2017-01-01

A Behavioral and Neuroimmune System Model of the Effects of Chronic Low-Level Lead Exposure in Young Male C57BL/6J Mice

Mayra Gisel Flores-Montoya
University of Texas at El Paso, mgflores3@utep.edu

A BEHAVIORAL AND NEUROIMMUNE SYSTEM MODEL OF THE EFFECTS OF CHRONIC LOW-LEVEL LEAD EXPOSURE IN YOUNG MALE C57BL/6J MICE

MAYRA GISEL FLORES-MONTOYA

Doctoral Program in General Psychology

APPROVED:

_____
Christina Sobin, Ph.D., Chair

_____
Edward Castañeda, Ph.D.

_____
Wendy Francis, Ph.D.

_____
Katherine Serafine, Ph.D.

_____
Charlotte Vines, Ph.D.

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

DEDICATION

I dedicate this dissertation to my grandmother Maria de la Luz Chavez Sosa. Maria is the best example of a person that against all odds achieved wonderful things. Maria was born in a small town in Chihuahua, Mexico and grew up in an economically disadvantaged family. Nonetheless, her fearlessness, enthusiasm, intelligence, and love for her family allowed her to be successful and live a wonderful life. Maria raised five children Rosy Montoya (my mother), Olga Montoya, Manuel Montoya, Oscar Montoya, and Samuel Montoya. While raising her children she managed to complete a career as a teacher and was passionate about teaching Spanish and math to children and adolescents. She has been an example for all the members of my family; we admire her tenacity, tenderness, and intelligence. I am aware that if it was not for my grandmother's will to grow and help her children succeed I might not have found a passion in education and might not have had the resources to achieve my goals. Maria has lived a successful life full of achievements that while might not be recognized around the world have made her and her family immensely happy and have left a legacy of people that live and teach her values.

A BEHAVIORAL AND NEUROIMMUNE SYSTEM MODEL OF THE EFFECTS OF CHRONIC LOW-LEVEL LEAD EXPOSURE IN YOUNG MALE C57BL/6J MICE

by

MAYRA GISEL FLORES-MONTOYA, M.A.

DISSERTATION

Presented to the Faculty of the Graduate School of
The University of Texas at El Paso
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Psychology
THE UNIVERSITY OF TEXAS AT EL PASO
August 2017

ACKNOWLEDGEMENTS

I would like to acknowledge my deepest appreciation for my mentor and Committee Chair Dr. Christina Sobin. I have had the privilege to develop my scientific thinking and career under the guidance of Dr. Sobin. During the past eight years she has been extremely supportive of my intellectual development and has constantly challenged my intellect so that I can grow into a successful scientist. Her precision, dedication, intelligence, and discipline are admirable and will continue to impact my decisions and my career in the future. I am immensely grateful to have had her as a mentor. Additionally, I would like to acknowledge the support of Dr. Charlotte Vines and Dr. Colin Bill for the development of ideas and completion of these studies and the support of my dissertation committee members. Also, I would like to acknowledge the assistance of Jordan Cedillo in the completion of these studies.

This research was made possible by grants from the National Institute of Child Health and Human Development (NICHD), National Institutes of Health, (R21HD060120, CS, PI); the National Center for Research Resources, a component of the National Institutes of Health (5G12RR008124); the Center for Clinical and Translational Science, The Rockefeller University, New York, New York; the Paso del Norte Health Foundation, El Paso, Texas; the University Research Institute, University of Texas, El Paso; and from the J. Edward and Helen M. C. Stern Professorship in Neuroscience, University of Texas, El Paso (CS). The funders had no role in the design, implementation, data analyses, or manuscript preparation for this dissertation.

ABSTRACT

Chronic low-level lead exposure reduces memory in children however the brain mechanisms mediating these effects are not known. In previous studies we showed that early lead exposure reduced olfactory memory and exploratory behavior in young mice, and reduced microglia cell density in hippocampus/dentate gyrus. The present studies aimed to identify additional behavioral tests that were sensitive to early low-level lead exposure in young mice; and to examine whether microglia upregulated factors known to promote cell migration. Seventy-two C57BL/6J male mice were exposed to 0 ppm (controls), 30 ppm (low-dose), or 430 ppm (high-dose) of lead acetate via dams' milk from PND 0 to 28. Behavioral studies and a microglial cell study were conducted. For behavioral studies, mice were tested for developmental milestones (body weight, eye-opening, and righting reflex), muscle strength (inverted screen test), exploratory activity and/or anxiety (open field), and olfactory memory (odor habituation/dishabituation task). For microglia studies, levels of major histocompatibility complex (MHC) II and C-C chemokine receptor 7 (CCR7) in hippocampal and brain microglia were quantified with fluorescence activated cell sorting analyses (FACS). For behavioral studies, it was found that chronic low-level lead exposure increased body weight and decreased exploratory activity. For microglial cell studies, it was found that chronic low-level lead exposure decreased MHC II and CCR7 surface expression in hippocampal microglia. Exploratory analyses revealed additional effects in macrophages and immune cells. These results suggested that chronic low-level lead exposure disrupts exploratory behavior, alters body weight, and disrupts the early neuroimmune system in young mice. Future studies should examine whether alterations in memory and exploratory activity are mediated either by trafficking of microglial cells outside of brain and/or disruption of MHC II and CCR7.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

