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LOW LEVEL LEAD EXPOSURE AND INFLAMMATORY MARKERS IN THE BRAINS OF C57BL/6J MICE

MIGUEL ANTONIO CERVANTES

MASTER'S PROGRAM IN PUBLIC HEALTH

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LOW LEVEL LEAD EXPOSURE AND INFLAMMATORY MARKERS IN THE BRAINS OF C57BL/6J MICE

BY

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THESIS

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ABSTRACT

Background and Significance: Prior studies have indicated that early chronic low-level lead exposure may be associated with adverse effects on motor and cognitive functions. The mechanisms by which low-level lead affects brain function are unknown. **Objectives:** The current study aimed to (1) determine whether early chronic low-level lead exposure altered the expression of pro- or ant-inflammatory cytokines and markers of oxidative stress in mouse brain studied at two ages, pre-adolescence (PND 28) and early adulthood (PND 40); and (2) in the same pre-adolescent and early adulthood mice, compare and contrast the levels of pro- or ant-inflammatory markers in three brain regions including anterior cerebrum, posterior cerebrum and cerebellum. **Hypothesis:** We hypothesized that early chronic exposure to low-level lead triggers abnormal expression of pro- or ant-inflammatory markers consistent with an inflammatory response and oxidative stress in younger and older mice; and that posterior cerebrum would be more susceptible to the effects of chronic low-level lead than anterior cerebrum and cerebellum. **Methods:** Two studies were conducted one in 28 day and one in 40 day old C57BL/6J mice. For each study, mice were assigned to one of three experimental groups including a control group (no lead exposure), a low dose group (exposed to 40 ppm lead acetate), and a high dose group (exposed to 230 ppm lead acetate). Mice were exposed to lead acetate (99.4% pure) via dams' drinking water from birth until weaning. The gene expression levels of four pro-inflammatory markers (TNF-α, IFN-γ, IL-6, iNOS), two immune-suppressive markers (IL-10 and Hmox-1) and an oxidative stress indicator GPR-78 were measured in homogenized brain sections from anterior cerebrum, posterior cerebrum and cerebellum using qRT-PCR. These markers were selected because they have roles in brain development and

inflammatory responses (Bauer, Kerr, & Patterson, 2007; Ahamed & Siddiqui, 2007; Anthony & Campbell, 2002; Bokara, et al., 2008; Cabell, et al., 2004; Deverman & Patterson, 2009; Gastaldello Moreira, de Magalhaes Rosa, Moraes Barros, Vassilieff, & Vassillieff, 2001) **Results:** There were no statistically significant differences observed in pre-adolescent mice. Young adult lead-exposed mice had significant differences in genetic expression of TNF-α, Hmox-1, and GRP-78. Lowest level lead exposure caused a statistically significant increase in genetic expression of TNF-α and Hmox-1 in the low dose group when compared to the control group. **Conclusions:** A mixed inflammatory response is activated due to chronic exposure to low-level lead in young adult mice. Additional studies are needed to further define the role of chronic low level lead exposure in interfering with normal brain function.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: BACKGROUND AND SIGNIFICANCE

The problem of lead exposure has been a topic of public health concern and controversy for many years. The use of lead throughout history has created multiple means of exposure throughout the world (Tong, von Schirnding, & Prapamontol, 2000). Lead has been used as an additive in smelting products, gasoline, household paint, plumbing, pottery, glass, and paint used to decorate children's toys and many other readily available items. Though the use of lead in gasoline, paint, and other items has been significantly reduced or banned in the United States (Agency for Toxic Substances and Disease Registry, 2010), lead continues to be an ubiquitous contaminant in the environment and a source for chronic low level exposure in children. Common sources of chronic low level lead exposure in children are drinking water, industry, residual lead dust from leaded gasoline combustion, low-income housing units, and pre-1950's housing units (Tong, von Schirnding, & Prapamontol, 2000).

Despite the Environmental Protection Agency's regulations requiring that lead traces in potable water be below 15 ppb (U.S. Environmental Protection Agency, 2009), individuals living in older U.S. homes may be exposed to lead through tap water as it is transported to the home via older plumbing systems that may contain and release lead into the water (Brown & Margolis, 2012). Studies have found that the amount of lead released from pipes into drinking water varies and is dependent on the type of water treatment used (Edwards & Dudi, 2004; Miranda, Kim, Hull, Paul, & Overstreet Galeano, 2007). In Washington D.C., water treatment was changed from free chlorine to chloramine in the year 2000 which caused an increase of lead released from pipes and higher incidence of Blood Lead Levels (BLLs) above 5 µg/dL (Centers for Disease Control and Prevention, 2004). The change in water treatment also

increased the incidence of BLLs above 10 μg/dL in children aged less than 1.3 years (Edwards, Triantafyllidou, & Best, 2009). BLL is used to measure the amount of lead in a person's body at the time of blood collection. The BLL of concern has been reduced from 60 μg/dL in 1960 (Centers for Disease Control and Prevention, 2007) to the current reference level of 5 μg/dL (Centers for Disease Control and Prevention, 2007; Centers for Disease Control and Prevention, 2014). It has been estimated that approximately one-half million children, aged 1-5 years old, have BLLs above the current "reference level" of 5 μg/dL (Centers for Disease Control and Prevention, 2014).

Despite these findings, the correlation of elevated BLLs in the population and water lead level has been inconsistent. A study that attempted to model child BLLs from lead-contaminated water found that water lead levels at 10-15 times above the recommended threshold set by the Environmental Protection Agency ($\geq$20 ppb) – were unlikely to increase incidence of BLLs above 10 μg/dL (Sathyanarayana, Beaudet, Omri, & Karr, 2006). Studies on water lead exposures however do not tend to report the incidence of child BLLs below current thresholds.

Industrial processes and the use of gasoline containing tetraethyl lead have released lead into the environment for many years resulting in a disproportionate accumulation of lead in various regions across the United States. Industries often use methods, such as smelting, to synthesize materials that contain lead. Two studies conducted in border regions along the U.S./Mexico border where smelters were located found that increased BLLs in children were associated with the children's home proximity to the smelters and one of the studies found that

lead traces >20ppb were still found years after the smelter had ceased operation (Diaz-Barriga, et al., 1997; Carriazales, et al., 2006).

Methods for measuring lead hazards in the environment include monitoring lead concentrations in the air and self-reported release of lead in the air by industries (Alliance for Healthy Homes and Environmental Defense, 2005; U.S. Environmental Protection Agency, 2010). Soil lead contaminations are also another means of lead exposure. One study suggested that, in addition to industry, soil lead contamination may be attributed to historically high traffic flow in communities due to the use of tetraethyl lead in gasoline from the 1920's until 1986 (Mielke, Powell, Gonzales, & Mielke Jr, 2007).

Another significant lead exposure source is in the household. These include housing units built prior to 1950 and low-income households. A study conducted on household lead in U.S. homes found that lead dust on the floor and windowsills was significantly increased in pre-1950's homes, areas where people smoked, and pre-1950's homes with chipping paint (Gaitens, et al., 2009). The same study also found that homes of non-Hispanic black individuals in the United States had significantly higher levels of lead dust than any other ethnic group (Gaitens, et al., 2009). Carpeted floors also posed a higher risk of exposure to lead as lead dust was more difficult to clean from a carpeted floor (Yiin, Lioy, & Rhoads, 2003).

The many means of exposure to lead create high variability in BLLs in the United States population and also make it difficult to identify a primary source to target for intervention. Some studies have been able to identify the lead sources in specific regions and have tested interventions to reduce the amount of lead in the environment. In one study, for example, childcare and community center playgrounds with top soil contaminated by lead in New

Orleans, Louisiana were covered with geotextile followed by a layer of river alluvium (Mielke, et al., 2010). The intervention succeeded in significantly reducing the soil lead levels, but did not eliminate the threat of chronic low level lead exposure since very low traces of lead could still be detected. Environmental interventions have demonstrated the need for a thorough understanding of molecular mechanisms activated by lead exposure in order to develop primary preventive methods that can be applied to children at risk of exposure.

Overview of the Effect of Lead on Health and Wellbeing

Lead is able to enter mammalian systems through direct contact, ingestion, or inhalation (Agency for Toxic Substances and Disease Registry, 2010). A percentage of the lead that enters the body is absorbed, while the remainder is excreted in urine, nails, hair and sweat (Centers for Disease Control and Prevention, 2005). Once lead is absorbed into the body, it is transported to the liver, renal cortex, aorta, brain, lungs, spleen, teeth, and bones via erythrocytes (Patrick, 2006; Sun, Ma, Chao, Liu, & Yuan, 2009).

Lead has been found to cause a number of adverse health outcomes that affect the nervous system, reproductive system, kidneys, blood and endocrine systems at BLLs above 10 µg/dL (Sanborn, Abelsohn, Campbell, & Weir, 2002). Children are more susceptible to the damaging effects of lead as their bodies absorb approximately 50 percent of the lead they intake (adults only absorb 10 percent) and because of the increased hand to mouth activity in children (Centers for Disease Control and Prevention, 2007). Clinical symptoms associated with lead toxicity include hypertension, decreased nerve conduction velocity, tremors, increased intracranial pressure, and abdominal pain amongst other symptoms (Vaziri, Ding, & Ni, 2001; Agency for Toxic Substances and Disease Registry, 2007). High body lead levels can also cause

anemia, severe central nervous system effects and even death (Agency for Toxic Substances and Disease Registry, 2007).

