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A Study of Hippocampal Microglia Distribution in Pre-adolescent Mice Chronically Exposed to Lead

Salvador Dominguez
University of Texas at El Paso, sdominguez13@miners.utep.edu

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SALVADOR DOMINGUEZ

Master's Program in Public Health

APPROVED:

Christina Sobin, Ph.D., Chair

Carla Campbell, Ph.D.

Charlotte Vines, Ph.D.

Charles Ambler, Ph.D.
Dean of the Graduate School

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SALVADOR DOMINGUEZ, B.S.

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Abstract

Background: Lead is a major unresolved child health hazard. Children are uniquely vulnerable to the effects of lead exposure, which include disruption of brain development and brain function. How lead exposure alters brain development and brain function is not currently known. Our laboratory has been developing a mouse model to understand effects of low-level lead exposure in the developing brain. In previous studies, we have shown that early chronic low level lead exposure diminished the number of microglia in the dentate gyrus region of the hippocampus, an area responsible for learning and memory. Building on the results of the previous study, we conducted a study looking at the effects of early chronic low level lead exposure on microglia in the entire hippocampal structure. The current study examined the microglia mean cell body number and distribution of microglia throughout hippocampus in pre-adolescent mice.

Goals of the Study: To determine whether microglia mean cell body number was diminished throughout the entire hippocampal structure of lead exposed mice as compared to controls; whether diminished microglia mean cell body number was unique to dentate gyrus in pre-adolescent mice with early chronic low-level lead exposure; and to examine whether microglial patterns of clustering around neurons differed in lead exposed animals.

Hypotheses: H1): as compared to control animals with no history of lead exposure, in lead exposed animals, microglia mean cell body number is less; H2): as compared to control animals with no history of lead exposure, in lead exposed animals microglia mean cell body number is greater in hippocampal regions that do not include dentate gyrus as compared to hippocampal regions that include dentate gyrus; H3): as compared to control animals with no history of lead

exposure, in lead exposed animals hippocampal microglia show greater clustering around neurons

Methods: The study examined the brains of 30 pre-adolescent C57BL/6J mice exposed to one of three possible lead exposure groups including controls (0 ppm), low-dose (30 ppm), and high-dose (330 ppm) animals. Microglia mean cell body number and spatial distribution data were collected using the Stereologer software system (Stereologer Resource Center, Gainesville, FL). Hippocampal volume was also measured. Generalized linear regression models were used to test hypotheses; all models controlled for sex with litter included as a random effect.

Results: Regression analyses showed that microglia mean cell body number was significantly reduced in lead exposed as compared to control animals (main effects for group, $F = 44.51$, $p < .001$ and sex, $F = 17.14$, $p < .001$); and a significant interaction of group by sex ($F = 5.77$, $p = .01$) was found. Parameter estimates and post-hoc tests showed that females in lead-exposed groups had significantly fewer microglia than males. Analyses of microglia mean cell body number in sections of hippocampus with and without dentate gyrus did not differ among groups. Clustering of microglia around neurons did not differ among groups.

Conclusion: The results replicated and expanded on our previous studies and showed that microglia mean cell body number was reduced throughout the hippocampal structure of pre-adolescent lead-exposed animals as compared to controls; and that the distribution of microglia around neurons did not differ in lead exposed animals as compared to controls. These results suggested that hippocampal microglial loss occurs in lead exposed mice before pre-adolescence. Further studies are needed to understand whether early chronic low-level lead exposure destroys microglia during development or somehow causes microglia to be trafficked out of the brain; and at which point in development these changes occur.

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Introduction

1.1 Lead Exposure in Children: A Continuing Problem

Exposure to higher levels of lead in children was a major problem in the United States before approximately 1980. Since that time, there have been many strides made to reduce child lead exposure. For example, in the 1970's the national average for child blood lead was reported to be 15 µg/dL (Blood Lead Levels United States, 1988-1991, 1994). As of 2014, that level had fallen to 1.2 µg/dL (Agency for Toxic Substances & Disease Registry, 2014). The decrease was accomplished by the enactment of various laws and regulations that prohibited the use of lead in gasoline and paint. The EPA worked on reducing lead in the water through the enactment of the Clean Water Act which prevents industry from discharging pollutants, such as lead, into the waters of the U.S (National Center for Environmental Health, 2013). The EPA also enacted the Safe Water Drinking Act which set standards for pollutants, such as lead, in the drinking water for water companies to follow. These regulations also required that individuals responsible for facilities (such as schools or daycare centers), or dwellings (such as landlords and building owners) built before 1978 become knowledgeable about the risks of lead contamination, educate others regarding the risks, and come up with methods to reduce possible exposure from these sources in children.

There is no denying the great advances that have been made in reducing child lead exposure. At the same time, it is also widely recognized that no level of lead exposure is "safe" for children (US EPA, 2013). Studies have suggested that as many as fifty percent of low-income minority children may be chronically exposed to environmental lead yielding blood levels between approximately 3 and 7 µg/dL (Sobin, Parisi, Schaub, & de la Riva, 2011). The recent events in Flint, Michigan are a reminder of the dangers that exist in major U.S. cities. After the city of Flint, Michigan switched their water supply from the Detroit-supplied Lake Huron water

to the Flint River water, lead began leaching into the water exposing many individuals, including children, to low levels of lead.

Among 1,473 children that were tested before the Flint, Michigan crisis, 2.4% of them had blood lead levels above the current "elevated" threshold (5μg/dL) (Hanna-Attisha, LaChance, Sadler, & Champney Schnepp, 2015). After the crisis, 4.9% of them had elevated blood lead levels. This is compared to a total sample of 2,202 children living outside of Flint in where no statistically significant change in blood lead level was observed during the same period. Following the events in Flint, Michigan, it has been estimated that as many as 1,500 cities across the U.S. are at risk of similar or worse environmental crises that would expose children to environmental lead through city water supplies (Safe Drinking Water Information System, 2016). The problem of early chronic low-level lead exposure in children persists and is a major unresolved child health disparity.

1.2 Current Sources of Lead Exposure in Children

There are many possible sources of child lead exposure particularly in lower-income neighborhoods. One major source continues to be lead-based paint used in dwellings, including apartment buildings and houses that were built before 1978 (when the lead paint laws were first enacted). It has been found that more than 80% of all homes that were built before 1978 had lead-based paint, with the older houses more likely to have a higher concentration of lead (National Center for Environmental Health, 2013). Houses that were built before 1950 pose an even greater risk for children, as they are even more likely to have lead-based paint than houses that were built after this period. Overall, it has been found that children who live in older housing are more likely to have an elevated blood lead level as opposed to children who live in newer housing, with 16.4% of poor children who live in older housing having elevated blood lead

levels (National Center for Environmental Health, 2013). Removal of deteriorated lead-paint, which also involves repainting over the deteriorated paint, is of great importance as children can be exposed to the dust created by this lead-based paint and can also ingest paint chips that are created by paint chipping off of the walls.

Although mandated in the 1970's, lead-based paint abatement efforts were never entirely successful. Because abatement requires methods that do not increase the likelihood of immediate or ongoing exposure, the EPA requires individuals and companies that perform abatement projects in dwellings built before 1978 to be certified and follow specific work practices to ensure the complete removal of these hazards. The financial burden however was left up to individual landlords and homeowners and there was little incentive other than moral imperative. Even today, if individuals determine that their living dwellings have lead-based paint, the EPA will recommend qualified professionals for removal but the cost is the responsibility of the landlord or home owner (US EPA, 2015). Particularly in lower income urban centers, many buildings remain unremediated for lead-based paint.

Another major source of child exposure is lead-contaminated soil. Lead-contaminated soil is of great concern as children are more likely to ingest, inhale, or come into contact with the contaminated soil than adults. For example, in particular New Orleans neighborhoods, the soil had been contaminated by vehicle leaded fuel and lead-based paint. A study was conducted examining the association between lead in 5,641 surface soil samples obtained from residential streets and 55,551 blood lead levels from children living in New Orleans. The mean blood lead level of the children from high traffic level streets was 7.18 μg/dL and 4.0 μg/dL from low traffic level streets. Overall, accumulation of lead in the soil was correlated with differences in blood

lead levels of children (Zahran et al., 2013). The blood lead level concentrations found in the children varied according to how far they lived from the contamination source.

Similar results were found in a study that examined the exposure risks associated with environmental lead produced by the Port Pirie lead smelter in Australia. A study was conducted examining the association between emissions from the lead smelter and blood lead levels in 13 infants living near the Port Pirie lead smelter (Simon, Maynard, & Thomas, 2006). The children, males and females, were followed from birth until they were 36 months old and blood lead levels were recorded throughout this period. All of the infants in the study were found to have a blood lead level higher than 10 μg/dL during at least one of the testing periods, with some of the infants having blood lead levels as high as 44 μg/dL as the study went on. Living in close proximity to the lead smelter was found to affect the blood lead levels of these infants.

Other possible sources include glazed ceramics that are used for cooking, eating, or drinking; home remedies; and inexpensive children's jewelry (Maas, Patch, Pandolfo, Druhan, & Gandy, 2005). For example, lead exposure due to imported candies and home remedies has been reported in Hispanic children in California (Courtney et al., 2002). Five children, ranging from ages 2-6 years, were found to have been in contact with either imported candies or home remedies that came from Mexico. The children were all found to have blood lead levels higher than 20 μg/dL after they were tested. The report stressed the importance of making health officials aware of the dangers posed by these imported candies and home remedies in regards to lead exposure and also discussed how other similar cases of lead exposure had been seen in other states. Of particular concern, may be chronic low level exposures in children that result from continuous contact with multiple low-level lead sources.