TABLE OF CONTENTS vi

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER 1: INTRODUCTION 1

1.1 Behavioral Effects of Chronic Low-Level Lead Exposure in Animals 3

1.1.1 Studies examining the behavior of adult animals 3

1.1.2 Summary of behavioral studies (adult animals) 14

1.1.3 Studies examining the behavior of young animals 16

1.1.4 Summary of behavioral studies (young animals) 21

1.2 Brain Mechanisms Underlying Disruptions of Behavior 25

1.2.1 Pathways of lead neurotoxicity 25

1.2.2 The hippocampus as a memory center 26

1.2.3 Effects of lead on the hippocampus 27

1.2.4 Functional roles of microglia in the brain 28

1.2.5 Effects of lead on microglia 29

1.3 The Lymph System 33

1.3.1 “Lymphatics” 33

1.3.2 The glymphatic system 34

1.3.3 The meningeal (or dural) lymphatic system 36

1.3.4 How are the “glymphatics” and “meningeal lymphatic vessels” related? 41

1.4 Can Microglia Traffic Towards Lymphatics? 41

1.5 Lead-Exposed Immune Cells Might Traffic Towards Lymphatics 42
1.6 Summary of Studies 42
1.7 Models and Hypotheses 45
1.7.1 Behavioral system model 45
1.7.2 Neuroimmune system model 45
CHAPTER 2: METHODS 47
2.1 Animals 47
2.2 Lead Exposure 47
2.3 Behavioral Studies 48
2.3.1 Developmental milestones 48
2.3.2 Inverted screen test 49
2.3.3 Open field test 49
2.3.4 Odor habituation/dishabituation test 50
2.3.5 Blood collection 51
2.3.6 Inductively coupled plasma mass spectrometry (ICP-MS) analyses of blood lead 52
2.3.7 Statistical analyses of behavioral data 52
2.4 Microglia Cell Study 54
2.4.1 Fluorescence activated cell sorting analyses (FACS) 55
2.4.2 Outcome variables measured 65
2.4.3 Statistical analyses of FACS data 66
CHAPTER 3: RESULTS 67
3.1 Behavioral Studies Results 67
3.1.1 Descriptive statistics of blood lead levels (BLLs) and behavioral outcomes 68
3.1.2 Tests of homogeneity of variance and distribution properties and selection of inferential statistics 74

3.1.3 Results of inferential tests of behavioral outcomes 76
3.2 Summary of Behavioral Studies Results....84
3.2.1 Developmental milestones....85
3.2.2 Inverted screen test 85
3.2.3 Open field....85
3.2.4 Odor habituation/dishabituation test....86
3.3 Neuroimmune System Studies Results....89
3.3.1 Descriptive statistics for flow cytometry data 89
3.3.2 Inferential statistics for flow cytometry data 90
3.4 Summary of Neuroimmune System Studies Results 106
3.4.1 Hippocampal microglia....106
3.4.2 Hippocampal macrophages....107
3.4.2 Hippocampal immune cells....107
3.4.4 Brain microglia 107
3.4.5 Brain macrophages....107
3.4.6 Brain immune cells 108
CHAPTER 4: DISCUSSION....109
4.1 Behavioral System Studies 109
4.1.1 Blood lead levels....110
4.1.2 Body weight....113
4.1.3 Open field....116
4.1.4 Memory....120
4.2 Neuroimmune System Studies....124
4.2.1 Microglial cells 125

4.2.2 Macrophages and immune cells 129
4.2.3 Strengths of neuroimmune system studies 130
4.2.4 Limitations 131
4.2.5 Conclusions 131
4.3 Is Behavior Associated with Changes in the Neuroimmune System in Chronic Low-Level Lead Exposed Mice? 132
4.3.1 Mouse studies showing a link between CCR7 and behavior 132
4.3.2 How could CCR7 modulate behavior? 134
4.3.3 Does reduced number of immune cells in brain alter behavior? 134
4.3.4 Conclusion 135
4.4 Final Remarks 136
4.5 Future Directions 137
LIST OF REFERENCES 139
APPENDIX A. SUPPLEMENTARY TABLES 156
APPENDIX B. SUPPLEMENTARY FIGURES 204
APPENDIX C. RELEVANT AUTHOR PUBLICATIONS 213
CURRICULUM VITA 238