It is widely known and accepted that acute lead exposures are dangerous and can be deadly (Centers for Disease Control and Prevention, 2007), but chronic exposure to low level lead in children may be of equal or greater concern. Low-level lead is thought to be detrimental to health despite the absence of diagnosable signs of toxicity. A number of clinical studies have suggested that low BLL negatively affect intellectual and academic performance, IQ, postural balance, reaction time, visual-motor integration, and fine motor skills in children (Bellinger, Stiles, & Needleman, 1992; Canfield, et al., 2003; Lanphear, et al., 2005; Lanphear, Dietrich, Auinger, & Cox, 2000; Nevin, 2009; Bhattacharya, Shukla, Dietrich, & Bornschein, 2006; Chiodo, Jacobson, & Jacobson, 2004; Despres, et al., 2005; Kordas, et al., 2006). In addition, a mean BLL of 0.73 μg/dL (maximum 2.2 μg/dL) in a study group was found to be correlated with attention-deficit/hyperactivity disorder (Nigg, Nikolas, Knottnerus, Cavanagh, & Friderici, 2010). Changes in cognition, motor ability, and behavior in children imply that lead is affecting the central nervous system at the molecular level at BLLs below the current threshold of action (Canfield, et al., 2003; Cecil, et al., 2008).

Recent studies of low-income minority children have shown that more than fifty percent had BLLs between 2.5 and 5.0 μg/dL (Sobin et al., 2009, Sobin et al., 2011) suggesting that the problem may affect millions of children nationwide. Similar to past studies, recent studies have also shown that these same low BLLs are associated with specific deficits in working memory and motor dexterity (Sobin, 2014). The brain mechanisms that account for these effects are not currently known.

Knowledge of the molecular mechanisms underlying brain changes following early chronic low-level lead exposure is essential for understanding findings from child behavioral studies, and is also critical for developing primary preventive measures to reduce effects in exposed children. It is important to note that in this report, "low-level" refers to chronic lead exposure resulting in BLLs below the current CDC "reference level" of 5 μg/dL.

Molecular Mechanisms of Lead Toxicity

Little is known regarding the molecular mechanisms that account for central nervous system effects during and following early chronic low-level lead exposure. A possible mechanism of lead toxicity in organ systems outside of the central nervous system is the accumulation of δ-aminolevulinic acid (δ-ALA) (Ahamed & Siddiqui, 2007). The accumulation of δ-ALA occurs in blood and urine as lead inhibits δ-aminolevulinic acid dehydratase function during heme synthesis in the mitochondrion (Ahamed & Siddiqui, 2007; Wang, Wu, & Zhang, 2005). The accumulation of δ-ALA was believed to cause oxidative stress when the unused δ-ALA molecules are oxidized to a number of reactive oxygen species (Hermes-Lima, 1995).

The brain is an extremely sensitive organ, which uses the blood brain barrier to control the flow of nutrients and molecules in and out of the brain, which enables the body to maintain an environment that promotes normal brain function and development (McCance & Huether, 2010). It was believed that excess δ-ALA due to lead exposure bypassed the blood brain barrier and increased oxidative stress in the brain, however a study conducted on adult rats found that excess δ-ALA in plasma had low blood brain barrier permeability and a mechanism in the choroid plexus released excess δ-ALA in cerebrospinal fluid into the peripheral blood supply which may protect the brain from excess δ-ALA in plasma (Ennis, et al., 2003).

While it is unknown how lead enters the brain or how lead causes alterations in normal brain function, it is known that lead is a heavy metal with no known nutritional value and has chemical properties similar to other metals, which are essential to normal biological function such as calcium, zinc, and iron (Flora, Gupta, & Tiwari, 2012). Lead's chemical properties may allow the metal to use transport mechanisms that the essential metals use to enter the brain to bypass the blood brain barrier and directly interfere with mechanisms taking place in the brain.

A study found that lead molecules are present in brain tissue of mice exposed to 0.1 mL or 0.2 mL of lead nitrate solution via vein injection (Sun, Ma, Chao, Liu, & Yuan, 2009). Studies of C57BL/6J mice reported specific morphological changes in microglia and loss of dentate gyrus volume in mice exposed to 40 ppm and 230 ppm lead acetate via the dam's milk from birth until weaning (Sobin, et al., 2013). Astrocytes appear to react to lead exposure at higher rates than neurons, which are mostly non-responsive, but, both types of cells are believed to be harmed (Cabell, et al., 2004; Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005b). The morphological changes in microglia and neural cells suggest that a neuroinflammatory response may be elicited by low-level lead which may explain the behavioral and motor dysfunctions observed in children. Neuroinflammation exacerbates neurodegenerative disorders such as Alzheimer's Disease (Rosenberg, 2005) and Parkinson's Disease (Hirsch & Hunot, 2009; Kasten-Jolly & Lawrence, 2011). Alzheimer's Disease and Parkinson's Disease are severe examples of neurodegenerative disorders triggered by neuroinflammation.

The neuroinflammatory response is believed to be generated by activated endothelium and microglia, which secrete a number of bioactive molecules in response to alterations in the brain's environment (Anthony & Campbell, 2002). The molecules secreted include pro-

inflammatory cytokines, which trigger the cascade of mechanisms making an inflammatory response, and anti-inflammatory cytokines that counter the effects of pro-inflammatory cytokines (Conti, et al., 2008). Unlike the inflammatory response in other parts of the body, anti-inflammatory cytokines are released simultaneously with pro-inflammatory cytokines to minimize damage to the brain (Strle, et al., 2001). It is also important to note that while these molecules play significant roles in immune and inflammatory responses in the brain, they have also been found to participate in reconstruction of neurons and programmed cell death during normal development (Sanes, Reh, & Harris, 2012).

It is possible that lead alters the normal function, release, and activation of a number of cytokines and biological markers of oxidative stress in the brain. The current study observed the effects of lead on the mRNA levels of Tumor Necrosis Factor α (TNF-α), Interleukin 6 (IL-6), Interferon γ (IFN-γ), Heme oxygenase 1 (Hmox-1), inducible Nitric Oxide Synthase (iNOS), Interleukin 10 (IL-10) and Glucose-regulated Protein of 78 kDa (GRP-78) (Ahamed & Siddiqui, 2007; Bokara, et al., 2008; Cabell, et al., 2004; Garcia-Arenas, Claudio, Perez-Severiano, & Rios, 1999; Gastaldello Moreira, de Magalhaes Rosa, Moraes Barros, Vassilieff, & Vassillieff, 2001; Jones, et al., 2008; Qian, Zheng, Weber, & Tiffany-Castiglioni, 2007; Strle, et al., 2001). These markers fall under three broad categories: pro-inflammatory cytokines (TNF-α, IL-6, iNOS and IFN-γ), anti-inflammatory cytokines (IL-10, Hmox-1), and a chaperone (GRP-78). The markers observed in the current studies were selected because they have been found to have roles in brain development and inflammatory response (Bauer, Kerr, & Patterson, 2007; Ahamed & Siddiqui, 2007; Anthony & Campbell, 2002; Bokara, et al., 2008; Cabell, et al., 2004; Deverman

& Patterson, 2009; Gastaldello Moreira, de Magalhaes Rosa, Moraes Barros, Vassilieff, & Vassillieff, 2001).

TNF-α is a key inflammatory cytokine that has been extensively studied for its role in the immune response. The TNF cytokine family is known to participate in developmental neuronal cell death (Sanes, Reh, & Harris, 2012). A study of mouse pups exposed to 0.1%, 0.5%, and 1% lead acetate in distilled water found that the presence of TNF-α increased with increasing lead exposure in the hippocampus (Li, et al., 2009).

Another inflammatory cytokine, IL-6, induces acute phase proteins during the inflammatory response (McCance & Huether, 2010). Aside from being known as an inflammatory cytokine, IL-6 is also categorized as a neuropoietic cytokine and is believed to have a role in regulation of neurogenesis (Erta, Quintana, & Hidalgo, 2012; Bauer, Kerr, & Patterson, 2007). The results of a study conducted to determine the effects of IL-6 on the brain suggested that the cytokine reduced behavioral dysfunction by suppressing microglial activation and increasing the number of oligodendrocytes in rats exposed to lipopolysaccharide (Pang, L.W, Zheng, Cai, & Rhodes, 2006). IL-6 is believed to have neuroprotective effects and reduced expressions of the cytokine during insult have been associated with increased oxidative stress and higher damage to neurons (Penkowa & Hidalgo, 2000). Lead exposure may reduce the expression of this cytokine and thus also reduce protective properties this cytokine activates in human glioma cells (Liu, Hsieh, & Yang, 2000).

It is possible that IFN-γ is released by microglia and blood-derived macrophages in the brain (Suzuki, Claflin, Wang, Lengi, & Kikuchi, 2005). The effect of IFN-γ has been studied on cultures of mouse cortical neurons. Findings from a study indicate that IFN-γ reduced

intracellular ATP levels and caused formation of dendritic beads and neuron damage *in vitro* (Mizuno, et al., 2008). Little is known about the effect of lead on IFN-γ expression in the brain, however studies have found neuritic beading which suggests the activation of IFN- γ (Jones, et al., 2008).

INOS is activated to induce nitric oxide generation during neuro immune responses (Zielinska, et al., 2015). iNOS generates nitric oxide by catalyzing a reaction which converts L-arginine to L-citrullinei. Nitric oxide is a byproduct of the reaction. Increased levels of nitric oxide have been found to be associated with neuronal loss. Garcia-Arenas and colleagues (1999) have reported that lead may increase the expression of iNOS and thus, increase the production of nitric oxide. The subsequent increase of iNOS may lead to higher oxidative stress and cause damage to brain function.

