to conventional cancer treatment drugs, without damaging normal cells (45). If this were to remain the case in states of inflammation, CND could be an effective treatment to reduce damage to cells while possibly preventing damage to the blood brain barrier. Overall, the results from analysis of copper suggest that there may be changes that occur to brain levels of the trace metal copper in the presence of chronic inflammation, such as that seen in the LDLr-/- mice.

Iron

When looking at the results for iron levels, both experiments' multivariate analyses for brain regions disclosed a significant p-value of p=.003 and <.001, respectively. Both tests of within subjects' effects revealed significance between regions, while the test of within subjects' contrasts for brain regions for both experiments were also significant (p<.001). Neither groups showed any significant differences when looking at the test of between subjects' effects or Tukey's HSD. Chronic inflammation has been shown to limit iron availability, but tissue-specific brain iron toxicity has been seen without increases in systemic iron, which could lead to assumptions that inflammation could affect brain iron homeostasis (15). In chronic disease states such as Parkinson's disease, Wilson's disease, and obesity, brain iron levels have shown to increase (28,30).

One interesting result from the present study that demands further research is that iron in the midbrain and striatum increased in the LDLr-/- mice and tended to decrease with the addition of CND treatment. As with the copper results, this could suggest that the CND treatment has promising effects on reducing inflammation and thus reducing the brain iron spike seen in the LDLr-/- 0mg CND mice. It may also suggest that in the presence of chronic inflammation such as that of the LDLr-/- mice, iron may increase in the brain, specifically in the midbrain and striatum. These findings are similar to other current research that has acknowledged that non-transferrin-bound iron uptake increases in response to proinflammatory stimuli, and that neurodegeneration may result in brain iron accumulation (46,47). This also could promote future research to look at the effects of inflammation on other brain regions, as this study only focused on the midbrain, striatum, and cortex. It is not fully known whether inflammation could directly

impact brain iron levels as we did not look at the blood brain barrier itself, but the results from this study can serve as a proposition for future research.

To our knowledge this is the first study of its kind to look at the effects of inflammation on brain trace metals manganese, copper, and iron. Although we did not look at the blood brain barrier directly, we can assume an inflammatory state based on the mouse models utilized. Future studies may want to look at the blood brain barrier directly, or include other metals such as zinc, which is another metal that is essential for proper brain function but is tightly regulated in the brain (48). Overall, this study found that in the presence of inflammation there are increased levels of manganese in the cortex, copper in the striatum, and iron in both the midbrain and striatum of mice. Some of these metals also decreased with addition of CND treatment, with copper being the most notable as a significant effect was seen between copper and treatment, which demands further research on the possible anti-inflammatory and protective effects of these nanoparticles. Some of the brain regions showed no differences in metals in the presence of inflammation, which may suggest that the blood brain barrier is able to defend itself against these particular states of inflammation. The findings of this study serve as a baseline of knowledge in this area of research and can promote future research to delve deeper into the consequences of inflammation on brain trace metal biology.

CHAPTER VI: EPILOGUE

The present study serves as a pilot study for future research, because although this study augments the inflammation literature, three main research gaps remain. The first gap following this study is that the present study lacked the use of female mice. Due to limited resources, the use of only male mice was justified as the brain tissue used in this study was received from another lab at the University of North Carolina at Greensboro in order to reduce the number of animals being studied for research purposes. We also know that in female mice, the estrous cycle could cause variability in animal studies due to the hormonal fluctuations, but the NIH encourages the use of males and females with sex as a biological variable in order to strengthen research (48). With this justification, there is still a need to look at the effects of inflammation on brain trace metals in female mice, as few studies look specifically at sex differences when focusing on inflammatory markers or trace metal biology, so whether there would be different results than those seen in male mice remains unknown (50).

The second gap resides in the trace element analyses. This study focused primarily on trace metals manganese, copper, and iron, which are three big "players" of brain function, but there is potential for also looking at zinc in the future. Zinc is an essential trace metal necessary for various functions, and its deficiency can result in improper growth and bone development, low immune function, and cognitive impairment (48). Zinc plays an important role in catalyzing reactions for many enzymes, stabilizing zinc-finger proteins, cellular signaling, and is evidently involved in Alzheimer's disease pathogenesis, which is a major reason the effects on inflammation on brain zinc levels should be studied in the future (48,51). In Alzheimer's disease pathogenesis, an imbalance can be seen in copper and zinc (51). It is suggested that zinc may affect amyloid metabolism, but on the other hand it can also help restore copper homeostasis and

modulate synaptic functioning, which is why homeostasis of zinc needs to be tightly regulated (51). When zinc levels are low, and levels of manganese, copper, and iron are high, it triggers the activation of an inflammatory and oxidative stress response (52). Since we know that neurodegeneration occurs when there is an imbalance of these metals in the brain, this study could have benefited from also looking at zinc levels in the brain in response to inflammation.

The third gap resides around the metals analyses. In order to strengthen the results of this study, we could have completed the atomic absorption spectroscopy in triplicates, rather than duplicates. Although the use of the GFAAS is highly beneficial due to its precision in determining trace metal concentrations, our model that we have access to (Model AA240, Agilent Technologies Inc., USA) is not the most recent model, and over time there may be variability in accuracy of results as lab equipment ages. When analyzing iron, we ran into an issue in which the AAS would essentially stop reading metal concentrations after around 20 samples. Because of this, we had to split up the number of samples run at one time, when with the other metals we were able to run all the samples at one time. This technical difficulty did not prevent us from running the samples properly, but it did cause variability in procedure, as samples for manganese and copper were inserted all at the same time, while the samples for iron were divided into groups, which could cause slight changes due to timing, as the AAS is a highly sensitive piece of equipment. In order to ensure that it was no longer running properly, we would use an internal standard by re-running the first sample and seeing if it was within +/-5%. If it was outside of this range, we would split the samples and run them in two groups.

A few strengths of the experiments were the use of standardized procedures such as dissection methods, protein analysis, and trace metal analysis. We also used an adequate number of mice for the study, in order to follow the reduction of animals used for research, while

practicing human anesthetizing techniques to advance animal welfare. Manganese, copper, and iron are three common metals in the brain that are affected by certain conditions, so the choice of assessing these three metals can also be seen as a strength of this study. Another strength of this study was utilization of two routes of inflammation, through both TNF-α injections in C57BL/6J mice and use of the LDLr-/- mouse model, as these would provide different forms of inflammation and different physical states in the mice. The LDLr-/- mice appeared to be obese, and are assumed to be atherosclerotic, which was not the case with the TNF-α mice. These different routes of inflammation could result in different effects on the blood brain barrier, so future research may want to look at the blood brain barrier directly to determine if this is the case. Future researchers may also want to utilize rat brain tissue, as it is larger and easier to dissect into more regions than just the midbrain, striatum, and cortex. This would allow researchers to look at the effects of inflammation on brain trace metals in other regions of the brain.

Overall, this study provides important information to further advance the knowledge on the effects of inflammation on brain trace metals manganese, copper, and iron. In the presence of inflammation, there were increases seen in specific metals in distinct brain regions, such as an increase of manganese in the cortex, copper in the striatum, and iron in the midbrain and striatum. Some of these increases seemed to decrease with the designated treatments of carbon nanodots, which promotes future research on this topic of research and the utilization of nanoparticles as an anti-inflammatory treatment. The continuation of work on this subject has the potential to enhance research on inflammation and neurodegeneration, and potentially reduce the progression of neurodegenerative diseases through reduction of inflammation.

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