LIST OF TABLES

Table 1. Summary of behavioral findings of chronic low-level lead exposed animals (behaviors measured in two or more studies) 24

Table 2. Summary of behavioral findings of chronic low-level lead exposed animals (behaviors measured in one study) 25

Table 3. BLLs means and standard deviations (SDs) in young C57BL/6J mice with and without early lead exposure. 68

Table 4. Eye-opening in C57BL/6J mice with and without early lead exposure 69

Table 5. Body weight means and SDs of young C57BL/6J mice with and without early lead exposure 69

Table 6. Means and SDs for righting reflex in young C57BL/6J mice with and without early lead exposure. 70

Table 7. Means and SDs for latency to fall from inverted screen test in young C57BL/6J mice with and without early lead exposure 70

Table 8. Means and SDs for open field behaviors in young C57BL6/J mice with and without early lead exposure 71

Table 9. Means and SDs for odor exploration time in habituation/dishabituation task in young C57BL/6J mice with and without early lead exposure. 73

Table 10. Summary of significant effects in BLLS and behavior in young C57BL6/J mice with and without chronic lead exposure. 88

Table 11. Summary of significant main effects and the interaction for habituation/dishabituation task (group x trial) in young C57BL6/J mice with and without chronic lead exposure. 88

Table 12. Summary of significant effects in hippocampal and brain cells in young C57BL6/J mice with and without chronic lead exposure.....108

Table 13. Means and standard deviations (SDs) for BLLs measured with ICP-MS in young C57BL/6J mice with and without early chronic lead exposure 110

LIST OF FIGURES

Figure 1. Representation of the lymphatic system before and after the discovery of the meningeal lymphatic vessels. 40
Figure 2. Representative image of gating strategy of single cells 40
Figure 3. Representative image of gating strategy of immune cells. 61
Figure 4. Representative image of gating stragegy for identifying microglia and macrophages ..62
Figure 5 Representative image of gating strategy for identifying MHC II “single positve”, CCR7 “single positive”, and “double positive” cells (MHCII/CCR7) in microglia.....63
Figure 6. Representative image of gating strategy for identifying MHC II “single positve”, CCR7 “single positive”, and “double positive” cells (MHCII/CCR7) in macrophages. 64
Figure 7. Blood lead levels measured in µg/dL in C57BL/6J young mice with and without early chronic lead exposure. 76
Figure 8. Body weights at PNDs 14, 21, and 28 measured in young mice with and without early lead exposure 77
Figure 9. Righting reflex measured at PNDs 14, 21, and 28 in young mice with and without early lead exposure. 78
Figure 10. Rearing number in the periphery of open field arena in young C57BL/6J mice with and without early chronic lead exposure 79
Figure 11. Rearing number in the center of open field arena in young C57BL/6J mice with and without early chronic lead exposure 80
Figure 12. Time exploring a cotton swab with water during the habituation phase of the odor habituation/dishabituation task in young C57BL/6J mice with and without early chronic lead exposure. 81
Figure 13. Time exploring a cotton swab with almond during the habituation phase of the odor habituation/dishabituation task in young C57BL/6J mice with and without early chronic lead exposure. 82

Figure 14. Time exploring a cotton swab with orange during Trial 3 and with social scent 1 Trial 1 during the dishabituation phase of the task in young C57BL/6J mice with and without early chronic lead exposure. 84

Figure 15. Representative histograms of effects of low-level lead exposure on hippocampal microglia on mean fluorescence intensity of CCR7 (overall analyses).....92

Figure 16. Representative histograms of effects of low-level lead exposure on hippocampal microglia mean fluorescence intensity of MHC II (double positive cells).....94

Figure 17. Hippocampal macrophages CCR7 “single positive” percentage of parent population in young C57BL6/J mice with and without early chronic lead exposure 99

Figure 18. Representative histograms of effects of high-level lead exposure on hippocampal macrophages on mean fluorescence intensity of MHC II (in double positive cells). 101

Figure 19. Representative histograms of effects of high-level lead exposure on brain macrophages on mean fluorescence intensity of MHC II (in double positive cells). 104

Figure 20. Hippocampal immune cell percentage of parent population in young C57BL6/J mice with and without early lead exposure 105

CHAPTER 1

INTRODUCTION

Lead is a heavy metal that is toxic to humans. Lead can alter a variety of body systems including the central nervous system (CNS), the immune system, the cardiovascular system, and the renal system (WHO, 2010). Children, as compared to adults, are far more vulnerable to the detrimental effects of lead and the CNS is exquisitely sensitive to its effects (CDC, 2013). Child exposure to high levels of lead (yielding blood lead levels, BLLs, between 70 to 150 µg/dL) can result in anemia, nephropathy, encephalopathy, and death (Gurer & Ercal, 2000, review). Child exposure to high levels of lead is less likely to occur in the U.S. however child chronic exposure to low-level lead is a major unresolved child public health problem particularly in lower socioeconomic neighborhoods (WHO, 2010).