On the other hand, anti-inflammatory cytokines are also produced during the inflammatory process to counteract the potentially damaging effects caused by pro-inflammatory cytokines and oxidative stress. The anti-inflammatory cytokine, IL-10, is important in reducing synthesis of pro-inflammatory cytokines and inhibiting the expression and activation of cytokine receptors (Strle, et al., 2001). The expression of this cytokine was observed to determine whether anti-inflammatory mechanisms were being activated to reduce the harms of pro-inflammatory cytokines induced by low-level lead exposure (Strle, et al., 2001). The control of pro-inflammatory cytokine production is necessary to reduce the potentially damaging effects of these cytokines to the brain.

Hmox-1 was found to inhibit pro-inflammatory responses and maintain expression of IL-10 induced by lipopolysaccharide exposure (Chauveau, et al., 2005). The Hmox-1 antioxidant

enzyme is activated in response to oxidative stress and breaks down heme groups into biliverdin, iron(II), and carbon monoxide (Schipper, 2000; Ryter & Tyrrell, 2000). Biliverdin metabolizes into bilirubin which serves as an antioxidant (Ryter & Tyrrell, 2000). Oxidative stress has been found to increase the presence of Hmox-1 during exposure to organic lead in cultured hippocampal astrocytes (Cabell, et al., 2004).

Finally, the 78-kDa glucose-regulated protein (GRP-78) is believed to be important in regulating mechanisms that reduce oxidative stress (Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a; Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005b; Qian, Zheng, Weber, & Tiffany-Castiglioni, 2007; White, et al., 2007). Studies have shown that lead may increase oxidative stress indirectly by binding to GRP-78 (Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a). Once lead binds to GRP-78, the protein protects neurons by removing lead molecules, but by doing so, it loses its ability to activate anti-oxidant mechanisms (Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a). GRP-78 is known to increase the expression of IL-6 during normal circumstances and is also thought to have an indirect role in the generation of reactive oxygen species (Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a). Once lead molecules bind GRP-78, the chaperone accumulates and increases reactive oxygen species generation, but cannot activate release of IL-6 (Qian, Zheng, Weber, & Tiffany-Castiglioni, 2007).

Study Rationale

The studies here described were part of a larger program of translational interdisciplinary research examining the effects on brain and behavior of early chronic low-level lead exposure (Flores-Montoya & Sobin, 2015; Flores-Montoya, Alvarez, & Sobin, 2015; Sobin, Flores-Montoya, Gutierrez, Parisi, & Schaub, 2015; Sobin, et al., 2013; Sobin, Parisi, Schaub,

Gutierrez, & Ortega, 2011; Sobin, Gutierrez, & Alterio, 2009). An understanding of molecular mechanisms activated by chronic low-level lead exposure is needed to understand the results of child behavioral studies and to provide the data needed to assist in adjusting policies that set guidelines for the use and handling of lead. Understanding inflammatory immune response in the brain is also necessary to develop primary preventive measures that can ameliorate exposure to low lead levels. The majority of studies conducted to date on neuroinflammatory responses to lead have examined effects of lead exposures that would produce BLLs higher than the current CDC "threshold of concern" of 5 μg/dL and studies at lowest level exposures are needed. Currently, no preventive measures exist for the effects of chronic low-level lead. Medical treatment is available only for severe acute cases (Patrick, 2006). The findings of this study may be useful in guiding further studies that attempt to develop preventive measures targeting neuroinflammation.

In many prior studies of neuroimmune response following lead exposure *in vitro* methods were used (Garden & Moller, 2006). *In vitro* studies alone may provide only a partial understanding of neuroinflammatory responses. These studies examined isolated cells such as microglia and astrocytes and could not suggest possible synergistic mechanisms involving other cytokines (Harry & Kraft, 2008).

In the studies here described, cytokine gene expression was examined in two studies of brain tissue from pre-adolescent and adult mice exposed to low and higher levels of lead as compared with controls. Total cytokine gene expression, and the balance of cytokine gene expression in defined brain regions, were examined. As discussed above, there were a number of possible markers of mechanisms related to inflammatory responses secondary to lead-

induced neurotoxicity (Harry & Kraft, 2008). The inflammatory markers measured in the study were selected because of their major roles in pro-inflammatory and anti-inflammatory responses. Study 1 (pre-adolescent mice) examined pro-inflammatory IL-6, TNF-α, IFN-γ, and iNOS, the GRP78 chaperone, and the anti-inflammatory markers IL-10 and Hmox-1 in anterior and posterior brain regions. Study 2 (young adult mice) examined the same markers in anterior, posterior and cerebellar regions.

CHAPTER II: STUDY AIMS AND HYPOTHESIS

Specific Aims

Aim 1: To determine whether chronic lead exposure alters the *levels* of cytokine gene expression and/or oxidative stress marker gene expression in mouse brain at pre-adolescence and/or at early adulthood.

Aim 2: To determine whether chronic lead exposure alters the *balance* of cytokine gene expression and/or oxidative stress marker gene expression in mouse brain at pre-adolescence and/or early adulthood.

Hypotheses

It was hypothesized that chronic exposure to low-level lead contributes to brain disruption through its effect on the balance of the expression of pro-inflammatory and anti-inflammatory markers. Specifically, lead would cause a dose-dependent reduction of IL-6 through the inhibition of GRP-78. Pro-inflammatory cytokines (TNF-α, IFN-γ, and iNOS) would also be expressed at higher than normal concentrations as the brain responds to lead exposure. In response to the increase in oxidative stress and pro-inflammatory cytokine expression, anti-oxidant (Hmox-1) and anti-inflammatory (IL-10) marker expression would also increase to reduce the harm of oxidative stress and inflammatory mechanisms. The posterior cerebrum would be most susceptible to abnormal genetic expression of markers due to lead exposure. Since previous studies had not compared different effects in pre-adolescent and young adult mice, no hypotheses were proposed for possible age effects.

CHAPTER III: METHODS AND MATERIALS

Ethical Treatment of Animals

The experimental animal study was approved by the University of Texas at El Paso Institutional Animal Use Committee (Protocol #A-200804, C. Sobin, PI)

Experimental Protocol

Two experimental studies were conducted using a total of forty C57BL/6J mice (Jackson Laboratories, Sacramento, CA). In Study 1, brain tissue from 16 C57BL/6J mice (10 males/6 females) was harvested. The mice in Study 1 were sacrificed at 28 days of age, the age just preceding the onset of adolescence. In Study 2, brain tissue from 24 C57BL/6J mice (17 males/7 females) was harvested. The mice in Study 2 were sacrificed at 40 days of age, the age when mice reach sexual maturity. Both studies included two experimental groups and one control group. The experimental mice were exposed to lead through the milk of the dams. The dams were exposed to lead through the addition of lead acetate to their drinking water. The exposures to lead lasted from birth until weaning (post-natal day 21). The experimental groups consisted of a group exposed to 40 ppm lead acetate (low dose group) and a group exposed to 230 ppm lead acetate (high dose group). The third group served as the control and was not exposed to any lead acetate. The mice lived in a 12 hour light to 12 hour dark day cycle. All mice lived in cages equipped with a paper/plastic hut, bedding, and nestlet enrichment.

After 28 (Study 1) or 40 days (Study 2), the mice were sacrificed to conduct immunological studies on brain tissues. The brain tissue was harvested within one minute of sacrifice and was fresh frozen on dry ice. All brain tissue was stored at -80^{o}C until RNA extraction. Blood samples obtained from the heart and randomly selected brains from each

group were sent to the New Mexico State University in Las Cruces, New Mexico to obtain blood and brain lead levels of mice in the study using Inductively Coupled Plasma Mass Spectrometry.

RNA Extraction from Fresh Frozen Brain

RNA was extracted using Ambion's RiboPure™ Kit (Life Technologies, 2006). Each fresh frozen brain was cut into three segments: the anterior cerebrum, the posterior cerebrum, and the cerebellum. After the cerebellum was removed, the anterior and posterior portions were divided into two equal portions. The samples were sliced within one minute and were quickly transferred to glass homogenizers where the tissue was homogenized for less than thirty seconds to break down large tissues. TRI Reagent was immediately added after initial homogenization.

The anterior cerebrum included the following brain structures: basal forebrain, striatum, ventral striatum and septum. The posterior cerebrum included the midbrain, hippocampus, amygdala, thalamus, and hypothalamus. The medulla and pons were analyzed with the cerebellum.

The following procedures were followed as stated in the RiboPure™ Kit protocol and were included to describe specific amounts of reagents added as they were adjusted to the size of brain tissue used. Each of the three brain segments were homogenized with 400 µL of TRI Reagent® individually. After the brain segment was homogenized, 100 µL of chloroform (without isoamyl alcohol) was added to the homogenate and vortexed for 15 seconds. The mixture was then allowed to incubate for five minutes at room temperature.

After incubation, the mixture was centrifuged at 12,000 x g at room temperature for ten minutes. The mixture separated into three phases (as described in the protocol): an organic

phase, an interphase, and an aqueous phase. The top aqueous phase layer was transferred to a new 1.5 microcentrifuge tube. Once in a new tube, 100 µL of 100% ethanol was added to the aqueous phase and vortexed immediately for five seconds. The sample was then transferred to a filter cartridge and collection tube supplied in the kit.