1.3 Studies of the Effects of Low Level Lead Exposure in Children

Concerns about the effects of low level lead exposure in children have stimulated a large body of child clinical research. Of particular concern, have been the effects of chronic low-level lead exposure on a child's cognitive and behavioral functions.

Low-level lead exposure in children has been known to result in cognitive deficiencies that extended to early adulthood, with IQ being one of the most common measures to be assessed (Childhood Lead Poisoning, 2010). For example, a study that was conducted among 3,176 Swedish children between 1978 and 2007 measured blood lead level, IQ and school performance. Each child was tested for blood lead levels between the ages of 7 and 12 and this was compared with school performance for each of the children at age 16. In addition, IQ was assessed at age 18 using the IQ test mandated for Swedish men and women available for military service. The average blood lead level for children was 3.4 μg/dL (reported as 34.0 μg/L). Lead exposure was significantly associated with worse performance in school and lower IQ (Skerfving, Löfmark, Lundh, Mikoczy, & Strömberg, 2015). At age 18, effects on cognitive functions persisted, with an average decrease of 0.51 IQ points for every increase of blood lead level from 0.5- 5.0 μg/dL. Low-level lead exposure was also associated with increased behavioral problems including attention problems and antisocial behavior.

Similar results were seen in a prospective observational cohort study conducted among 1,135 children from the United Kingdom (Chandramouli, Steer, Ellis, & Emond, 2009). This study aimed to observe the effects of low level lead exposure on behavioral and cognitive function. Each child was tested for blood lead levels at 30 months of age. These blood lead levels were compared with the Strengths and Difficulties Questionnaire (Goodman, 1997), Development and Well-being Assessment (Jones et al., 2008), Anti-social Behaviour Interview (Loeber, Stouthamer-Loeber, Kammen, & Farrington, 1989), and the SAT (QCA, 1999), at age 7

and 8. The Strength and Difficulties Questionaires, Anti-Social Behavior Interview, and Development and Well-being Assessment assessed behavior and the SAT assessed cognitive function. The majority of the children assessed (94%) had blood lead levels lower than 10 μg/dL. Blood lead levels ranging from 5-10 μg/dL were found to be associated with a decrease in SAT scores for the children in reading, writing, and spelling. These same blood lead levels were also found to be associated with hyperactivity and antisocial behavior.

Cognitive and behavioral deficits were also seen in a cross sectional study conducted among 299 Italian adolescents that were exposed to low levels of lead (Lucchini et al., 2012). Each of the female and male children in this study were between ages 11 and 14. Blood lead levels were compared with the Wechsler Intelligence Scale for Children, which assessed verbal and performance IQ, and the Conners-Wells' Adolescent Self-Report Scale-Long Form, which assessed behavioral problems. The average blood lead level of the children in this study was 1.71 μg/dL. A decrease in cognitive function was observed in all of the children. Lower blood lead levels were associated with the greatest decrease in IQ, with both performance and verbal IQ being affected by these low blood lead levels. These results mirrored those that had been seen in earlier studies where lower blood lead levels were found to result in a significant decrease in IQ. Low level lead exposure was also found to be associated with ADHD, although the association was not as strong as the one found for IQ.

Other reports have suggested that effects of early child lead exposure seemed to persist into adulthood. For example, a prospective cohort study that was conducted among a cohort of 249 infants in Boston compared the effects of low level lead exposure on cognitive function during adulthood (ages 28-30). Each subject was tested for blood lead levels at ages 4 and 10 years. These blood lead levels were compared with results from the Adult Wechsler Abbreviated

Scale of Intelligence, which assessed overall IQ, verbal IQ, and performance IQ at adulthood. The average blood lead level for the children in this study was 3.0 μg/dL when they were assessed at age 4 and 10 μg/dL at age 10. Children who had blood lead level concentrations lower than 10 μg/dL had the highest decrease in intellectual function and this was associated with a lower IQ during adulthood (Mazumdar et al., 2011).

Thus, multiple longitudinal studies have confirmed the association between low level lead exposure and cognitive and behavioral deficiencies in children that persist into adulthood. Although no brain imaging studies in children exposed to low levels of lead were conducted, the clinical neurocognitive studies of children have repeatedly suggested that low-level lead exposure alters brains function.

1.4 The Ill Effects of Lead Exposure in the Human Brain

Following on the results of the clinical neurocognitive studies of children, it is important to consider the effects of lead on brain function. While the ill effects of lead exposure on different organs in the human body have been widely studied, less research has been conducted examining the effects of lead exposure on the human brain and how it enters the brain.

One path by which lead enters the brain is via the blood-brain barrier (Struzyńska, Walski, Gadamski, Dabrowska-Bouta, & Rafałowska, 1997). Lead is able to cross the blood-brain barrier because the structure of lead mimics the structure of calcium and in some mechanistic processes will substitute for calcium ions. This has many implications for different molecular mechanisms that in the healthy brain and body depend on calcium. This is true for cation channels in the brain. These cation channels are voltage-insensitive calcium ions channels that store calcium ions and regulate a variety of biochemical processes by controlling intracellular calcium concentrations (Kerper & Hinkle, 1997b). These cation channels are

activated when intracellular calcium ion stores are depleted at which point uptake of extracellular calcium ion occurs. When lead is present however, it will compete with extracellular calcium and lead has been shown to bind to these receptors like calcium ions. Once in cation channels lead crosses the blood brain barrier and is taken up into the brain (Kerper & Hinkle, 1997). It is by this mechanism that lead enters the brain capillary endothelial cells.

When lead enters the brain, it can alter brain structure and function. For example, a study was conducted which examined three different populations of adults that were exposed to lead through workplace or environmental exposure (Stewart & Schwartz, 2007). The study included 1,109 former United States agricultural workers, 803 current and former inorganic lead workers in Korea, and 1,140 50- to 70-year-old Baltimore residents. The effects of lead on the brain were examined through the use of brain magnetic resonance imaging (MRI). The mean blood lead level for the agricultural workers was 4.6 μg/dL, 32 μg/dL for the Baltimore residents, and 3.5 μg/dL for the Korean subjects. The structural changes in the brain that were seen in the MRI suggested to the authors that lead could increase apoptosis, change cellular structure, increase oxidative stress, and activate an inflammatory response in the brain of the subjects. The authors of the study discussed the importance of conducting a second set of brain MRIs on the subjects along with use of other imaging technology to fully understand the structural and functional changes that occur due to lead exposure as there is potential that these changes could contribute to the onset of neurodegenerative conditions later on in life, such as ALS/Parkinsonian dementia, and others.

Lead exposure was also found to result in a loss of structural volume in the brain. This was demonstrated in a longitudinal study that studied the brains of adults who were exposed to lead during childhood (Cecil et al., 2008). The study was comprised of 157 adults between the

ages of 19 and 24. Brain MRIs were taken to assess any changes in the brain due to lead exposure. These participants had a mean blood level of 13.3 μg/dL when they were tested as children. Significant reduction in the gray matter volume of specific regions in the brain was found in the adults exposed to lead during childhood. The regions that were the most significantly affected by lead were the prefrontal cortex, including the ventrolateral prefrontal cortex and the anterior cingulate cortex. Loss of volume in these areas has been associated with a variety of negative cognitive and behavioral effects that have been seen in individuals exposed to lead, such as deficiencies in intellectual and executive functioning, antisocial behaviors, attention deficit hyperactivity disorders, and other cognitive and behavioral deficiencies (Bush, Luu, & Posner, 2000).

1.5 Animals Models of Low-Level Lead Exposure

Building on the results of the child studies and the findings regarding the effects of lead in the human brain it is important to discuss the findings of animal studies. Animal models are important as they enable researchers to carefully manipulate the levels of lead exposure and directly examine the effects in the brain. Animal models are also important for better understanding observational findings from child studies. Of particular interest are animal models that look at the effects of low level lead exposure on behavioral and cognitive function.

For example, an *in vivo* study examined the brains of rats exposed to lead to see its effects on the hippocampus (Schneider et al., 2005). Postnatal day 25 male Lewis rats were separated into two groups of lead-exposed and control animals. The exposed rats had mean blood lead levels of 20 μg/dL. The rat brains were examined through the use of immunohistochemistry. Proliferation of progenitor cells in the hippocampus was found to be inhibited due to chronic lead exposure. This is of particular concern as these progenitor cells can

eventually differentiate into dentate granule or glial cells, which are essential for the hippocampus. The dentate granule and glial cells are responsible for maintaining neurogenesis throughout adulthood (Parent et al., 1997). Due to this, the study concluded that inhibition of these progenitor cells as a result of lead exposure could result in changes in hippocampus structure and function. This inhibition is of particular concern because the hippocampus supports memory and learning and thus changes in hippocampal structure and function could directly impact these critical functions (Deng, Aimone, & Gage, 2010).

Another mouse study exposed C57BL/J6 mice to water containing 27 ppm of lead to examine its effects of behavioral function (Leasure et al., 2007). The mice were separated into three groups which included low dose (27 ppm), moderate dose (55 ppm), and high dose (109 ppm). The mice were exposed to lead prenatally through the dam and from birth up to postnatal day 10 through lead in the drinking water. Behavioral deficiencies in the mice were assessed through the use of the amphetamine-induced motor activity at 1 year of age. The blood lead level of the mice was less than 10 μg/dL. Behavioral deficiencies related to low level lead exposure were observed in the exposed mice. The exposed mice were found to be less active than the control mice in the exploratory activity and running wheel activity. The exposed mice were found to have a heightened sensitivity to amphetamine stimulation which is similar to what is seen in ADHD. This result seemed to indicate that low level lead exposure could be a factor in the onset of ADHD in children.