Recent events in Flint, Michigan are a stark reminder that chronic low-level lead exposure continues to pose a serious threat for children nationwide. In April 2014, the water supply in Flint, Michigan, was changed from Lake Huron to the Flint River. The problem was that water from the Flint River had a chemical composition (high chloride to sulfate mass ratio and no corrosion inhibitor) that caused increased leaching of lead from lead pipes into the water supply. This resulted in an increase in child BLLs. Specifically, from 2014 to 2016, the percentage of children with BLLs above 5 micrograms per deciliter (µg/dL), the current threshold for “elevated” in the U.S., increased from 2.9% to 4.9% and to 6.6% in highest exposure neighborhoods (Hanna-Attisha, LaChance, Casey Sadler, & Champney Schnepp, 2016).

This is just one example of how large numbers of children can be exposed to low-level lead. Lead-contaminated water is just one of many potential sources of child lead exposure. Over 9,600 industries in United States emit from 10 to 10,000 pounds of lead per year into the environment (EPA, 2006) and air-borne lead can create high risk of exposure for children living in close proximity to these industries. Other possible sources of lead exposure include contaminated soil, lead paint in old housing, lead pipes, children's jewelry, toys, and cosmetics (EPA, 2015). In fact, lead is ubiquitous in our modern industrial environment.

Once lead is ingested or inhaled it can alter brain function. Over 50 clinical studies have shown that children exposed to chronic low-levels of lead have diminished memory, poorer cognitive-set shifting, and reduced fine-motor dexterity (e.g. Bellinger & Needelman, 2003; Canfield et al., 2003; Franko, Palome, Brown, Kennedy & Moore, 2000; Gilbert & Weiss, 2006; Jusko et al., 2008; Landrigan et al., 2006; Lanphear, Burgoon, Rust, Eberly & Galke, 1998; Lanphear et al., 2005; Needleman, Schell, Bellinger, Levinton & Allred, 1990; Needleman, Riess, Tobin, Biesecker & Greenhouse, 1996; Schnaas et al., 2000; Sobin, Flores-Montoya, Gutierrez, Parisi & Schaub, 2015; Wasserman et al., 2000). Given the well-documented detrimental effects of chronic low-level lead exposure on brain and behavior, the Centers for Disease Control and Prevention (CDC) for many years have stated that for children, there is no "safe" level of lead exposure (CDC, 2012). Recent studies in our laboratory have shown that 14% of children living in lower socioeconomic neighborhoods in the El Paso Border region have BLLs $\geq$ 5 µg/dL, and over 50% have BLLs between approximately 2.5 and 7 µg/dL (Sobin et al., 2015.) These are BLLs at which deficits in memory have been detected.

Currently, the only approach to intervention is source removal. The environmental sources of low-level lead exposure are often multiple however and result from current conditions

as well as historical accumulation, and can be very difficult to identify and remove. The brain mechanisms underlying effects on memory of chronic low-level lead exposure are not understood. It is necessary to understand how chronic low-level lead exposure alters behavior and brain in order to identify approaches other than source removal for primary prevention and early intervention in children. New animal models are needed to understand these deficits.

1.1 Behavioral Effects of Chronic Low-Level Lead Exposure in Animals

1.1.1 Studies examining the behavior of adult animals. In an early study in adult rats, developmental milestones and exploratory activity were examined following early lead exposure (Reiter, Anderson, Laskey, & Cahill, 1975). Two generations of Sprague-Dawley rats were pre-treated with one of three lead treatment levels. These included 0 parts per million (ppm) (controls), 5 ppm (low-dose), and 50 ppm (high-dose). After this, rats were mated and the first generation (F1) of rats were exposed to the same treatment via dams' milk. After weaning, rats continued the same treatment until post-natal day (PND) 180. At PND 90, the F1 rats were mated and their pups (F2) received the same treatment exposure. Rats from the F1 and F2 generations (N = 115) were tested for developmental milestones. Five pups from 9 to 11 litters from the F1 and F2 generations were tested behaviorally beginning at PND 120.

The developmental milestones tested included the startle response, righting reflex, and the day at which animals (males and females) opened their eyes. For the startle response, a toy clicker was used to make a clicking sound behind the head of the rats. After hearing this noise, rats that had developed the response were expected to present a muscle contraction on their limbs. For the righting reflex test, rats were held upside down (holding them from the neck and the tail) and were placed approximately 30 centimeters (cm) above a table and were released.