The filter and collection tube was centrifuged at 12,000 x g for 1 minute at room temperature to pass all the liquid through the filter and ultimately bind the RNA to the filter. The flow-through liquid was discarded and then the filter replaced in the collection tube. An aliquot of 250 µL of Wash Solution was be added and the tube was again centrifuged at 12,000 x g for 1 minute. The flow through was discarded and a second wash was conducted with another 250 µL of Wash Solution. Once again, the tube was centrifuged at 12,000 x g for 1 minute and the filter was placed in a new collection tube. Once the filter was in a new tube, 40 µL of Elution Buffer was added to recover the RNA from the filter. Once the Elution Buffer was added, the tube was incubated for 2 minutes. After incubation, the tube was centrifuged at 12,000 x g for 90 seconds.

Due to the possibility of DNA presence in the extracted RNA, DNase obtained from Invitrogen was added to digest any remaining DNA. To each RNA sample, one µL of 10X DNase I Reaction Buffer and one µL of DNase I Amplification grade was added. The RNA and DNase mixture was then incubated at room temperature for 15 minutes. At the end of the incubation period, one µL of 25 mM EDTA solution was added and the mixture was heated to 65^{o}C for ten minutes to inactivate the DNase. After treatment with DNase, RNA concentration was obtained using the ND-1000 machine from NanoDrop Technologies.

cDNA Synthesis

The synthesis of cDNA was conducted immediately after RNA extraction to reduce possible RNA degradation. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems.

A master mix was created containing all the items in Table 2 according to the number of reactions that were conducted. Ten µL of the prepared master mix was combined with ten µL of extracted RNA in a 0.2 mL tube. The tubes were then centrifuged briefly to collect contents at the bottom of the tubes and were then loaded into a GeneAmp® PCR System 9700 thermal cycler manufactured by Applied Biosystems. The thermal cycler followed the cycling program described in Table 3. After the program was completed, the tubes containing cDNA were stored at -80^{o}C until they were used for gene expression analysis.

Gene Expression Analysis

Gene expression analysis was used to quantify inflammatory markers using Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) techniques. TaqMan® probes were ordered from Applied Biosystems for each of the biomarkers analyzed and β-actin served as the reference gene. Table 4 describes each biomarker and its corresponding primers. A 96-well qRT-PCR plate was prepared with each well containing an amount of cDNA equivalent to 500 ng of RNA, 1 µL TaqMan® probe, 10 µL TaqMan® Universal Master Mix, and an amount of nuclease-free water that brought the total volume in the well to 20 µL. Once the plate was filled with the samples to be analyzed, it was placed in an iCycler iQ^{TM} Optical Module thermal cycler manufactured by BIO RAD. The cycling program that the thermal cycler followed is

described in Table 5. Data was collected during Step 2 of Cycle 3. Duplicate wells of all samples were run to ensure that reading was consistent for each sample.

Data and Statistical Analysis

qRT-PCR data was analyzed by Relative Quantification using a reference gene (β-actin). The analysis was completed by using the ΔC_T Method using a reference gene (Kasten-Jolly, Heo, & Lawerence, 2011; Bio-Rad Laboratories, Inc., 2005), which produces normalized expression of target genes relative to the reference gene (β-actin). The following equation was used to obtain normalized expression of target genes:

$$C_T = Thermocycler\ output\ data$$

$$\Delta C_T = 2^{C_T\,\beta-actin\ -\ C_T\,target\,gene} \times 1000$$

IBM SPSS Statistics 22.0 software was used for all analyses. Possible group differences in the balance of cytokine gene expression in different brain regions were tested using a 3 x 2 (3 lead exposure groups by 2 brain regions tested), or a 3 x 3 (3 lead exposure groups by 3 brain regions tested) two-way mixed method ANOVA. Type III sum of squares were used to determine statistically significant differences. Tukey's Honest Significant Difference was conducted on significant ANOVA results.

CHAPTER IV: RESULTS

Study 1: Pre-adolescent Mice

qRT-PCR analyses were conducted on the anterior cerebrum, posterior cerebrum, and cerebellum of pre-adolescent mice (N = 16). Analysis of data obtained from cerebellar tissue could not be conducted due to low yields of RNA obtained from the harvested tissue. The low RNA yields did not produce accurate readings by the iCycler iQ™ Optical Module thermal cycler. Table 5 displays the mean genetic expression and standard deviation of each targeted biomarker relative to the reference gene, β-actin. The following results of the 3x2 (3 exposure groups x 2 brain regions) two-way mixed method ANOVA conducted for Study 1 were obtained for each biomarker. The main effect of brain region (not considering lead exposure) was analyzed to determine if any significant difference in expression of markers was observed between brain regions. Then statistical analysis was conducted taking brain region and lead exposure group into consideration. Any significant difference was further analyzed by Tukey post hoc tests.

TNF-α

A total of 14 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 4). There was a significant main effect of brain region tested in the expression of TNF-α ($F_{1, 11}$ = 18.7, p = 0.001; η_p^2 = 0.63). There was no significant difference in genetic expression of TNF-α when considering both, brain region and lead exposure group ($F_{2, 11}$ = 0.17, p = 0.85).

IL-6

A total of 14 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 4). There was a significant main effect of brain region tested in the expression of IL-6 ($F_{1, 11}$ = 34.9, p

< 0.001; $\eta_p^2 = 0.76$). There was no significant difference in genetic expression of IL-6 when considering both, brain region and lead exposure group ($F_{2, 11} = 3.37$, $p = 0.072$), however the Type III SS for the exposure group effect was significant ($F_{2, 11} = 8.87$, $p = 0.005$; $\eta_p^2 = 0.62$).

IFN-γ

A total of 12 brain samples were analyzed ($n_{CONTROL} = 6$, $n_{LOW\ DOSE} = 4$, and $n_{HIGH\ DOSE} = 2$). There was no significant main effect of brain region tested in the expression of IFN-γ ($F_{1, 9} = 0.45$, $p = 0.84$). There was no significant difference in genetic expression of IFN-γ when considering both, brain region and lead exposure group ($F_{2, 9} = 1.15$, $p = 0.36$).

Hmox-1

A total of 12 brain samples were analyzed ($n_{CONTROL} = 5$, $n_{LOW\ DOSE} = 3$, and $n_{HIGH\ DOSE} = 4$). There was no significant main effect of brain region tested in the expression of Hmox-1 ($F_{1, 9} = 1.49$, $p = 0.25$). There was no significant difference in genetic expression of Hmox-1 when considering both, brain region and lead exposure group ($F_{2, 9} = 0.61$, $p = 0.56$).

iNOS

A total of 13 brain samples were analyzed ($n_{CONTROL} = 6$, $n_{LOW\ DOSE} = 4$, and $n_{HIGH\ DOSE} = 3$). There was no significant main effect of brain region tested in the expression of iNOS ($F_{1, 10} = 4.93$, $p = 0.051$). A significant difference in genetic expression of iNOS was observed when considering both, brain region and lead exposure group ($F_{2, 10} = 10.26$, $p = 0.004$; $\eta_p^2 = 0.67$), however Type III SS for the exposure group effect was not significant ($F = 0.76$, $p = 0.49$). Tukey's post hoc analyses also showed no significant difference between any of the exposure groups.

IL-10

A total of 13 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 3). There was a significant main effect of brain region tested in the expression of iNOS ($F_{1, 10}$ = 5.68, p = 0.038; η_p^2 = 0.36). A significant difference in genetic expression of IL-10 was observed when considering both, brain region and lead exposure group ($F_{2, 10}$ = 6.74, p = 0.014, η_p^2 = 0.57), however Type III SS for the exposure group effect was not significant ($F_{2, 10}$ = 0.14, p = 0.88). Tukey's post hoc analyses also showed no significant difference between any of the exposure groups.

GRP-78

A total of 15 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 5, and $n_{HIGH\ DOSE}$ = 4). There was a significant main effect of brain region tested in the expression of GRP-78 ($F_{1, 12}$ = 6.34, p = 0.027; η_p^2 = 0.35). There was no significant difference in the genetic expression of GRP-78 when considering both, brain region and lead exposure group ($F_{2, 12}$ = 0.36, p = 0.71).

Study 2: Young Adult Mice

qRT-PCR analyses were conducted on anterior cerebrum, posterior cerebrum, and cerebellum of young adult mice (N = 21). Table 6 displays the mean genetic expression and standard deviation of each targeted biomarker relative to the reference gene, β-actin. Due to low RNA yields of some cerebellar tissues, the expression of iNOS, IL-10, and GRP-78 was not collected on some of the test samples. A 3x3 two-way mixed method ANOVA was conducted for TNF-α, IL-6, IFN-γ, and Hmox-1. A 3x2 two-way mixed method ANOVA was chosen to analyze group differences in genetic expression of iNOS, IL-10 and GRP-78 because a 3x3 two-way mixed method ANOVA would have reduced the sample size to one sample in some situations due to

the absence of data on some test samples as explained above. The main effect of brain region (not considering lead exposure) was analyzed to determine if any significant difference in expression of markers was observed between brain regions. Then statistical analysis was conducted taking brain region and lead exposure group into consideration. Any significant difference was further analyzed by Tukey post hoc tests.

TNF-α

A total of 12 brain samples were analyzed ($n_{CONTROL}$ = 3, $n_{LOW\ DOSE}$ = 3, and $n_{HIGH\ DOSE}$ = 6). There was no significant main effect of brain region tested in the expression of TNF-α ($F_{2, 18}$ = 0.44, p = 0.71). A significant difference in the genetic expression of TNF-α was observed when considering both, brain region and lead exposure group ($F_{4, 18}$ = 3.08, p = 0.043; η_p^2 = 0.41). The Type III SS for the exposure group effect was also significant ($F_{2, 9}$ = 8.15, p = 0.010; η_p^2 = 0.64). Tukey's post hoc analyses showed a significant difference ($p < 0.05$) between the control group and the low dose group; and also between the low dose group and the high dose group (Figure 1).