Another study that was conducted on mice exposed them to low levels of lead to gauge the effects on cognitive function (Bijoor, Sudha, & Venkatesh, 2012). The mice were separated into two groups which included control (0 ppm) and lead treated (50 ppm). The mice were exposed to lead prenatally through the dam and from birth up to postnatal day 45 through lead in

the drinking water. The mice were then assessed through the use of the step avoidance test and dark chamber test at postnatal day 45. The mean blood lead level of the mice was 10.65 μg/dL. An association between low level lead exposure and cognitive deficiencies was found in the exposed mice. The exposed mice had a harder time making their way through the dark chamber, despite going through it multiple times, as opposed to the control mice. Low level lead exposure was found to result in deficits in learning and memory in the mice as seen by the results of the dark chamber test. Low level lead exposure resulted in a significant decrease in neurobehavioral function as seen by how the exposed mice were not able to avoid the electric shock in the step avoidance test. This was suggested to be due to the exposed mice having impaired learning processes. The exposed mice were slower at remembering the negative effects of the test and kept repeating the same actions that would lead to the adverse effect.

Our lab has also conducted studies on mice to study the effects of low level lead exposure. Our lab was the first to report methods for obtaining low blood lead levels in mice that mimic those found in approximately fifty percent of in low-income children examined in our clinical studies (BLLs between approximately 3 and 6 μg/dL). One of these studies aimed to assess if low level lead exposure impaired olfactory recognition memory in lead exposed mice at pre-adolescence (Flores-Montoya, Alvarez, & Sobin, 2015). The mice were separated into three groups which included control (0 ppm), low-dose (30 ppm), and high-dose (330 ppm). The mice were exposed to lead prenatally through the dam and from birth up to postnatal day 28 through lead in the drinking water. Olfactory recognition memory in the mice was assessed through the use of the novel odor recognition task at postnatal day 28. The blood lead levels for the mice in this study ranged from 0.02 to 20.31 μg/dL. An increase in blood lead level was associated with

a decrease in time spent by the mice exploring the novel odor as opposed to the familiar odor. This suggested an impairment in spatial memory and object recognition of the exposed mice.

Another one of these studies also looked at the effects of low level lead exposure on exploratory behavior (Flores-Montoya & Sobin, 2015). The mice were separated into three groups which included control (0 ppm), low-dose (30 ppm), and high-dose (230 ppm). The mice were exposed to lead from birth up to postnatal day 28 through lead in the drinking water. The mice were assessed through the use of the nose-poke task, open field task, and rotarod task at postnatal day 28. The mice had blood lead levels ranging from 1.98 to 14.84 μg/dL. Chronic exposure to low levels of lead during development in the mice was associated with reduced exploratory activity in the mice, as shown by how the exposed mice performed on the three tasks. The low-dose mice performed worse than the control mice in the three tasks, showing the effects of lead on exploratory activity.

Of particular interest is the study that our lab conducted examining the differences in neuroimmune and brain structure in mice that have been chronically exposed to lead (Sobin et al., 2013). The mice were separated into three groups which included control (0 ppm), low-dose (30 ppm), and high-dose (330 ppm). The mice were exposed through lead in the dam's drinking water from birth up to postnatal day 28. Stereological methods were used to assess microglia volume and number and dentate gyrus volume after they were sacrificed at postnatal day 28. The exposed mice had mean blood lead levels of 4.12 μg/dL for the low exposure group, 10.31 μg/dL for the higher exposure group, and 0.03 μg/dL for the control group. Low level lead exposure was not found to result in an increased neuroinflammatory response as five out of the six neuroinflammatory markers showed no changes in response to lead exposure. Microglia volume however was found to be significantly larger in the mice exposed to low levels of lead as the

microglia volume exceeded that of the controls by 40.53 μm^3 and as visually quantified during stereological assessment.

One of the most interesting findings of the study was that microglia mean cell body number was significantly decreased in the low-level lead exposure measure as opposed to the control group, with 1,842 fewer cells. A reduction in the dentate gyrus volume was also noted in both the low level and higher exposure groups, although the differences were not significant between the two groups. The reduction in dentate gyrus volume seen in the low-level exposure group is concerning as the dentate gyrus is known to be associated with learning and memory, since it is part of the hippocampus, and thus a reduction in the dentate gyrus volume, especially during development, could have great consequences on learning and memory later on in life (Ming & Song, 2005). The increase in microglial volume and reduction in number of microglia in mice that were exposed to low levels of lead is of particular concern as microglia have been shown to have an important role in the development of the brain. The mechanisms by which microglia number are reduced in lead exposed animals are not yet understood. This study highlighted the importance of conducting further research on the effects of low level lead exposure on microglia.

1.6 Microglia and Lead Exposure

As discussed earlier, research has suggested how lead enters the brain but very little else is known regarding the effects of lead on the brain and brain function. Of the many types of cells in the brain, microglia have critical roles in brain development and brain protection. Despite research that has been conducted on the effects of lead on humans, very little is known regarding the effects of lead on microglia. With regard to understanding mechanisms of neurotoxicity from

early chronic low level exposure, it may be important to discuss microglial function and how alteration of these cells might impact the brain.

Microglia are primarily found in the grey matter of the brain, with the highest concentration being found in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Lawson, Perry, Dri, & Gordon, 1990). Microglia have been primarily known for the immune function they serve in the central nervous system, which involves immune surveillance and phagocytosis, but recently researchers have discovered that they serve many other roles in the brain during neurogenesis, synaptogenesis, and myelination, all of which contribute to the maintenance of proper brain homeostasis (Pierre et al., 2016). These will be discussed in more detail below.

Microglia have been shown to be a factor in the development of neuronal circuitry during development and the maintenance of neuronal cells. For example, one study conducted on rat, human, and monkey brains examined microglia in the brain to observe their role in the development of neuronal circuitry (Cunningham, Martínez-Cerdeño, & Noctor, 2013). Brain tissue for the monkeys was obtained from the fetus of macaque monkeys of both genders, prenatal human brain tissue was obtained from a donation, and embryonic and postnatal rats were used to obtain brain tissue from rats. The brains were immunostained so that the microglia could be examined with a confocal microscope. Microglia are crucial for controlling neurogenesis in the developing brain by maintaining the proper balance of neuronal cells. Microglia accomplish this by actually reducing the number of neuronal cells through phagocytosis to make sure that the appropriate number of neuronal cells are maintained in developing brain circuits. These studies were among the first to show that stimulating microglial

activation resulted in a reduced neuronal cell number while decreasing the number of microglia in the brain was associated with an increase in the number of neuronal cells in the brain.

Another study conducted on mouse brains found similar results with regard to the role of microglia in the brain (Squarzoni et al., 2014). Mouse embryonic brains of both genders were used. The brain tissue was immunostained so that the microglia could be examined under electron microscopy. Disrupting microglial activity resulted in an uncontrolled outgrowth of dopaminergic axons, along with an irregular distribution of neocortical interneurons. Microglia were shown to play a role in the outgrowth of dopaminergic axons and the positioning of neocortical interneurons, both of which are essential for the normal development of neuronal circuitry in the brain (Squarzoni, Thion, & Garel, 2015).

Microglia have also been shown to play a role in synaptogenesis by mediating changes in synaptic circuitry and function through the process of synaptic pruning. Synaptic pruning contributes to the maintenance of synaptic circuitry and function by eliminating synapses that contain errors and leaving only those that are functional (Chechik, Meilijson, & Ruppin, 1999). A study conducted on Cx3cr1 knockout mice and control mice aimed to examine the role of microglia in synaptogenesis. The brain of the mice were assessed at postnatal day 13 to 16 by immunohistochemistry. A reduction of microglia in developing mouse brains was associated with a reduction in the process of synaptic pruning (Paolicelli et al., 2011). This alteration in the process of synaptic pruning due to reduction of microglia was found to have a great impact in the affected mouse brain. The affected mouse brains were shown to have an excess of dendritic spines and undeveloped synapse which could potentially result in a neurodegenerative disorder.

Microglia also play a role in the myelination of the central nervous system by aiding the development of oligodendrocytes which are responsible for myelination of the central nervous

system (Bradl & Lassmann, 2010). A study conducted on developing mouse brains aimed to examine the role of microglia in regards to myelination. Prenatal C57BL/6 mouse brains were studied using immunohistochemistry. Microglia were found to enhance oligodendrogenesis through the release of cytokines, specifically IL-1β and IL-6 in mouse brains (Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014). Inhibition of IL-1β and IL-6 in the mouse brain was found to be associated with a decrease in the rate of oligodendrogenesis as compared to brains in where these cytokines were not inhibited. IL-1β has been shown to enhance the rate of gliogenesis, which is important to the development of oligodendrocytes (Wang et al., 2007). IL-1β is a proinflammatory cytokine that is vital for an organism's defense responses to challenges such as infection or injury (Dinarello, 1996). It is also a key factor in a host's response to pathogens. IL-1β has also been shown to worsen damage during chronic disease and tissue injury when it becomes neurotoxic. IL-1β is produced and released by two different molecules, which are pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) (Eder, 2009). IL-6 has been shown to play a role in the proliferation and differentiation of oligodendrocyte progenitor cells (Wang et al., 2007).