Animals that have developed the righting reflex were expected to turn mid-air and land with their four paws on the table. The day of full eye-opening was also recorded.

For examination of exploratory ambulation, male rats were tested on a residential maze (76 cm x 60 cm) with *ad libitum* access to food and water. Rats were tested from PND 120 to 125. The maze was equipped with infrared light beams that allowed the automatic recording and rating of animal behavior. Exploratory ambulation was recorded on a Xerox microcomputer every hour for five days.

For examining the stimulant effects of d-amphetamine on locomotion, on day five immediately after testing, rats were challenged with 4 mg/kg of d-amphetamine and 20 minutes (min) after injection they were returned to the residential maze. The outcome variable was the number of times that the animals broke the laser beams.

For the statistical analyses of developmental milestones and exploratory ambulation, t-tests were used. (No significant differences between the F1 and F2 generations were detected thus data were averaged for both generations). With regard to developmental milestones, it was found that eye-opening for the low-dose group was delayed by one day as compared to controls; the righting reflex was delayed by one day for the low-dose group and two days for the high-dose group as compared to controls. No differences for the startle response were found. Also, the lead-exposed animals had decreased exploratory ambulation as compared to controls. Similarly, after the amphetamine challenge, locomotion was decreased in lead-exposed animals as compared to controls. These results suggested that chronic low-level lead exposure delayed the development of the nervous system, impaired locomotion, and reduced the sensitivity of animals

to the stimulatory effects of d-amphetamine. It was also suggested that changes in exploratory ambulation might be a result of motor pathway/s disruption.

In a similar study, adaptation to a novel environment and exploratory ambulation of adult rhesus monkeys exposed chronically to low-levels of lead were examined (Ferguson & Bowman, 1990). Eight rhesus monkeys were exposed to one of two treatments via nasogastric intubation. The control group (n = 4) was exposed to sodium and the experimental group (n = 4) was exposed to 0.7 mg/kg of lead of body weight, dissolved in a milk formula, from PND 8/9 to 29. From PND 42 to 356, the lead-exposed group received 3.0 mg/kg of lead of body weight as described earlier and the control group received an equivalent dose of sodium (BLLs for all animals were $\leq$ 5 µg/dL at two years old; authors did not report specific BLLs at the time of testing.)

For examining exploratory ambulation, the open field (OF) task was used. An arena (2.4 m x 2.0 m x 2.2. m) equipped as a play-room for the monkeys was used for testing. The arena had wire mesh ledges, ramps, and a bar that traversed the room. On the floor of the OF, 27 sectors of equal size were created by demarcating them with chalk. Animals were habituated to the arena from three months old to one year old. Animals were then allowed to mature to four years old and were tested behaviorally.

Animals were tested once a week during six sessions. They were given 10 min to enter the OF, if they did not enter it within the time allotted, they were pushed into it. Once in the OF, they were allowed to explore it for 15 min and behavior was recorded with an Apple AIIE microcomputer. At a later time, raters trained to reliability rated monkeys' behavior. The outcome variables analyzed were the latency to enter the arena (recorded manually with a timer),

urinations and defecations (measures of anxiety), number of sectors entered, and frequency and duration of 17 behaviors (described below) every 5 min. Immediately after monkeys completed behavioral testing, blood was extracted via femoral venipuncture and was later analyzed for BLLs with gas furnace atomic absorption spectrometry (GFAAS).

Behavior was analyzed with repeated measures analyses of variance (ANOVA) with treatment as a between subjects factor and day of testing as a within subjects factor. Only five out of the 17 behaviors recorded occurred frequently enough to be analyzed. These included time inactive, exploratory activity (every time they manipulated the environment with their mouth, hands, and feet), exploratory ambulation (number of quadrants crossed), self-directed behavior (play activity), and vocalizations.

It was found that the lead-exposed monkeys had increased latencies to enter the OF as compared to controls. Also, the lead-exposed monkeys displayed less inactive time, and had increased exploratory activity, and increased quadrants crossed as compared to controls. No significant effects were detected for urinations, defecations or vocalizations. These results suggested that chronic low-level lead exposure delayed adaptation to a novel environment and increased the exploration of a novel environment in adult monkeys. The authors suggested that the adult lead-exposed animals had increased exploration of the environment because they had memory impairments, i.e. they might have needed more time to encode environmental information as compared to controls.