IL-6

A total of 15 brain samples were analyzed ($n_{CONTROL}$ = 4, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 7). There was a significant main effect of brain region tested in the expression of IL-6 ($F_{2, 24}$ = 11.70, $p < 0.001$; η_p^2 = 0.49). There was no significant difference in the genetic expression of IL-6 when considering both, brain region and lead exposure group ($F_{4, 24}$ = 0.39, p = 0.81). The Type III SS for the exposure group effect was significant ($F_{2, 12}$ = 8.08, p = 0.006; η_p^2 = 0.57)

IFN-γ

A total of 15 brain samples were analyzed ($n_{CONTROL}$ = 4, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 7). There was a significant main effect of brain region tested in the expression of IFN-γ ($F_{2, 24}$ = 19.0, $p < 0.001$; η_p^2 = 0.75). There was no significant difference in the genetic expression of IFN-γ when considering both, brain region and lead exposure group ($F_{4, 24}$ = 1.69, p = 0.19), however Type III SS for the exposure group effect was significant ($F_{2, 12}$ = 9.38, p = 0.004; η_p^2 = 0.61).

Hmox-1

A total of 14 brain samples were analyzed ($n_{CONTROL}$ = 3, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 7). There was a significant main effect of lead exposure in the expression of Hmox-1 ($F_{2, 11}$ = 10.4, p = 0.003). There was a significant main effect of brain region tested in the expression of Hmox-1 ($F_{2, 22}$ = 6.70, p = 0.005; η_p^2 = 0.38). There was also a significant difference in the genetic expression of Hmox-1 when considering both, brain region and lead exposure group ($F_{4, 22}$ = 6.86, p = 0.001; η_p^2 = 0.56). The Type III SS for the exposure group effect was also significant ($F_{2, 11}$ = 10.4, p = 0.003; η_p^2 = 0.65). Tukey HSD post hoc analyses showed a significant difference ($p < 0.01$) between the control and low dose groups; and also between the low dose and high dose groups (Figure 2).

iNOS (cerebellum not analyzed)

A total of 16 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 6). There was a significant main effect of brain region tested on the expression of iNOS ($F_{1, 13}$ = 8.36, p = 0.013; η_p^2 = 0.39). There was no significant difference in the genetic expression of iNOS when considering both, brain region and lead exposure group ($F_{2, 13}$ = 1.43, p = 0.27).

IL-10 (cerebellum not analyzed)

A total of 10 brain samples were analyzed ($n_{CONTROL}$ = 3, $n_{LOW\ DOSE}$ = 3, and $n_{HIGH\ DOSE}$ = 4). There was no significant main effect of brain region tested on the expression of IL-10 ($F_{1, 7}$ = 3.14, p = 0.12). There was no significant difference in the genetic expression of IL-10 when considering both, brain region and lead exposure group ($F_{2, 7}$ = 0.35, p = 0.72), however the Type III SS for the exposure group effect was significant ($F_{2, 7}$ = 7.15, p = 0.02; η_p^2 = 0.67).

GRP-78 (cerebellum not analyzed)

A total of 16 brain samples were analyzed ($n_{CONTROL}$ = 6, $n_{LOW\ DOSE}$ = 4, and $n_{HIGH\ DOSE}$ = 6). There was a significant main effect of brain region tested on the expression of GRP-78 ($F_{1, 11}$ = 56.4, p < 0.001; η_p^2 = 0.84). There was a significant difference in the genetic expression of GRP-78 when considering both, brain region and lead exposure group ($F_{2, 11}$ = 4.60, p = 0.035; η_p^2 = 0.46). The Type III SS for the exposure group effect was also significant ($F_{2, 11}$ = 7.15, p = 0.015; η_p^2 = 0.54). Tukey HDS post hoc analyses determined that there was a significant difference (p < 0.05) between the low dose and high dose groups (Figure 3).

CHAPTER V: DISCUSSION

Numerous studies have observed the effects of lead on specific inflammatory molecules (Garcia-Arenas, Claudio, Perez-Severiano, & Rios, 1999; Li, et al., 2009; Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a; Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005b; Qian, Zheng, Weber, & Tiffany-Castiglioni, 2007), but few have observed the expression of multiple markers of innate immunity in brains of mice exposed to low levels of lead (Kasten-Jolly, Heo, & Lawerence, 2011; Sobin, et al., 2013). In order to better understand the inflammatory response elicited by chronic low level lead exposure, the current studies analyzed the expression of multiple markers of inflammation that fell under one of four categories to determine how exposures to chronic low levels of lead affected the expression of those markers and whether balance of those markers in the brain was also affected. Expression of pro-inflammatory cytokines (TNF-α, IL-6, and IFN-γ), oxidant regulating enzymes (Hmox-1 and iNOS), an anti-inflammatory cytokine (IL-10), and a chaperone (GRP-78) were observed in brains of pre-adolescent and young adult C57BL/6J mice.

The studies aimed to determine whether chronic lead exposure altered the *levels* of inflammatory cytokine gene expression and/or oxidative stress marker gene expression in mouse brain at pre-adolescence and/or at early adulthood. The second aim was to determine whether chronic lead exposure alters the *balance* of inflammatory cytokine gene expression and/or oxidative stress marker gene expression in mouse brain at pre-adolescence and/or early adulthood. Cytokines are expressed at varying levels due to the numerous roles and functions that they have in immune responses and neurogenesis (Khan, 2008; Deverman & Patterson, 2009). The pro-inflammatory molecules tested in this study (TNF-α, IFN-γ and IL-6) are usually

expressed at higher concentrations during brain disease and can contribute to neural damage through their prolonged activation (Wilms, et al., 2010; Mizuno, et al., 2008; Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005a). In the brain, anti-inflammatory mechanisms are usually activated at the same time as pro-inflammatory ones to prevent extensive damage from the pro-inflammatory molecules (Strle, et al., 2002). It has been found that IL-10 induces the release and expression of Hmox-1 to reduce oxidative stress (Lee & Chau, 2002). Prior studies have found that expression of IL-6 had an inverse relation to expression of GRP78 during lead exposure (Qian, Zheng, Weber, & Tiffany-Castiglioni, 2007; Liu, Hsieh, & Yang, 2000).

Study 2: Genetic Expression and Distribution of TNF-α, Hmox-1, and GRP78

In young adult mice, the genetic expression of TNF-α (Figure 1), Hmox-1 (Figure 2), and GRP78 (Figure 3) showed statistically significant differences when both lead exposure group and brain segment were analyzed.

The expression of TNF-α and Hmox-1 followed similar expression patterns in the anterior and posterior cerebrum in young adult mice. The two markers were most highly expressed in the low dose group. These patterns suggest that lead was distributed throughout the anterior and posterior cerebrum in the days that followed the end of lead exposure in the mice in this study. Lead exposure in young adult mice ended at post-natal day 21, the same time as pre-adolescent mice.

In the anterior portion of the brain, expression of TNF-α and Hmox-1 was significantly higher in the low dose group when compared to the control group. This interaction was interesting because it did not follow the expected dose response pattern. Through some currently unknown mechanism, it appears that chronic lowest level lead exposure elicits an

extended and more highly expressed inflammatory response. Additional testing should be conducted in lowest level lead exposed animal models to further investigate this finding. The expression difference of TNF-α and Hmox-1 between the control and high dose group was not significant.

GRP78 was expressed in a manner that was different from that of TNF-α and Hmox-1, however the statistically significant differences of GRP78 were found between low dose and high dose groups. There was no statistical significance between the control group and either of the experimental groups.

Summary of Observed Inflammatory Response

As hypothesized, pro-inflammatory (TNF-α) and anti-inflammatory (Hmox-1) mechanisms were activated simultaneously in Study 2, however no statistically significant findings were made in Study 1. The findings in Study 2 support previous studies describing the activation of both pro- and anti-inflammatory mechanisms during neuroimmune activation (Deverman & Patterson, 2009; Harry & Kraft, 2008; Kasten-Jolly & Lawrence, 2011; Strle, et al., 2001). The simultaneous activation of pro- and anti- inflammatory mechanisms allows for the activation of pro-inflammatory mechanisms in response to lead exposure and the anti-inflammatory response may ameliorate some of the deleterious effects of extended activation of the pro-inflammatory processes.

The expression of neuroinflammatory markers suggests an activated immune response that support findings of microglial disruption by previous studies (Sobin, et al., 2013). Cytokines and inflammatory molecules have been found to have varying roles during neurogenesis. Studies have found that chronic exposure to low-level lead alters mouse exploratory function,

olfactory recognition, and aggression (Flores-Montoya & Sobin, 2015; Flores-Montoya, Alvarez, & Sobin, 2015; Kasten-Jolly, Pabello, Bolivar, & Lawrence, 2012). It is unclear however, whether these changes in behavior are a result of extended activation of neuroimmune processes causing damage to the brain or if interference caused by low-level lead exposures prevent normal brain development.

A study conducted by Giedd et al. found that cerebral volume peaks a 14.5 years in human males and 11.5 years in human females. The study further found that by age 6, the brain is at approximately 95% of that peak in both males and females (Lenroot & Giedd, 2006; Giedd, et al., 1999). By interfering with normal expression and balance of neuroinflammatory processes, lead may affect normal brain development during childhood, a vital time period for that development. This may explain loss of gray matter in portions of the prefrontal cortex in the brains of adults exposed to lead during childhood (Cecil, et al., 2008).