Given the many functions of microglia, it is also important to discuss the mechanisms by which microglia become activated. Microglial activation in the brain is dependent upon two different factors, which are adenosine triphosphate (ATP) and the P2Y receptor (Nimmerjahn, Kirchhoff, & Helmchen, 2005). A study conducted on mouse brains examined the mechanisms by which microglia become activated (Davalos et al., 2005). Adult transgenic Cx3cr1 mouse brains were used. The brain tissue was immunostained so that the microglia could be examined through *in vivo* two-photon microscopy. ATP was found to contribute to the activation of

microglia through the release of non-hydrolyzable ATP, which resulted in microglia migrating to the source of the ATP release and responding rapidly to it. When apyrase, which catalyzes the hydrolysis of ATP, was applied to mouse brain tissue there was no microglial response that could be observed (as compared with mouse brain in which no apyrase had been applied). This suggested that microglia activation was influenced by the release of non-hydrolyzable ATP since no microglial response could be seen when hydrolysis of ATP occurred. In addition to ATP release, P2Y receptors have also been suggested to play a role in the microglial response. The injection of two P2Y inhibitors in the mouse brain was found to result in a significant reduction in the number and motility of microglial processes in response to an injury. This suggested that P2Y receptors are critical for the activation of microglia in response to an injury. The authors of this study also suggested the release of ATP is thought to be involved in the activation of P2Y receptors, showing the interplay between these two factors and the importance of these two factors to ensure a proper microglial response.

Despite the many critical functions of microglia and their role in healthy brain function, microglia can also become neurotoxic in the brain in response to certain conditions. For example, microglia have been shown to become neurotoxic as a result of a chronic brain inflammatory response. Microglia are known to be a source of a variety of pro-inflammatory factors, such as tumor necrosis factor α (TNF- α), prostaglandin E_2, interferon-γ, and oxidative stress, all of which can potentially cause damage to neurons (Block & Hong, 2005). Proper microglia function is essential for normal brain function, but uncontrolled and over activated microglia have been shown to result in severe neurotoxic consequences if left unchecked. One study conducted in rats infused with low doses of an inflammagen, lipopolysaccharide (LPS), aimed to gauge the effects of LPS of on microglial activity (Gao et al., 2002). Adult male Fischer 344 rats

were used. The rat brains were infused with LPS for two weeks. Brain tissues were then prepared using immunohistochemical methods to allow visualization of microglia under a confocal microscope. LPS was found to trigger a neuroinflammatory response in the microglia which resulted in the overall destruction of nigral dopaminergic neurons. Immunofluorescent analysis of the mouse brain was able to confirm this result and it was also compared with the controls, where no destruction was seen. This destruction of the nigral dopaminergic neurons was preceded by the release of TNF- α, nitric oxide (NO), and superoxides, all of which were released by microglia, showing the neurotoxic capacities of these factors. Destruction of these dopaminergic neurons from chronically activated microglia has been shown to result in a number of different neurodegenerative conditions, including Parkinson's disease (Qian & Flood, 2008).

Another study also examined microglial neurotoxicity. An *in vitro* study was conducted in rats and mice exposed to diesel exhaust particles (DEP) (Block et al., 2004). This study was conducted to gauge the effects of DEP on microglia and how this resulted in microglial neurotoxicity. Adult female Fischer 344 pregnant rats and eight-week-old B6.129S6-Cybb^{tm1Din} mice were used. The rat and mouse brains were immunostained to examine the microglia under a microscope. DEP was found to result in microglial neurotoxicity in the rats that were exposed. The rats and mice that were injected with at least 5 μg/ml of DEP were shown to have destruction of dopaminergic neurons, when compared to control rats and mice. This destruction was found to occur due to oxidative insult that was produced by microglia activated by the hazard (DEP). This activation of microglia resulted in these cells producing free radicals, such as ROS, which have been known to be toxic to neuronal cells, such as dopaminergic cells.

Microglia are vital components of the brain and have been shown to play a variety of roles in the overall function of the brain. To build on past studies from our laboratory which

found reduced number of microglia in the dentate gyrus region of the hippocampus in lead exposed mice, a follow-up study was conducted to examine the numbers and distribution of microglia throughout the hippocampus, and to compare the distribution of microglia in hippocampal regions with and without dentate gyrus.

1.7 Goals of the Study

The goals of this study are to 1) examine whether early chronic low level lead exposure is associated with the differences in the number and distribution of microglia throughout the hippocampus of pre-adolescent mice; 2) determine whether hippocampal microglia cluster differently in lead exposed, as compared to control, mice.

1.8 Hypothesis

H1: As compared to control animals with no history of lead exposure, hippocampal microglia are fewer in animals chronically exposed to lead from birth to pre-adolescence (PND 28).

H2: As compared to control animals with no history of lead exposure, microglia distribution in hippocampal regions with and without dentate gyrus differs in animals chronically exposed to lead from birth to pre-adolescence (PND 28).

H3: As compared to control animals with no history of lead exposure, hippocampal microglia cluster around neurons in animals chronically exposed to lead from birth to pre-adolescence (PND 28)

Materials and Methods

This study will follow the same methods used in our laboratory's original study examining microglia in dentate gyrus of lead exposed, as compared to control, mice. Male and female C57BL/6J mice were exposed to one of three possible lead exposure groups which included, control (0 ppm), low-dose (30 ppm), and high-dose (330 ppm). The mice were exposed to lead through water tainted with lead acetate crystals in the dams' drinking water. The mice were exposed over a 28-day period and this study was carried in accordance to Institutional Animal Care and Use Committee (IACUC) standards. After this 28-day period, mice were transcardially perfused, and the brain tissue harvested. Brains were sectioned at 40 μm slices in the coronal plane through the entire brain, which included the brain stem and cerebellum. These slices were then prepared for IBA-1 immunostaining and incubated in PBS containing normal donkey serum, Triton-X and biotin-SP-AffiniPure donkey anti-rabbit IgG for one hour, and then in phosphate-buffered saline (PBS) containing avidin-biotinylated horseradish peroxidase complex (Vectastin elite ABC kit, Vector Lab) for another hour. After all of this, the sections were then mounted on gelatin-covered slides and then counterstained with a FD NeuroTechnologies cresyl violet solution.

The region of interest that was used for this study was the whole hippocampus as it aimed to build upon the findings of our first microglia study which looked only at the dentate gyrus region of the hippocampus. The total number of mice that were used for this study was based on the number successfully used in our previous study and included 30 total with 10 mice from each of the three different exposure groups. The beginning and the end of the mouse hippocampus was obtained using the Allen Mouse Brain atlas. Sectioning of the brain was done at 40 μm, with approximately 25 sections per hippocampus. The sampling interval was two, yielding

approximately twelve to thirteen sampled sections per mouse. The Stereologer software system (Stereologer Resource Center, Gainesville, FL) was installed on a Dell Optiplex tower computer and connected to a Nikon Eclipse E600 microscope fitted with an X–Y–Z motorized stage controller, linear encoder microcator (z-axis gauge), high resolution color video camera and .50 C-mount.

The data collection process involved the collection of microglia cell number, spatial distribution, and hippocampus volume. Microglia cell number and spatial distribution were recorded using the 100x/1.40 oil lens, and hippocampus volume was recorded using the 10x/0.25 lens. The main experimenter (SD) was responsible for the data collection.

2.1 Statistical Analyses

Descriptive and inferential statistics were analyzed with SAS Version 9.3. (SAS Institute Inc., Cary, North Carolina) and SPSS Version 23 (IBM, Armonk, New York). The Stereologer software generates Excel spreadsheet data for each subject. These data were transformed into summary statistics for group comparisons. Prior to transformation, all raw data were graphed and checked for accuracy and completeness. Transformed data were then entered into spreadsheet format.

Generalized linear regression (SAS PROC GLIMMIX) was used for all models. All regression models included two fixed effects (exposure group and sex) and one random effect (litter). Prior to the main analyses, generalized linear regression was used to test for differences in hippocampal volume across exposure groups with litter included as random effect. For the first hypothesis, generalized linear regression controlling for sex with litter included as a random effect tested whether lead exposure group predicted the density (mean cell body number) of microglia in hippocampus. For the second hypothesis, generalized linear regression controlling

for sex with litter included as a random tested whether the density of microglia across levels of the hippocampus (with and without dentate gyrus) differed by lead exposure group. For the third hypothesis, generalized linear regression was used to compare spatial distribution (mean volume density) of microglia around neurons in control as compared with lead exposed mice (whether more microglia clustered around neurons in lead exposed animals). For all models, F and p values associated with Type III sum of squares were evaluated first. When significant main and/or interaction effects were observed, tests of parameter estimate differences were checked for significance; and last, Tukey-Kramer corrected post-hoc tests of least square mean differences were evaluated. Statistical significance was determined using $p < .05$.

Results

Thirty C57BL/6J mice were used for this study. Mice were exposed from birth to PND 28 to one of three lead exposure groups via the dams' drinking water: 30 ppm *n*= 10 (6 males), 330 ppm *n*= 10 (4 males), 0 ppm *n*= 10 (8 males). Brains were harvested at PND 28 and brain sections were prepared using immunohistochemical methods that allowed visualization of microglia via light microscope.