In another study of adult animals with early chronic low-level lead exposure, exploratory ambulation, the stimulating effects of d-amphetamine on locomotion, gross motor coordination, and running wheel behavior were examined in chronic low-level lead exposed

adult mice (Leasure et al., 2008). Two lead exposure models were developed, a gestational lead exposure model (GLE) and a post-natal lead exposure model (PLE). For the GLE model, C57BL/6J mice pups, 12-18 litters per group, were exposed to one of four treatment conditions, including 0 ppm (controls), 27 ppm (low-dose) (mean BLL 3 µg/dL at PND 21), 55 ppm (moderate) (mean BLL 9 µg/dL at PND 21) and 109 ppm (high-dose) (mean BLLs 15 µg/dL at PND 21) of lead acetate via dams' drinking water, two weeks before mating and until PND 10 of the pups. For the PLE model, pups were exposed to one of three treatment conditions, including 0 ppm (controls), 27 ppm (low-dose) (mean BLL 10 µg/dL at PND 21), and 55 ppm (moderate) (mean BLL 25 µg/dL at PND 21) of lead acetate via dams' drinking water from PND 0 to 21. Mice from the GLE but not the PLE model were tested behaviorally when they matured to one year old.

For examining exploratory ambulation and the possible stimulating effects of d-amphetamine on locomotion, mice were tested on the OF task. This task consisted of an Optovarimax behavioral monitor (40 cm x 40 cm x 40 cm) equipped with laser beams that detected ambulation. Mice were habituated to the arena for 15 min and they were tested for 30 min. The number of times that mice crossed the laser beams were detected automatically every 5 min during testing. After this, mice received a 3 mg/kg d-amphetamine challenge and they were immediately placed back in the OF and were allowed to explore the arena for three hours.

For examining gross motor coordination, mice were tested on the rotarod (RR) task. This machine had a rotating rubber barrel and a laser beam that automatically detected when an animal fell from the barrel. Animals were trained to stay on the barrel while it rotated at a constant speed of 5 rotations per minute (rpm) for 90 sec. After training, mice were tested in three trials with an inter-trial interval (ITI) of one hour. During each trial, the rotating barrel was

set at 50 rpm for 90 sec and then the speed was increased by 0.1 rpm every 30 sec. The outcome variable was the latency (in sec) for the animals to fall from the rotating barrel.

For running wheel behavior, mice were placed individually, for five consecutive days, in home cages that contained a running wheel (Whatman type) and *ad libitum* access to food and water. The running wheels had counters that recorded the distance travelled by the mice (in meters). The outcome variable was the total distance travelled by mice. At a later time, animals from the GLE and PLE models were sacrificed and BLLs were analyzed with a Lead Care I device (Environmental Sciences Associates, Inc., Chelmsford, MA).

For the statistical analyses, possible differences between groups with regard to BLL and body weight in mice from the GLE and PLE models were analyzed with two-way ANOVA (treatment group x sex). Behavior for mice in the GLE model was analyzed with one-way ANOVA and one-way repeated measures ANOVA.

With regard to BLL, the lead-exposed groups had increased BLL as compared to controls in both the GLE and PLE models, however differences in BLL between groups were not detected after PND 30 in the GLE model and after PND 60 in the PLE model. With regard to body weight, it was found that male but not female mice from the GLE and PLE models had increased body weight as compared to controls. With regard to behavior, it was found that for the OF task, during the first 30 min of exploration, the lead-exposed male mice, but not female mice, had decreased exploratory ambulation as compared to controls. And the low-dose male mice had decreased exploratory ambulation as compared to the high-dose male mice. Additionally, after the amphetamine challenge, it was found that lead-exposed male mice, but not female mice, had increased locomotion as compared to controls. This suggested that lead-exposed male mice were

more sensitive to the effects of d-amphetamine on locomotion than unexposed mice. For the RR task, lead-exposed male mice, but not female mice, stayed for shorter periods of time on the rotating barrel as compared to controls. For running wheel activity, no differences between the groups were detected.

These results suggested that chronic low-level lead exposure affected male but not female mice with gestational chronic exposure to lead. Specifically, in male mice, increased body weight, decreased exploratory ambulation, increased exploratory ambulation after an amphetamine challenge, and decreased gross motor coordination were detected. It was suggested that these effects might have not occurred simply because of increased body weight in male mice, since all treatment groups had travelled relatively equal distances on the running wheel. Instead, it was suggested that chronic low-level lead exposure might have altered motor pathway/s and thus exploratory ambulation in male mice.

In a study of adult Wistar rats, exploratory ambulation and object recognition memory were examined in female adults exposed to low-levels of lead (Azzaoui, Ahami, & Khadmaoui, 2009). Fourteen Wistar rats were exposed to one of two levels of lead nitrate, including 0 ppm (control, n = 7) and 20 ppm (low-dose, n = 7). Rats were exposed to treatment via drinking water from PND 90 to180. BLLs were not reported. Rats were tested behaviorally every 14 days from the beginning to the end of the intoxication period.