Strengths

The studies, presented, were among the first to observe the *in vivo* expression of multiple pro-/anti-inflammatory markers in animal models exposed to chronic low levels of lead. There are many different markers involved in brain development and neuroinflammatory responses. The markers observed in the current studies were selected because they have been found to have roles in brain development and inflammatory response (Bauer, Kerr, & Patterson, 2007; Ahamed & Siddiqui, 2007; Anthony & Campbell, 2002; Bokara, et al., 2008; Cabell, et al., 2004; Deverman & Patterson, 2009; Gastaldello Moreira, de Magalhaes Rosa, Moraes Barros, Vassilieff, & Vassillieff, 2001). Despite not being able to define specific mechanisms of detrimental effects caused by chronic low level lead exposure in the brain, this study can be

used to focus future research on the pathways associated with the expression of iNOS and IL-10 in pre-adolescent mice and TNF-α, Hmox-1 and GRP78 in young adult mice.

Another strength of the current study was that the measurement of inflammatory marker expression was conducted for two age groups. This is significant because the observed expression of some of the genetic markers showed that chronic exposure to lowest levels of lead continue to affect neuroinflammatory responses after the exposure is halted.

The studies also measured genetic expression of inflammatory markers in three different brain segments. Few studies have analyzed expression of inflammatory markers in different brain regions after chronic low level lead exposure (Kasten-Jolly, Pabello, Bolivar, & Lawrence, 2012; Sobin, et al., 2013; Kasten-Jolly, Heo, & Lawerence, 2011). Measurement of the expression of the selected inflammatory markers in three different brain regions allowed for the observation of what can potentially be different exposure and response patterns in the anterior cerebrum, posterior cerebrum, and cerebellum of pre-adolescent and young adult mice tested.

Finally, the study exposed test animals to concentrations of lead that typically result in BLLs that fall below the current threshold of action as described by the CDC. The current study showed that TNF-α and Hmox-1 were expressed more highly in young adult mice exposed to the lowest levels of lead. These findings suggest that lowest level lead exposures may cause an extended and more heightened neuroinflammatory response that can cause further damage or obstruction to normal brain function.

Limitations

A high variability in some of the results can be explained by the following factors: low RNA yields, smaller than expected sample sizes, and varying lead levels within each exposure group. RNA yields in some of the samples were too low despite homogenizing brain tissue quickly in TRI Reagent. This led to smaller sample sizes than expected when analyzing gene expression of some of the cytokines. The qRT-PCR equipment requires specific RNA concentrations in order to accurately detect the signals elicited by the TaqMan® Probes. In addition to RNA yields, some of the cytokines measured are expressed at minimal levels which may create additional difficulties in qRT-PCR detection. Use of alternate kits for the extraction of RNA from fatty tissues may increase the RNA yielded. It may also be beneficial that future studies pool brain tissue from 2-3 brains within the same exposure group in order to reduce error caused by low RNA yields. Lead levels in mice in the present studies were dependent on the amount of water that the dam drank and on the amount of dam milk consumed by the pup. This variability caused by this issue may be addressed by having larger sample sizes in each exposure group.

Some studies have found that males and females are affected differently to lead exposure. One study found that male mice exposed to low levels of lead had increased "aggressive behavior toward a stranger," while female mice demonstrated a lower exploratory behavior (Kasten-Jolly, Pabello, Bolivar, & Lawrence, 2012). The use of natural litters in these studies did not allow control for sex. Analysis of data by sex could not be conducted due to the lower number of female mice available. Future studies can be designed to observe differences

in expression of markers of inflammation in female and male mice exposed to chronic low levels of lead.

Implications of Findings to Public Health

The findings of the study suggest that lowest level lead exposures may trigger mechanisms throughout brain development that cause behavioral and motor dysfunctions in children.

Studies such as this one provide information that can be used to determine how chronic low level lead exposures may affect normal functioning of the brain and can have federal, state and local policies recognize that low level lead may be harmful to children, even at BLLs below 5 µg/dL. Changes in policy can allow for additional funding for improved epidemiological data collection and follow up. Currently, the CDC recommends that action and reporting be conducted on cases with BLLs ≥ 5 µg/dL, although it does recognize that there is no known safe threshold for lead (Centers for Disease Control and Prevention, 2014). The state of Texas, on the other hand, requires that all positive blood lead tests in children younger than 15 years of age be reported; regardless of whether the results are elevated or not (Texas Department of State Health Services, 2014). The stricter policy sets a standard that improves surveillance of BLLs in communities. The surveillance data, in turn, can be used to identify clusters of exposure for further epidemiological investigation and implementation of non-pharmaceutical control measures. Surveillance data can also be used to characterize the extent of lead availability throughout the United States and identify specific communities in need of environmental and community interventions.

Currently there are no identified primary preventive measures that can be used to prevent the effects of chronic low-level lead exposure in the pediatric brain. Research at the molecular level needs to continue to identify affected pathways or targets for preventive measures. The current study identified both pro- and anti-inflammation markers that are affected by chronic low-level lead exposure. The results observed in this study can be used to further investigate the specific neuroinflammatory mechanisms affecting normal brain development and/or function.

Conclusion

The brain is a highly complex organ requiring a homeostatic environment to function normally. During neurodevelopment, cytokines are involved in the normal restructuring of the brain by inducing programmed cell death as neuronal function becomes specialized (Victorio, Havton, & Oliveira, 2010). The findings in the present study suggested that chronic exposure to low levels of lead elicited an abnormal expression of a number of cytokines and biological markers of inflammation. The abnormal expression of cytokines and pro- and anti-inflammatory cytokines in the central nervous system have often been referred to as a double-edged sword, as it can be both protective and deleterious to the brain (Victorio, Havton, & Oliveira, 2010; Hu & Ivashkiv, 2009; Pang, L.W, Zheng, Cai, & Rhodes, 2006).

Previous studies have suggested that low-level lead exposures cause cognitive, behavioral, and motor dysfunctions in children (Bellinger, Stiles, & Needleman, 1992; Canfield, et al., 2003; Chiodo, Jacobson, & Jacobson, 2004; Lanphear, et al., 2005; Lanphear, Dietrich, Auinger, & Cox, 2000; Nevin, 2009). Environmental interventions such as soil remediation can significantly reduce environmental lead (Mielke, et al., 2010), however, because there may be

no safe threshold for lead exposure, primary preventive methods are required. In order to develop these measures, a better understanding of the molecular mechanisms activated by lead is key. The mouse models in this study provided an insight to innate immune responses to low levels of lead in the brain which may be presented similarly in human children. The study results suggest that the expression of cytokines is altered in the brain exposed to low levels of lead. These findings provide areas of further research that can assist in identifying targets for primary preventive measures. As this is one of the first studies to observe the expression of pro-/anti-inflammatory cytokines in brains of mice exposed to chronic low-level lead, further studies need to be conducted to support these findings. Future studies should be conducted to determine whether the genetic expression of markers observed is expressed similarly in antibody and protein levels through the use of enzyme-linked immunosorbent assay (ELISA) methodologies.

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TABLES

Table 1: cDNA Synthesis Master Mix

Component	**Volume (µL)**
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
Total	**10**

Table 2: cDNA Synthesis Thermal Cycler Program

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

Table 3: TaqMan® Probes for Brain Studies

Gene	Probe design is based on following Mus musculus sequences:	TaqMan® Probe Assay ID
GRP78	BC050927.1, AJ002387.1, AK004578.1, AK076079.1, AK077463.1, AK144650.1, AK144742.1, AK146647.1, AK148539.1, AK149447.1, AK151244.1, AK151647.1, AK151806.1, AK152020.1, AK152521.1, AK152958.1, AK159719.1, AK160821.1, AK166739.1, AK167011.1, AK168012.1, AK169034.1, AK192496.1, AK195133.1, AK197130.1, AK200033.1, AK219322.1, BC005785.1, BG076621.2, D78645.1, NM_022310.2, NM_022310.2, U89425.1	Mm00517691_m1
iNOS	BC062378.1, AF065919.1, AF065920.1, AF065921.2, AF065922.2, AF065923.2, AY090567.1, M84373.1, M87039.1, M92649.1, NM_010927.1, NM_010927.1, NM_010927.1, U43428.1	Mm01309901_m1
Hmox-1	BC010757.1, AK150934.1, AK151012.1, AK151627.1, AK153420.1, AK153516.1, AK154598.1, AK159521.1, AK169227.1, M33203.1, NM_010442.1, NM_010442.1, X13356.1	Mm00516007_m1
IL-6	NM_031168.1, AK089780.1, AK150440.1, AK152189.1, J03783.1, X06203.1, X54542.1	Mm00446191_m1
IL-10	BC120612.1, M37897.1, NM_010548.1, NM_010548.1	Mm00439616_m1
INF-γ	BC119063.1, AK089574.1, AY423847.1, BC119065.1, K00083.1, M28621.1, NM_008337.1, NM_008337.1, NM_008337.1	Mm00801778_m1
TNF-α	BC117057.1, AK153319.1, AK153800.1, AK154223.1, AK155964.1, AY423855.1, M11731.1, M13049.1, NM_013693.1, NM_013693.1, X02611.1	Mm00443258_m1
β-actin Endogenous control	NM_007393.1, AK078935.1, AK088691.1, AK145191.1, AK145196.1, AK145308.1, AK147787.1, AK149920.1, AK150545.1, AK150553.1, AK150558.1, AK150570.1, AK150662.1, AK150675.1, AK150711.1, AK150879.1, AK151010.1, AK151136.1, AK151145.1, AK151159.1, AK151166.1, AK151190.1, AK151202.1, AK151226.1, AK151277.1, AK151297.1, AK151350.1, AK151386.1, AK151398.1, AK151444.1, AK151468.1, AK151663.1, AK151712.1, AK151898.1, AK151956.1, AK151995.1, AK151999.1, AK152043.1, AK152052.1, AK152083.1, AK152218.1, AK152433.1, AK152470.1, AK152591.1, AK152615.1, AK152651.1, AK152671.1, AK152813.1, AK152841.1, AK153461.1, AK159759.1, AK159834.1, AK160029.1, AK166349.1, AK166498.1, AK167117.1, AK167825.1, AK167960.1, AW537707.1, BC040513.1, BG063688.2, BG063722.2, BG063855.2, J04181.1, M12481.1, NM_007393.1, NM_007393.1, X03672.1, X03765.1	Mm00607939_s1

All TaqMan® Probes used the FAM™ reporter dye.