Table 1 shows the means and standard deviations for blood lead level at sacrifice, weight at sacrifice and thickness of prepared sections by exposure group. The mean blood lead levels show that differences in blood lead levels were achieved. Among the control animals, (0 ppm) mean blood lead level was 0.03 µg/dL±0.01 for males and 0.02 µg/dL ±0.00 for females; among the low-dose animals (30 ppm) mean blood lead level was 3.68 µg/dL±0.67 for males and 3.03 µg/dL±0.65 for females; among the high-dose animals (330 ppm) mean blood lead level was 15.42 µg/dL ±3.88 for males and 12.79 µg/dL ±1.54. Importantly, the thickness of individual hippocampal sections sampled for these studies was very similar across groups. Among controls mean section thickness was 20.45±0.64 for males and 20.05±0.71 for females; among the low-dose group mean section thickness was 20.23 ±1.14 for males and 20.28±1.60 for females; among high-dose animals mean section thickness was 20.26±0.79 for males and 21.02±0.19 for females. The area sampling fraction (ASF) and section sampling fraction (SSF) are values used to estimate the mean density of microglia throughout the entire hippocampal structure. ASF (0.073) refers to the total area sampled divided by the total area on all sampled sections; SSF (0.50) refers to the number of sections sampled divided by the total number of sections throughout the reference space; both ASF and SSF were the same for all animals.

Tables 2 and 3 show the means and standard deviations of the outcome variables tested. As the table shows, mean hippocampal volume, measured as micrometers $(\mu)^3$, was lower in the low-dose males and females (males, 1501176697.67±94290904.59; females, 1775998187.83±159011849.87) and high-dose males and females (males, 1764272042.54± 99508009.00; females, 1775998187.83±159011849.87) when compared to the control males and females (males, 1764272042.54± 99508009.00; females, 1775998187.83±159011849.87). These results indicated that low-dose and high-dose exposure resulted in a decrease in hippocampal volume.

Table 2 also shows that microglia mean cell body number in the entire hippocampus was noticeably lower in the low-dose males and females (males, 15527.26±4016.98; females, 15692.89±767.17) and high-dose males and females (males, 17690.00±4761.19; females, 15008.98±5179.71) as compared to control males and females (males, 24034.02±6436.75; females, 19827.74±4505.13). Microglia mean cell body number in hippocampal sections with and without dentate gyrus also appeared to be similar across groups. Thus, low-dose and high-dose exposures also resulted in decreased hippocampal microglia mean cell body number. Moreover, similar reduction of microglia mean cell body number was seen in both the low dose and high dose mice in hippocampus sections with and without dentate gyrus.

With regard to microglia clustering around neurons, microglia volume density in the entire hippocampus was similar in the three exposure groups. This was also true when hippocampal sections with and without dentate gyrus were individually examined. These results suggested that low-dose and high-dose exposure did not appear to alter microglia clustering around neurons.

Generalized linear regression models were used to test whether the observed differences were statistically significant. All models were first tested including litter as a random effect and the significance of litter was evaluated using tests of parameter solutions for the variable litter. In 4 of 7 models, the amount of variance contributed by litter was not detectable (as indicated by the SAS results statement "G matrix not positive definite"). In the remaining models, the parameter solutions were not statistically significant. For the final analyses, the models were re-calculated excluding the random effect.

Hypothesis 1 predicted that lead exposure significantly altered microglia mean cell body number in hippocampus. Table 4 summarizes the Type III fixed effects solutions and parameter estimates for the main effects of group and sex and the interaction. Type III fixed effects for group, sex and the interaction were statistically significant (group, $F_{2/24} = 44.51$ p = < 0.001; sex, $F_{1/24} = 17.14$ p = < 0.001; group x sex, $F_{2/24} = 5.77$ p= 0.009). The fixed effects parameter estimates for high and low dose animals differed from controls (high dose, t = -4.32, p = < 0.001; low dose, t = -3.52, p = 0.002). Males and females differed significantly (males, t = 3.70, p = 0.001; females, t = -3.70, p = 0.001).

With regard to the fixed parameters estimate differences for the interaction, low dose but not high dose males differed from male controls; and low dose but not high dose females differed from female controls (low dose males/females, t=-3.14, p = 0.005).

Tests of least square mean differences for mean microglia cell body number for groups, sex and group x sex were also significant (males vs. females, diff = 22.41, t= -4.14, p= <.001, 95% C.L. -33.57 – -11.24) (low-dose vs. controls, diff = -63.21, t= -9.07, p = <.001, 95% C.L. -80.61 – -45.80; high-dose vs. controls, diff = -55.81, t= -7.92, p = <.001, 95% C.L. -73.40 – -38.23) (high-dose males vs. control males, diff = -63.44, t= -7.36, p = <.001, 95% C.L. -108.19 –

-61.95; low-dose males vs. control males, diff = -85.07, t= -11.38, p = 0.003, 95% C.L. -90.09 – -36.79; low-dose females vs. control females, diff = -41.35, t= -3.52, p = 0.019, 95% C.L. -77.72 – -4.98; high-dose females vs. control females, diff = -48.19, t= -4.32, p = <.001, 95% C.L. -82.64 – -13.74). Thus, microglia mean cell body number was significantly altered by low and high level lead exposure and the effects were approximately the same regardless of level of lead exposure. In other words, the microglia mean cell body number in low-dose and high-dose mice was similarly diminished. Overall, females had less microglia mean cell body number than males.

In addition to testing microglia mean cell body number throughout the entire hippocampus we were also interested in examining whether microglia mean cell body number differed among groups more specifically in hippocampus sections that included dentate gyrus and hippocampus sections that did not include dentate gyrus. Tables 5 and 6 summarize the Type III fixed effects solutions and parameter estimates for the main effects of group and sex and the interaction. Results for these different regions of hippocampus were similar to the results obtained for whole hippocampus. Type III fixed effects for group, sex, and group x sex in dentate gyrus regions were statistically significant (group, $F_{2/24}$ = 20.64 p = < 0.001; sex, $F_{1/24}$ = 7.36 p = 0.01; group x sex, $F_{2/24}$ = 3.80 p= 0.04). The fixed effects parameter estimate differences for high and low dose animals in the regions of hippocampus including dentate gyrus differed from controls (high dose, t = -2.15, p = 0.042; low dose, t = -2.52, p = 0.019), and lead exposure groups did not differ. Also, males and females differed significantly as well (males, t = 2.14, p = 0.043; females, t = -2.14, p = 0.043)

With regard to the parameter estimate differences for the interaction in hippocampal dentate gyrus region showed that, low-dose but not high-dose, males differed from male

controls; and low dose but not high dose females differed from female controls (low-dose males/females, t= 2.08, p = 0.048).

Tests of least square mean differences between groups, sex and group x sex were also significant for the hippocampal region that included dentate gyrus (males vs. females, diff = 9.87, t= 2.71, p= 0.012, 95% C.L 2.36 – 13.38) (low-dose vs. controls, diff = -29.25, t= -6.34, p = <.001, 95% C.L. -40.77 – -17.34; high-dose vs. controls, diff = -15.94, t= -3.34, p = 0.007, 95% C.L. -27.86 – -4.02) (low-dose males vs. control males, diff = -38.84, t= -7.94, p = <.001, 95% C.L. -53.96 – 23.72). Microglia mean cell body number was significantly altered by low and high level lead exposure in sections that included dentate gyrus and the effects were shown to be stronger in the low-dose group. The microglia mean cell body number in low-dose, as compared to high-dose, mice was greatly diminished.

Type III fixed effects for group and sex in the sections that did not include dentate gyrus also were statistically significant (group, $F_{2/24}$ = 27.30 p = <.001; sex, $F_{1/24}$ = 10.00 p = 0.004). In this model, the interaction was not statistically significant. The fixed effects parameter estimate differences for high and low dose animals in the sections with no dentate gyrus differed from controls (high dose, t = -3.57, p = 0.002; low dose, t = -2.15, p = 0.042). Males and females differed significantly as well for this group (males/females, t = 3.11, p = 0.005). Tests of least square mean differences between groups and sex were also significant for this region of hippocampus (males vs. females, diff = 3.98, t= 3.16, p= 0.004, 95% C.L 4.37 – 20.80) (low-dose vs. controls, diff = -31.07 t= -5.99, p = <.001, 95% C.L. -44.01 – -18.13; high-dose vs. controls, diff = -36.99, t= -7.20, p = <.001, 95% C.L. -49.81 – -24.17). Thus, microglia mean cell body number was significantly altered by low and high-dose lead exposure in sections that did not include dentate gyrus and the effects were shown to be stronger in the low-dose group. The

microglia mean cell body number in low-dose as compared to high-dose mice was greatly diminished.

Hypothesis 2 predicted that in lead exposed as compared to control animals, microglia mean cell body number was greater in hippocampal portions without as compared to with dentate gyrus. Thus, this hypothesis predicted a significant interaction between group and microglia mean cell body number in hippocampal regions without vs. with dentate gyrus. Because a significant interaction between Group and Sex was found in the previous models examining microglia mean cell body number (see Hypothesis 1 results above), the model for hypothesis 2 was first tested using three main effects (sex, groups, and DG/noDG), two-way interaction Group x Sex, and the three-way interaction Group x Sex x DG/NoDG. In this first model, the three-way interaction was not found to be significant so the model was re-calculated without the three-way interaction and including two two-way interactions (Group x Sex and Group x DG/NoDG). Table 7 summarizes the Type III fixed effects solutions and parameter estimates for the main effects of groups, sex, hippocampal region (DG/noDG), and the interactions Group x Sex and Group x DG/noDG. Type III fixed effects for group, sex, DG/NoDG, and the interactions were statistically significant (group, $F_{2/51} = 41.42$ p = < 0.001; sex, $F_{1/51} = 16.66$ p = < 0.001; DG/no DG, $F_{1/51} = 53.04$ p = < 0.001; group x sex, $F_{2/51} = 5.91$ p= < 0.001; group x DG or no DG, $F_{2/51} = 8.51$ p= < 0.001).