For examining exploratory ambulation, rats were tested on the OF task. They were first habituated to an open top wooden box (100 cm x 100 cm x 40 cm) that had its floor marked into 25 quadrants. After habituation, rats were placed on the center of the arena and exploratory

ambulation was recorded for 7 min. The outcome variable was the number of times that the rats crossed each quadrant with all four paws.

For examining object recognition memory, rats were tested with the novel object recognition (NOR) task. Rats were habituated to a square arena (40 cm x 50 cm x 50 cm) for 5 min. After habituation, rats were placed back in the arena that had two identical objects located in the upper left and upper right corners of the arena. Animals were allowed to explore these objects for 5 min. After a 24 hour ITI, rats were placed back in the arena, but this time one of the objects was replaced by a novel object (NO). The time that the rats spent exploring each object was recorded. Object exploration was quantified when the animal had its nose ≤ 1 cm of proximity to the object. If rats had an intact memory, they were expected to spend more time exploring the NO as compared to the familiar object (FO).

For the statistical analyses of exploratory ambulation, a one-way repeated measures ANOVA with treatment group as the between subjects factor and day of testing as the within subjects factor were used. For the NOR task, first a discrimination ratio (DR) was calculated with the following formula = (Time exploring NO / Time exploring NO + Time exploring FO), a DR above 0.50 would mean that the rats spent more time exploring the NO as compared to the FO. Differences between groups with regard to the DRs were also examined with one-way repeated measures ANOVA as described above.

For exploratory ambulation, no significant differences between groups were found within the first 12 weeks of testing. However at the end of the testing period, it was found that the lead-exposed rats had increased number of quadrants crossed as compared to controls. For the NOR task, no significant differences between groups were found within the first two to six weeks of

testing. However during weeks eight and 10, low-level lead-exposed rats had diminished DRs as compared to controls. These results suggested that chronic low-level lead exposure increased exploratory ambulation and diminished object recognition memory in female adult Wistar rats.

In a similar study, exploratory ambulation, gross motor coordination, visuo-spatial memory, and aggressive behavior of chronic low-level lead exposed adult mice were examined. Sixty-six Balb/c mice were exposed to 0 ppm (controls, n = 30) or 20 ppm (low-dose, n = 36) of lead acetate via dams' drinking water from gestational day 8 until PND 21 (Kasten-Jolly, Pabello, Bolivar, & Lawrence, 2012). BLLs were not reported. Animals were allowed to mature to adulthood and then they were tested behaviorally. PND of testing was not reported.

To quantify exploratory ambulation, mice were tested in dim light sound-attenuating chambers (65 cm x 55 cm x 65 cm) equipped with 16-beam activity monitors (42 cm x 42 cm x 30 cm). Mice were habituated to the chambers for one hour; after an ITI (inter-trial interval) of 5 min they were returned to the chambers and were allowed to explore them for 15 min. A PC computer and a Digiscan software automatically recorded and rated mouse behavior. The outcome variables examined were total distance travelled, resting time, rotations, habituation, and speed. Additionally, in order to measure anxiety, the time spent and distance travelled in the periphery of the arena (thigmotaxis) vs. the center of the arena were measured. (Thigmotaxis is a behavior displayed when animals are anxious, Simon, Dupis, & Costenin, 1994.)

For examining gross motor coordination, animals were tested on the RR (rotarod) task. The machine used for this study had four compartments (48 cm x 11 cm x 30 cm) and a rotating rubber barrel. Mice were tested on three consecutive trials for 180 sec with ITIs of 20 min. Mice were first habituated to the rotating barrel for one hour. After habituation, they were tested.

During the first 60 sec of testing, the speed of the rubber barrel increased from 0 rpm to 15 rpm and it was maintained at 15 rpm for 110 sec. During the last 10 sec of testing, the speed of the barrel decreased from 15 rpm to 0 rpm. The outcome variable was the latency to fall from the barrel. The Smartrod Windows software 1.7 was used to record automatically this latency.

For examining visuo-spatial memory, mice were tested on the Morris Water Maze (MWM) task. Mice were placed in a 1.5 meters (m) diameter pool. Nontoxic white paint was used to create opaque water. A hidden platform was located 1.5 cm below the surface of the water. Mice were trained for four consecutive days, during eight trials, with ITIs of 45 min (except for trials 4 and 5 that had ITIs of 60 min), to locate the hidden platform via swimming. During each training trial, mice were given 60 sec to locate the hidden platform and were allowed to stay on it for 30 sec. Mice that did not locate the platform within the allotted 60 sec were placed on it manually.