Table 4: qRT-PCR Thermal Cycler Program

	Cycle 1 (1 repeat)	**Cycle 2 (1 repeat)**	**Cycle 3 (40 repeats)**	
	Step 1	***Step 1***	***Step 1***	***Step 2 (Data Collection)***
Temperature	50^0C	95^0C	95^0C	60^0C
Time	2 minutes	10 minutes	15 seconds	60 seconds

Table 5: Study 1 - Mean Expression of Markers

		TNF-α	IL-6	IFN-γ	Hmox-1	*iNOS**	*IL-10**	GRP78
Anterior	**Control**	2.958E-02	2.233E-02	2.212E-04	4.764	0.15011	2.079E-03	219.77
	Std Dev	7.985E-03	2.580E-03	6.127E-05	1.784	0.02995	5.239E-04	84.47
	Low Dose	2.403E-02	2.535E-02	2.628E-04	6.272	0.15364	2.587E-03	295.68
	Std Dev	2.108E-02	4.572E-03	1.270E-04	1.551	0.03663	1.651E-04	106.39
	High Dose	4.250E-02	1.627E-02	2.321E-04	6.477	0.14039	1.420E-03	281.49
	Std Dev	3.065E-03	3.935E-03	7.272E-05	1.151	0.00828	1.794E-04	144.68
Posterior	**Control**	4.850E-02	1.887E-02	1.815E-04	5.258	0.16477	2.509E-03	353.84
	Std Dev	1.354E-02	1.886E-03	6.905E-05	2.792	0.03206	4.690E-04	95.83
	Low Dose	4.790E-02	1.465E-02	2.924E-04	9.066	0.12769	2.109E-03	356.35
	Std Dev	2.119E-02	6.662E-03	1.471E-04	2.192	0.04290	8.140E-04	206.30
	High Dose	5.972E-02	9.570E-03	2.579E-04	6.735	0.19864	2.941E-03	432.08
	Std Dev	6.206E-03	1.477E-03	1.643E-04	3.634	0.04045	8.893E-04	124.20

**** Significant Brain Portion x Lead Exposure interaction***

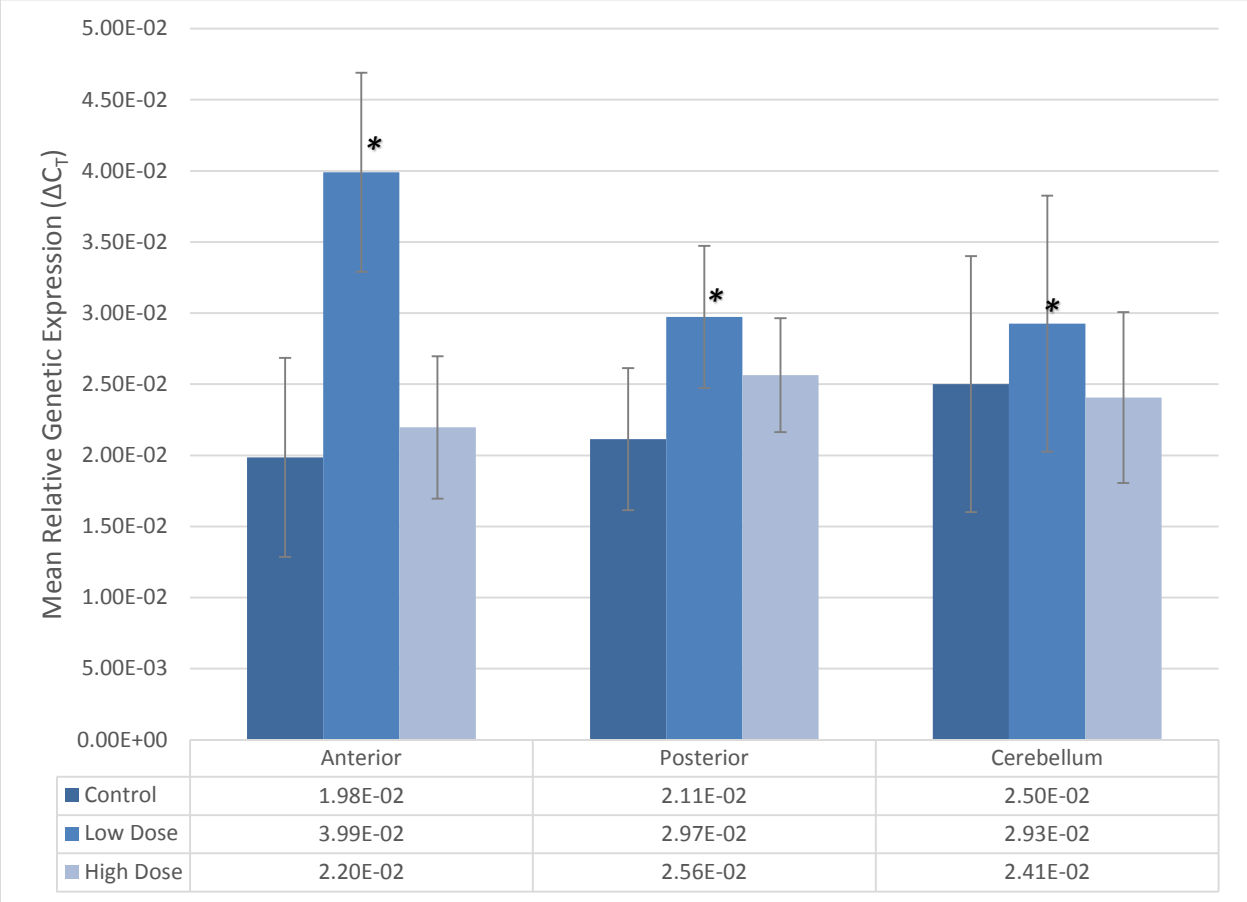
Table 6: Study 2 - Mean Expression of Markers

		*TNF-α**	IL-6	IFN-γ	*Hmox-1**	iNOS	IL-10	*GRP78**
Anterior	**Control**	1.985E-02	1.556E-02	8.63E-05	4.5634	0.08937	1.498E-03	187.46
	Std Dev	5.069E-03	4.227E-03	4.41E-05	1.0044	0.06388	2.992E-04	25.89
	Low Dose	3.991E-02	1.333E-02	1.94E-04	10.8736	0.06261	1.663E-03	109.45
	Std Dev	3.448E-03	1.327E-03	8.00E-05	0.6676	0.03142	6.842E-04	53.56
	High Dose	2.196E-02	1.455E-02	1.96E-04	5.6164	0.10343	2.108E-03	158.18
	Std Dev	5.657E-03	1.812E-03	3.59E-05	1.5942	0.03244	7.129E-04	45.80
Posterior	**Control**	2.113E-02	1.396E-02	1.85E-04	4.8003	0.09731	1.637E-03	259.12
	Std Dev	1.729E-03	6.481E-03	3.56E-05	2.4964	0.03382	4.181E-04	68.78
	Low Dose	2.973E-02	1.086E-02	2.20E-04	6.5778	0.11612	2.269E-03	270.63
	Std Dev	4.821E-03	2.223E-03	8.00E-05	1.0942	0.02028	7.863E-05	15.84
	High Dose	2.564E-02	1.320E-02	1.88E-04	5.1386	0.15667	2.659E-03	372.51
	Std Dev	4.182E-03	1.198E-03	4.56E-05	0.6682	0.02656	2.388E-04	59.22
Cerebellum	**Control**	2.500E-02	1.083E-02	2.06E-04	7.1416	*Cerebellum not considered for iNOS, IL-10 and GRP 78 due to the reduction of sample size caused during the statistical analysis if cerebellum were considered.*		
	Std Dev	4.054E-03	3.724E-03	7.46E-05	1.4074			
	Low Dose	2.925E-02	7.960E-03	3.69E-04	7.7071			
	Std Dev	5.986E-03	1.108E-03	3.04E-05	0.7707			
	High Dose	2.406E-02	7.426E-03	3.21E-04	6.0895			
	Std Dev	8.024E-03	1.201E-03	9.00E-05	1.9565			