The fixed effects parameter estimate difference for high and low dose animals differed from controls (high dose, t = -5.33, p = < 0.001; low dose, t = -3.15, p = 0.003). Males and females differed significantly (males/females, t = 3.66, p = 0.001). Microglia mean cell body number distribution in sections with or without dentate gyrus differed significantly (DG, t = -5.62, p = <.001; No DG, t = 5.62, p = <.001).

With regard to the parameters estimates for the interactions, low dose but not high dose males differed from male controls; and low dose but not high dose females differed from female controls (low dose males/females, t= 3.14, p = 0.003). Microglia mean cell body number distribution in sections with dentate gyrus of high-dose mice but not low-dose mice differed from controls (high-dose DG, t= 3.67, p = <.001).

Tests of least square mean differences for microglia mean cell body number between groups, sex, DG/no DG, group x sex, and group x DG/no DG were also significant (low-dose vs. controls, diff = -30.32 t= -8.75, p = <.001, 95% C.L. -38.69 – -21.96; high-dose vs. controls, diff = -26.65 t= -7.61, p = <.001, 95% C.L. -35.10 – -18.20) (Males vs. females, diff = 10.93, t= 4.08, p= <.001, 95% C.L. 5.55 – 16.31) (DG vs. No DG, diff = -17.90, t= -7.28, p = <.001, 95% C.L. -22.83 – -12.97). Microglia mean cell body number distribution in sections with dentate gyrus was significantly altered by high-dose lead exposure and there was no significant effect in low-dose animals. High-dose animals were shown to have a greater number of microglia in sections with dentate gyrus as compared to sections without dentate gyrus.

Hypothesis 3 predicted that clustering of hippocampal microglia around neurons would be greater in exposed as compared to control animals. Table 8 summarizes the Type III fixed effects solutions and parameter estimates for the main effects of group and sex and the interaction. Type III fixed effects for group, sex and the interaction were not statistically significant (group, $F_{2/24}$ = 0.26 p = 0.613; sex, $F_{1/24}$ = 0.56 p = 0.577; group x sex, $F_{2/24}$ = 0.96 p= 0.396). Microglia were not found to be clustering around damaged neurons in both the low-dose and high-dose mice

While not a goal of the study, we also tested whether hippocampal volume was significantly altered by lead exposure. Table 9 summarizes the Type III fixed effects solutions

and parameter estimates for the main effects of group and sex and the interaction. The Type III fixed effect for group was significant (group, $F_{2/24}$ = 15.22 p = <.001), while the fixed effects of sex and the interaction were not statistically significant. The fixed effects parameter estimate differences for high and low dose animals differed from controls (high dose, t = -2.32, p = 0.029; low dose, t = -5.60, p = <.001). Tests of least square mean tests differences between groups were also significant (low-dose vs. controls, diff = -2447.93, t= -5.52, p = <.001, 95% C.L. -3556.34 – -1339.51; high-dose vs. controls, diff = -1508.68, t= -3.40, p = 0.006, 95% C.L. -2617.09 – -400.26) Hippocampal volume was significantly altered by both low-dose and high-dose lead exposure. Importantly, reduction of hippocampal volume in both low-dose and high-dose mice did not differ, which suggested that the developmental effects on hippocampal volume were approximately equivalent in low- and high-dose lead exposure.

Table 1. Descriptive Statistics (mean ± SD) for Male and Female Mice by Exposure Group

Group	**0 ppm**		**30 ppm**		**330 ppm**	
	Males	Females	Males	Females	Males	Females
Blood Lead Level (µg/dL)	0.03±0.01	0.02±0.00	3.68±0.67	3.03±0.65	15.42±3.88	12.79±1.54
Sacrifice Weight (g)	15.02±1.05	14.33±0.15	13.63±0.70	11.88±1.05	13.75±1.66	12.68±1.95
Section Thickness(μm^3)	20.23±1.14	20.28±1.60	20.26±0.79	21.02±0.19	20.45±0.64	20.05±0.71

Table 2. Descriptive Statistics (mean ± SD) of Spatial Distribution (Microglia Number Volume Density) for Male and Female Mice by Exposure Group

Group	Hippocampal Volume		Microglia Number (Entire Hippocampus)		Microglia Number (Dentate Gyrus)		Microglia Number (No Dentate Gyrus)	
	Males	Females	Males	Females	Males	Females	Males	Females
0 ppm	$1.76x10^9 \pm 9.95x10^7$	$1.77x10^9 \pm 1.59x10^7$	$2.40x10^4 \pm 6.43x10^3$	$1.98x10^4 \pm 4.50x10^3$	$1.04x10^4 \pm 2.02x10^3$	$8.80x10^3 \pm 2.89x10^3$	$1.33x10^4 \pm 4.95x10^3$	$1.07x10^4 \pm 1.19x10^3$
30 ppm	$1.50x10^9 \pm 9.42x10^7$	$1.54x10^9 \pm 6.12x10^7$	$1.55x10^4 \pm 4.01x10^3$	$1.56x10^4 \pm 767$	$6.53x10^3 \pm 2.09x10^3$	$6.83x10^3 \pm 698$	$8.99x10^3 \pm 2.28x10^3$	$8.85x10^3 \pm 675$
330 ppm	$1.64x10^9 \pm 4.23x10^7$	$1.59x10^9 \pm 7.19x10^7$	$1.76x10^4 \pm 4.76x10^3$	$1.50x10^4 \pm 5.17x10^3$	$8.83x10^3 \pm 3.53x10^3$	$7.19x10^3 \pm 3.34x10^3$	$8.85x10^3 \pm 1.31x10^3$	$7.81x10^3 \pm 2.40x10^3$

Table 3. Descriptive Statistics (mean ± SD) for Outcome Variables of Male and Female Mice by Exposure Group

Group	**0 ppm**		**30 ppm**		**330 ppm**	
	Males	Females	Males	Females	Male	Females
MNVD	$1.28x10^{9}±7.55x10^{7}$	$1.28x10^{9}±1.14x10^{8}$	$1.33x10^{9}±7.53x10^{7}$	$1.34x10^{9}±1.20x10^{8}$	$1.40x10^{9}±7.91x10^{7}$	$1.41x10^{9}±1.26x10^{8}$
MNVD (DG)	$1.15x10^{4}±1.26x10^{3}$	$1.23x10^{4}±2.11x10^{3}$	$1.01x10^{4}±2.85x10^{3}$	$1.11x10^{4}±2.44x10^{3}$	$1.25x10^{4}±1.58x10^{3}$	$1.32x10^{4}±2.32x10^{3}$
MNVD (No DG)	$1.18x10^{4}±2.01x10^{3}$	$1.00x10^{4}±2.48x10^{3}$	11826.70±1464.29	$9.00x10^{3}±2.30x10^{3}$	$1.19x10^{4}±2.79x10^{3}$	$1.09x10^{4}±3.03x10^{3}$

MNVD = microglia number volume density; DG= dentate gyrus

Table 4. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Hippocampal Microglia Cell Body Number

Type III fixed effect				***Solution for fixed effects***					
	F	*p*			Est	SE	DF	t value	*p*
Group	44.51	<.0001		*Intercept*	198.28	9.9568	24	19.91	<.0001
Sex	17.14	0.0004		330 ppm	48.1876	11.1424	24	-4.32	0.0002
Group x Sex	5.77	0.0090		30 ppm	-41.3486	11.7631	24	-3.52	0.0018
				0 ppm	0.00	-	-	-	-
				Males	42.0628	11.3658	24	3.70	0.0011
				Females	0.00	-	-	-	-
				330 ppm x Males	-15.2526	14.0862	24	-1.08	0.2897
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-43.7190	13.9389	24	-3.14	0.0045
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				Females	-42.0628	11.3658	24	-3.70	0.0011
				Males	0.00	-	-	-	-
				330 ppm x Females	15.2526	14.0862	24	1.08	0.2897
				30 ppm x Females	43.7190	13.9389	24	3.14	0.0045
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Table 5. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Hippocampal Microglia Number in Hippocampal Dentate Gyrus Sections

Type III fixed effect				***Solution for fixed effects***					
Hippocampal Microglia Number (Dentate Gyrus included)									
	F	*p*			Est	SE	DF	t value	*p*
Group	20.64	<.0001		*Intercept*	88.0236	6.6341	24	13.27	<.0001
Sex	7.36	0.0121		330 ppm	-16.0817	7.4835	24	-2.15	0.0419
Group x Sex	3.80	0.0368		30 ppm	-19.6608	7.8168	24	-2.52	0.0190
				0 ppm	0.00	-	-	-	-
				Males	16.1681	7.5522	24	2.14	0.0427
				Females	0.00	-	-	-	-
				330 ppm x Males	0.2863	9.5460	24	0.03	0.9763
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-19.1821	9.2205	24	-2.08	0.0483
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				Females	-16.1681	7.5522	24	-2.14	0.0427
				Males	0.00	-	-	-	-
				330 ppm x Females	-0.2863	9.5460	24	-0.03	0.9763
				30 ppm x Females	19.1821	9.2205	24	2.08	0.0483
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Table 6. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Hippocampal Microglia Distribution in Hippocampal Sections That Do Not Include Dentate Gyrus