**** Significant Brain Portion x Lead Exposure interaction***

FIGURES

Figure 1: Genetic Expression of TNF-α in Young Adult C57BL/6J Mice

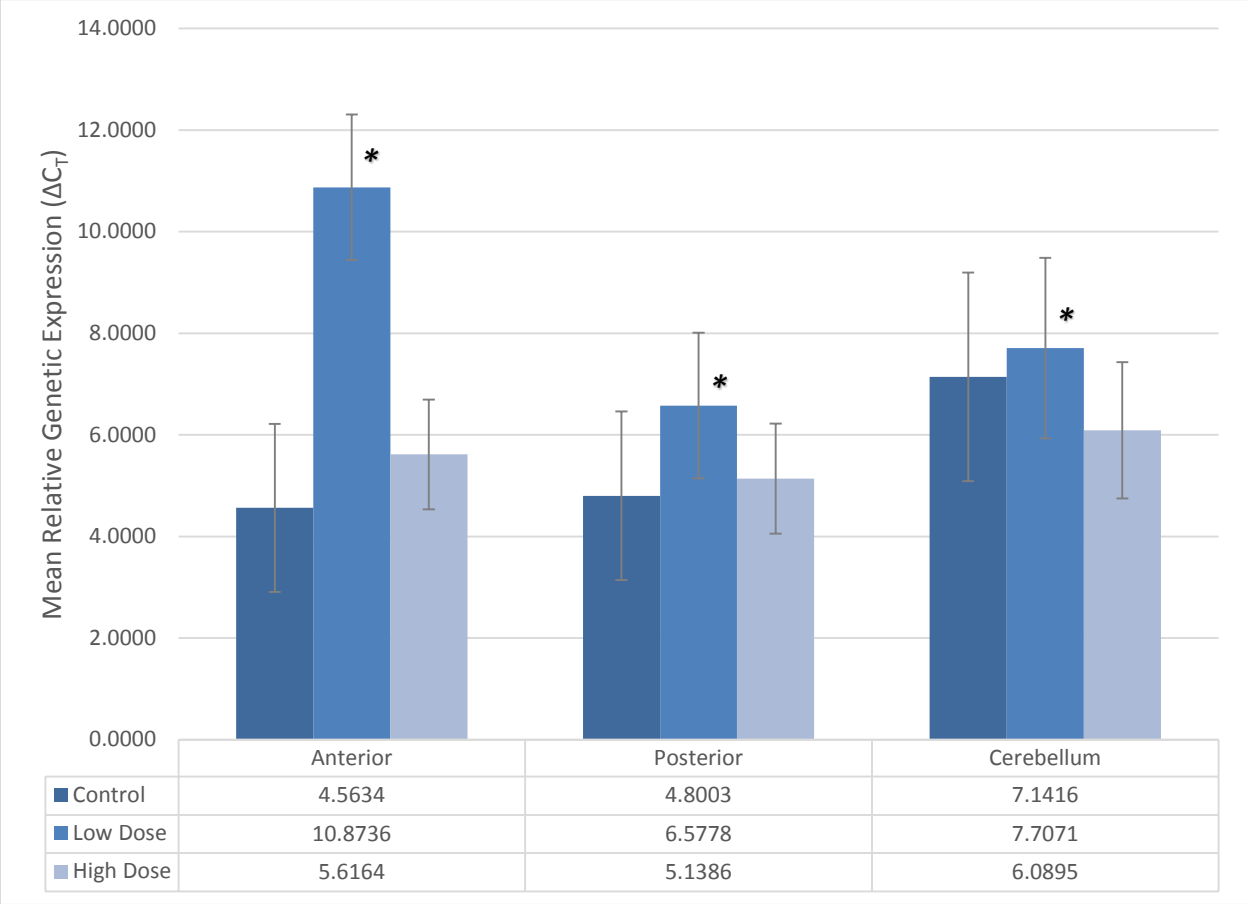


	Anterior	Posterior	Cerebellum
Control	1.98E-02	2.11E-02	2.50E-02
Low Dose	3.99E-02	2.97E-02	2.93E-02
High Dose	2.20E-02	2.56E-02	2.41E-02

Error bars represent 95% Confidence Intervals.

*A significant difference was observed between the low dose group and the control group. The low dose group was also statistically different from the high dose group.

Figure 2: Genetic Expression of Hmox-1 in Young Adult C57BL/6J Mice

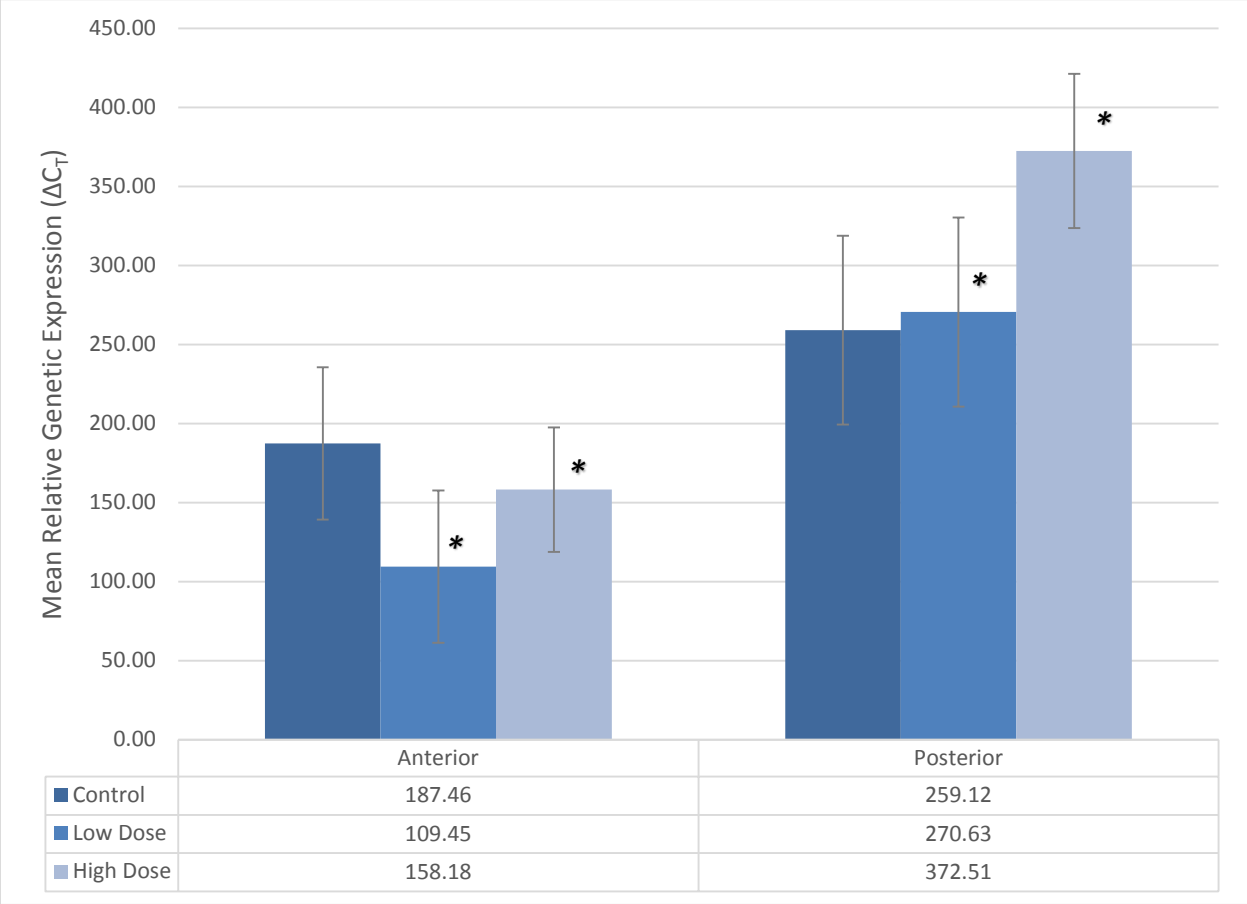


	Anterior	Posterior	Cerebellum
Control	4.5634	4.8003	7.1416
Low Dose	10.8736	6.5778	7.7071
High Dose	5.6164	5.1386	6.0895

Error bars represent 95% Confidence Intervals.

*A significant difference was observed between the low dose group and the control group. The low dose group was also statistically different from the high dose group.

Figure 3: Genetic Expression of GRP78 in Young Adult C57BL/6J Mice



	Anterior	Posterior
Control	187.46	259.12
Low Dose	109.45	270.63
High Dose	158.18	372.51

Error bars represent 95% Confidence Intervals.

*Significant difference observed between low dose and high dose groups.

CURRICULUM VITA

Miguel A. Cervantes received a Bachelor of Science Degree from the University of Texas at El Paso. His research experience includes: low level lead exposure and inflammatory markers in brains of C57BL/6J mice, assessing work-associated health injuries among migrant farm workers, participating in an animal trial of a novel DNA vaccine for *Leishmania mexicana,* and he also developed and piloted the protocols and procedures for a zoonotic surveillance study identifying *L. mexicana* and *T. cruzi* in animal samples obtained from West Texas using PCR techniques. He is currently a member of the Epidemiology Response Team for the Texas Department of State Health Services Health Service Region 9/10 where he responds to public health emergencies. His previous public health experience was in HIV/STI surveillance and intervention with the City of El Paso Department of Public Health. He completed federal internships at CDC's Coordinating Office for Terrorism Preparedness and Emergency Response (now the Office of Public Health Preparedness and Response) in Atlanta, GA and in HRSA's Area Health Education Center Branch in Washington DC. He completed his master's practicum at the El Paso Independent School District Coordinated School Health Program. Miguel was an oral presenter at the Society for Advancement of Chicanos and Native Americans in Science National Conference in 2009 and as a poster presenter for the NEUROTOX National Conference in 2010.

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