Hippocampal Microglia Number (No Dentate Gyrus)									
	F	*p*			Est	SE	DF	t value	*p*
Group	27.30	<.0001		*Intercept*	107.29	7.3242	24	14.65	<.0001
Sex	10.00	0.0042		330 ppm	-29.1392	8.1651	24	-3.57	0.0016
Group x Sex	2.84	0.0782		30 ppm	-18.7210	8.7055	24	-2.15	0.0418
				0 ppm	0.00	-	-	-	-
				Males	26.0536	8.3852	24	3.11	0.0048
				Females	0.00	-	-	-	-
				330 ppm x Males	-15.6978	10.2695	24	-1.53	0.1394
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-24.6958	10.3653	24	-2.38	0.0255
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				Females	-26.0536	8.3852	24	-3.11	0.0048
				Males	0.00	-	-	-	-
				330 ppm x Females	15.6978	10.2695	24	1.53	0.1394
				30 ppm x Females	24.6958	10.3653	24	2.38	0.0255
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Table 7. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Microglia Clustering in Sections With or Without Dentate Gyrus

Type III fixed effect				***Solution for fixed effects***					
	F	*p*			Est	SE	DF	t value	*p*
Group	41.42	<.0001		*Intercept*	111.60	5.6409	51	19.78	<.0001
Sex	16.66	0.0002		330 ppm	-34.5848	6.4931	51	-5.33	<.0001
DG or no DG	53.04	<.0001		30 ppm	-21.5254	6.8324	51	-3.15	0.0027
Group x Sex	5.91	0.0049		0 ppm	0.00	-	-	-	-
Group x DG or no DG	8.51	0.0006		Males	20.4619	5.5959	51	3.66	0.0006
				Females	0.00	-	-	-	-
				DG	-26.8554	4.7745	51	-5.62	<.0001
				No DG	0.00	-	-	-	-
				330 ppm x Males	-6.9833	6.9727	51	-1.00	0.3213
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-21.6092	6.8737	51	-3.14	0.0028
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x DG	22.8515	6.2271	51	3.67	0.0006
				330 ppm x No DG	0.00	-	-	-	-
				30 ppm x DG	4.0136	6.1961	51	0.65	0.5200
				30 ppm x No DG	0.00	-	-	-	-
				0 ppm x DG	0.00	-	-	-	-
				0 ppm x No DG	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Table 8. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Hippocampal Microglia Clustering

Type III fixed effect				***Solution for fixed effects***					
	F	*p*			Est	SE	DF	t value	*p*
Group	0.26	0.6132		*Intercept*	11433	1418.29	24	8.06	<.0001
Sex	0.56	0.5771		330 ppm	-1392.36	1637.70	24	-0.85	0.4036
Group x Sex	0.96	0.3963		30 ppm	830.40	1737.05	24	0.48	0.6369
				0 ppm	0.00	-	-	-	-
				Males	237.20	1585.70	24	0.15	0.8823
				Females	0.00	-	-	-	-
				330 ppm x Males	1527.66	2047.13	24	0.75	0.4628
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-998.71	2047.13	24	-0.49	0.6301
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				Females	-237.20	1585.70	24	-0.15	0.8823
				Males	0.00	-	-	-	-
				330 ppm x Females	-1527.66	2047.13	24	-0.75	0.4628
				30 ppm x Females	998.71	2047.13	24	0.49	0.6301
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Table 9. Type III Fixed Effects and Parameter Estimates for Associations between Lead Exposure Group and Hippocampal Volume

Type III fixed effect				***Solution for fixed effects***					
	F	*p*			Est	SE	DF	t value	*p*
Group	15.22	<.0001		*Intercept*	17643	307.51	24	57.37	<.0001
Sex	0.03	0.8743		330 ppm	-1233.75	532.62	24	-2.32	0.0294
Group x Sex	0.67	0.5200		30 ppm	-2630.95	469.72	24	-5.60	<.0001
				0 ppm	0.00	-	-	-	-
				Males	-117.26	687.61	24	-0.17	0.8660
				Females	0.00	-	-	-	-
				330 ppm x Males	549.87	887.70	24	0.62	0.5415
				330 ppm x Females	0.00	-	-	-	-
				30 ppm x Males	-366.06	887.70	24	-0.41	0.6837
				30 ppm x Females	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-
				0 ppm x Females	0.00	-	-	-	-
				Females	117.26	687.61	24	0.17	0.8660
				Males	0.00	-	-	-	-
				330 ppm x Females	-549.87	887.70	24	-0.62	0.5415
				30 ppm x Females	366.06	887.70	24	0.41	0.6837
				0 ppm x Females	0.00	-	-	-	-
				330 ppm x Males	0.00	-	-	-	-
				30 ppm x Males	0.00	-	-	-	-
				0 ppm x Males	0.00	-	-	-	-

Discussion

4.1 Study Overview

This study was conducted to extend the results of our earlier microglia study, which found a reduction in microglia mean cell body number in the dentate gyrus as a result of early chronic low-level lead exposure (Sobin et al., 2013). The goal of this study was to test if microglia was reduced not only in the dentate gyrus but the entire hippocampal structure as a result of lead exposure, and to determine whether the distribution of microglia was altered by lead exposure. The study used three lead exposure groups to test the hypotheses including controls (0 ppm), low-dose (30 ppm), and high-dose (330 ppm) animals. Hypothesis 1 predicted that lead exposure significantly reduced microglia mean cell body number in hippocampus. Hypothesis 2 predicted that in lead exposed as compared to control animals, microglia mean cell body number was greater in hippocampal portions without as compared to with dentate gyrus. Hypothesis 3 predicted that clustering of hippocampal microglia around neurons would be greater in exposed as compared to control animals.

4.2 Summary of Major Findings

Regression models showed that microglia mean cell body number in the entire hippocampus was lower in both the low-dose and high-dose animals, and low- and high- dose animals did not differ. When microglia mean cell body number was compared in hippocampal regions with and without dentate gyus, differences were found in the high dose group only, whereby high-dose animals had a greater number of microglia in sections with dentate gyrus as compared to sections without dentate gyrus. Finally, tests of whether microglia tended to cluster around more around neurons in lead exposed animals were not significant. In other words, the distribution of microglia (microglia volume density) throughout the hippocampus and within

hippocampal sections with and without dentate gyrus was the same across groups, suggesting that these levels of early chronic lead exposure did not promote clustering of microglia around neurons at preadolescence. Two additional findings were also observed. Hippocampal volume was significantly lower in both the low-dose and high-dose animals and lead exposed groups did not differ suggesting that low-dose and high-dose exposure resulted in a similar reduction of hippocampus volume. Also, females were found to have significantly fewer microglia than males. These findings will be discussed in detail below.

4.3 Lead Exposure Altered Number of Microglia in Hippocampus

Low-dose and high-dose animals were found to have fewer microglia in the entire hippocampus. The same was found when hippocampal sections with and without dentate gyrus were separately considered. These effects were found to be approximately the same regardless of exposure. In addition, PND 28 females had significantly fewer microglia than males.

The decrease of microglia in the entire hippocampus replicated the findings of our earlier microglia study. In that study, we found that microglia mean cell body number in the dentate gyrus, which is a part of the hippocampus, was significantly decreased in the both the low-dose and high-dose animals (Sobin et al., 2013). This study replicated our earlier findings and expanded on them by showing that microglia in lead exposed animals are in fact reduced throughout the entire hippocampal structure.

This is particularly concerning because microglia constitute the immune system for the brain and central nervous system. In addition, as discussed in the Introduction, microglia serve a role in many other important functions that include neurogenesis, synaptogenesis, and myelination, all of which are critical for proper brain development and brain function (Pierre et al.,2016). A loss of microglia could be expected to alter the development of neural pathways and

in turn, disrupt neural networks required for functions such as memory, perception and learning (Szalay et al., 2016). A decrease of hippocampal microglia could also be of concern because the hippocampus is understood to be the key brain structure responsible for learning and memory (Deng, Aimone, & Gage, 2010). This decrease of hippocampal microglia could potentially disrupt normal learning and memory of children who have been exposed to lead through its effects on microglia and hippocampal function. Further studies would be needed to examine the effects of microglia loss as a result of early chronic lead exposure on learning and memory.

4.4 Lead Exposure Altered Microglia Distribution in Hippocampus Sections with and without Dentate Gyrus, but Only in Animals Receiving the Higher Chronic Exposure Dose

Given our earlier findings that lead exposure reduced microglia mean cell body number in dentate gyrus, we wanted to test the possibility that lead exposure had caused microglia to migrate away from dentate gyrus and towards other regions of hippocampus that did not contain dentate gyrus. Thus, we hypothesized an interaction between group and DG/noDG for a model testing microglia number, whereby lead exposed animals would have greater numbers of microglia in hippocampal regions without versus with dentate gyrus, as compared to controls. The findings did not support this hypothesis. For low-dose animals and control animals, (and controlling for overall reduction of microglia mean cell body number in lead exposed animals) microglia cell body number were fewer in hippocampal regions with dentate gyrus (that is, more microglia in regions without dentate gyrus). However, interestingly among high dose animals, the microglia mean cell body number were nearly equivalent in both regions of hippocampus.

Microglia distribution has been shown to be heterogenous throughout the hippocampal structure in the absence of injury or disease. A study was conducted which examined the distribution of microglia in adult male mice brains using a light microscope (Jinno, Fleischer,

Eckel, Schmidt, & Kosaka, 2007). Immumohistochemical methods were used to prepare the mouse brains for visualizing microglia. In this study of adult animals, stereological analysis of microglia distribution showed that there were more microglia in sections that included dentate gyrus as compared to sections that did not include dentate gyrus. The authors speculated that greater microglia in dentate gyrus contributed to microglial control of hippocampal neuronal activity. In our pre-adolescent mice, we also found heterogenous distribution, but in the opposite direction as that reported in adult mice, perhaps suggesting that the distribution of microglia in hippocampus changes with development. Additional studies are needed to explore this possibility.

Very interestingly, our study showed that high-dose exposure altered this heterogenous distribution (numbers of microglia in hippocampal regions with and without dentate gyrus were nearly equivalent in high-dose mice), which could have serious implications for hippocampal neuronal activity. Given how this heterogenous distribution is vital for the modulation of hippocampal neuronal activity, early chronic high-dose exposure could impact this important function. This could be one way that lead exposure affects downstream functions of memory and learning in children. As mentioned earlier, complex neural networks are required for functions such as memory, perception, and learning, and thus the modulation of neuronal activity is vital for these networks to work effectively. Thus, a change in hippocampal distribution in areas with and without dentate gyrus as a result of early chronic-high dose lead could result in learning and memory deficiencies in exposed children. Further studies would need to be conducted to examine why high-dose exposure resulted in a nearly equivalent microglia distribution in both regions of hippocampus and why there was no significant effect in the low-dose mice. Our lab is

currently conducting studies to understand immune factors that could influence the distribution of microglia following early chronic lead exposure.

4.5 Lead Exposure Did Not Result in Clustering of Microglia Around Damaged Neurons

Again, given our previous findings, we considered the possibility that microglia were missing from the dentate gyrus in lead exposed animals as a secondary effect of lead exposure on neurons. In other words, if lead exposure was disrupting or damaging neurons in the hippocampus, this could trigger microglia to cluster around the compromised neurons, causing microglia to cluster abnormally in dentate gyrus and also pull microglia away from critical areas of dentate gyrus into regions of hippocampus without dentate gyrus. Regression analyses showed however that low-dose and high-dose animals did not differ from controls with regard to the clustering of microglia around neurons in hippocampus. The distribution of microglia in hippocampus sections with and without dentate gyrus were found to be similar across all three exposure groups, indicating that there was no significant clustering of microglia around neurons. Previous studies have suggested that microglia cluster around neurons when they become damaged, for example, in cases of brain injury (Vilhardt, 2005) (Lull & Block, 2010). These studies showed that microglia would proliferate in response to neuronal injury and would migrate toward these cells to attempt to repair them. The findings may indirectly suggest that neurons in the brains of our lead exposed animals were not disrupted or damaged by lead exposure. Alternatively, these results could suggest that chronic lead exposure might alter the ability of microglia to respond to neuronal disruption caused by these low- and high-dose exposures. Further studies are needed to explore these possibilities. If further studies reveal that lead exposure causes microglia to lose their responsiveness to neuronal disruption or damage this also would be of particular concern. The ability of microglia to respond to brain insults is critical

for the maintenance for healthy brain function (Pierre et al., 2016). More specifically, the brains of children who have been chronically exposed to lead might lack the ability to appropriately respond to possible chronic insult and this in turn might be expected to alter development of the brain throughout childhood, perhaps with life-long consequences. It is important to conduct further studies to assess whether neurons are disrupted by early chronic lead exposure, and if so, why microglia are not clustering around damaged neurons and how this might be affecting the developing brain.

4.6 Lead Exposure Resulted in a Reduction of Hippocampus Volume

Low-dose and high-dose animals were found to have a significant reduction of hippocampal volume. In the analyses, group explained differences in hippocampal volume. Importantly, low-dose and high-dose exposure groups were found to have similar effects on reduction of hippocampal volume. This decrease in hippocampal volume replicated findings of our earlier microglia study. In that study, we found that dentate gyrus volume was significantly decreased in both the low- and high-dose animals and the differences between the two groups was not significant. This study supported and replicated our earlier findings and expanded on them by showing that not only was dentate gyrus volume reduced but the entire hippocampal structure was affected as well.

This finding also has implications for cognitive function in low-level exposed children. A reduction of hippocampal volume has been found to be associated with negative effects on cognitive function in an earlier study (Frodl et al., 2006). This study recruited 68 adult male and female patients, mean age 40, to examine their hippocampal volume through the use of structural high resolution MRI. Visual and verbal memory were tested through the Rey Auditory Verbal Learning Test (RAVLT) which measured these two variables. Cognitive function was assessed

through the Wicsconsin Card Sorting Test (WCST). In this study, individuals with a reduced hippocampal volume were found to have visual and verbal memory impairment. In addition, these individuals also experienced symptoms of cognitive dysfunction, which included difficulties with executive functioning.

Thus, a reduction of hippocampal volume following early chronic lead exposure in children could potentially result in them experiencing deficits in cognitive function and thus affecting their ability to learn throughout childhood. In our child studies, children were found to have working memory deficits following early chronic lead exposure (Sobin, Flores-Montoya, Gutierrez, Parisi, & Schaub, 2015). This study recruited 206 males and 211 females, ages 5 to 12 years. Working memory was assessed through the Rapid Visual Processing task (RVP). Low-level lead exposed children had poorer working memory as evidenced by their poor scores on the RVP. This deficit in working memory could potentially result in these children having problems with reading comprehension, sciences, and problem-solving in math.

One mechanism that might explain this reduction in hippocampal volume were discussed in the Introduction (Deng, Aimone &Gage 2010). This previous research suggested that lead inhibited proliferation of progenitor cells during development of the hippocampus, many which become dentate granule cells and glial cells. Further studies need to examine whether fewer dentate granule cells and/or glial cells account for the loss of hippocampal volume observed in this study."

Given how there was no significant difference in lead exposure groups in regard to reduction of hippocampal volume, it is important to prevent both early chronic low- and high-dose exposure in children to prevent this alteration of hippocampal volume. These findings provide further support for the findings of many other studies that have suggested that for

children, there is no “safe” level of lead exposure. The effects of low-dose lead exposure may be just as detrimental as those of high-dose exposure.

4.7 Contribution of Sex Differences to Outcomes

Our study also suggested that sex was a contributing factor to microglia mean cell body number throughout the hippocampus. Overall, pre-adolescent female animals had fewer microglia across groups, with the low- and high-dose females having significantly less microglia than the control females. Thus, it appeared that lead exposure exaggerated this sex-based characteristic.

These findings replicated the findings of earlier studies which have found that female rats have fewer microglia in the brain before PND 28 (Schwarz, Sholar, & Bilbo, 2012). The researchers also suggested that sex differences in regard to microglia number might worsen brain outcomes following challenges such as neurodegeneration or recovery from injury during development. Other studies have found that early life brain injuries could cause significantly greater damage to male brains than female brains (Wynne et al., 2011). This study used adult male and female mice that were exposed to an early life respiratory infection. Hippocampal function in these mice was assessed through microarray analysis conducted on RNA from the mouse hippocampal tissue. Males were found to have greater injury in regard to hippocampal function than females when both were assessed.

Another study conducted on male and female Wistar albino rat pups examined sex differences that might occur in response to brain injury in regard to hippocampal function (Llorente et al., 2009). Maternal deprivation, which involved separating the dams from the pups, was used to induce stress in pups that would negatively impact brain structure and function. Immunohistochemical methods were used to examine brain tissue under fluorescent illumination

microscopy for changes in hippocampal function. The stress induction was found to result in more marked changes in hippocampal function in males when compared to females. Males were found to be more vulnerable than females when possible challenges to brain function occur, although males had more microglia than females during this period.

Greater reduction of microglia in our lead-exposed females was particularly concerning because it suggested a new source of particular vulnerability and might suggest that females are more vulnerable to the effects of lead exposure in regard to microglia number in the hippocampus. These mouse findings could be very useful for translational child studies and more specifically, for refining cognitive assessments of children with low- and higher-doses of lead exposure. It might be of significance to include sex differences in cognitive assessments of children with low- and higher-doses in order to accurately assess the effects of lead exposure in male and female children. Thus, it is imperative to conduct further child and mouse studies to examine if females experience higher levels of memory deficiencies as a result of early chronic lead exposure or if males are more affected by possible brain insult that could occur following early chronic lead exposure.

Conclusion

The results of our study replicated and expanded on our previous studies and showed that microglia mean cell body number was reduced throughout the hippocampal structure of pre-adolescent lead-exposed animals as compared to controls; and that the clustering of microglia around neurons did not differ in lead exposed animals as compared to controls. These results suggested that hippocampal microglial loss occurs in lead exposed mice before pre-adolescence. Additional findings also indicated that sex differences could exaggerate in females the effects of early chronic lead exposure on hippocampal microglia. We also showed that hippocampal volume was reduced in pre-adolescent lead-exposed animals. Further studies are needed to understand whether early chronic low-level lead exposure destroys microglia during development or somehow causes microglia to be trafficked out of the brain; and at which point in development these changes occur.

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Vita

Salvador Dominguez born in El Paso, Texas. He is the second of two siblings, and was the first of his family to complete his college degree. He graduated in 2013 with a Bachelor of Science degree in Biomedical Sciences with Cum Laude honors. After graduation, Salvador was accepted into the Master of Public Health Program degree program at the University of Texas at El Paso (UTEP). He completed his coursework and participated in a practicum where he worked at the UT School of Public Health- El Paso regional campus conducting needs assessments of physical activity in the Segundo Barrio community of El Paso. He used these findings to draft an intervention aimed at increasing physical activity in the Segundo Barrio community.

Salvador plans to continue on to the Interdisciplinary Health Sciences PhD program at UTEP where he will continue working with Dr. Christina Sobin. He plans to continue conducting research on the effects of early chronic exposure on children and expand on the literature that exist in regard to the effects of early chronic lead exposure on microglia. He hopes to one day become an academic researcher and professor.

Permanent address: 8113 Mineola Dr
El Paso, TX, 79925

This thesis/dissertation was typed by Salvador Dominguez.