After the last training trial, mice were given an ITI of one hour and then they were tested. For testing, the hidden platform was removed from the water pool and mice were tested for 60 sec. If mice had an intact visuo-spatial memory, they were expected to spend more time in the zone where the platform was previously located as compared to other zones in the water maze. The automated Accutrak system was used to record behavior. The outcome variable was the time spent swimming in the area where the platform was previously located.

For examining aggressive behavior, male mice were tested with the resident-intruder test and were examined for litter-mate behavior at 10 months of age. For the resident intruder-test, male mice that were not litter-mates (intruders) were housed, for 10 min, in pairs, in cages with lead-exposed and unexposed male mice and mouse behavior was video-taped. The outcome

variables included number of bites, chasing, wrestling, time before the first aggressive behavior occurred, and behavior in the cage when mice interacted with their litter-mates. For litter-mate behavior, the number of animals injured in each treatment group were recorded (what constituted an injury was not reported in this study.)

For the statistical analyses of exploratory ambulation in the dim chambers, two-way ANOVAs were used, with treatment group and sex as the between subjects factors. For gross motor coordination in the RR, and for visuo-spatial memory in the MWM, mixed ANOVA models were used. For the RR, treatment group and sex were the between subjects factors and testing day the within subjects factor. For the MWM, treatment group and sex were the between subjects factors and trial the within subjects factor. When significant effects were detected, post-hoc analyses with Tukey's honestly significant difference (HSD) were used. For the resident-intruder test, a student t-test was used. For litter-mate behavior, the percentage of animals with injuries were compared between lead-exposed and unexposed groups.

For exploratory ambulation, it was found that the low-level lead-exposed mice travelled shorter distances, spent more time resting, and had diminished speed as compared to controls. It was also found that males travelled more distance, spent less time resting, and had increased speed as compared to females, regardless of treatment exposure. With regard to anxiety, it was found that low level lead-exposed females had increased thigmotaxis as compared to lead-exposed males and controls. For the RR task, no differences between groups were found. For the MWM task, it was found that the low-level lead-exposed animals spent less time swimming in the zone where the platform was previously located. For the resident-intruder task, it was found that lead-exposed male mice had diminished bouts of aggressive behavior and increased latency to display aggressive behaviors as compared to controls. On the other hand, for litter-mate

behavior, it was found that 28% of lead-exposed male mice injured their litter-mates whereas none of the control male mice injured their litter mates.

These results suggested that chronic low-level lead exposure diminished exploratory ambulation, increased anxiety in females but not males, decreased visuo-spatial memory, decreased male aggressive behavior towards intruders, and increased male aggressive behavior towards litter-mates in adult Balb/c mice.

1.1.2 Summary of behavioral studies (adult animals). As evidenced on the five studies described above, a wide-variety of behaviors were altered in adult animals as a result of chronic low-level lead exposure. Some of the findings were consistent among studies and some were not.

Two of the studies found that chronic low-level lead exposure diminished memory; one study found diminished visuo-spatial memory in adult male and female mice (Kasten-Jolly et al., 2012) and another study found diminished object recognition memory in female adult rats (Azzaoui et al., 2009). Visuo-spatial and object recognition memory are sub-served by different pathways that overlap in the hippocampus/DG (Kee, Teixeira, Wang & Frankland, 2007; Takeda et al., 2014). Thus, future studies could examine if chronic low-level lead exposure alters brain pathways and regions associated with these two types of memories.

Findings were mixed with regard to gross motor function. One study detected diminished gross motor function in lead-exposed males but not females (Leasure et al., 2008); another study did not detect effects in gross motor function in males or females (Kasten-Jolly et al., 2012).

Similarly, findings were mixed with regard to exploratory ambulation. Two studies detected increased exploratory ambulation (Ferguson et al., 1990; Azzaoui et al., 2009) whereas two studies detected decreased exploratory ambulation (Kasten-Jolly et al., 2012; Reiter et al.,

1975) and one study detected decreased exploratory ambulation in males but not females (Leasure et al., 2008).

Findings were also mixed with regard to the locomotor stimulant effects of d-amphetamine in exposed animals. One study detected increased locomotion promoted by d-amphetamine in males but not females (Leasure et al., 2008) whereas another study detected decreased locomotion in males (Reiter et al., 1975).

Several factors might have mediated mixed findings in gross motor function and exploratory ambulation including differences in the lead exposure period time, sex tested, e.g. some studies tested only males (Reiter et al., 1975) and some only females (Azzaoui et al., 2009), PND at which the animals were tested, and type of species tested. Even though mixed findings were detected, all of the studies suggested changes in exploratory ambulation. Future studies should examine basic motor functions in order to more thoroughly characterize possible motor deficits in animals.

Additionally, only one study examined developmental milestones (Reiter et al., 1975) and aggressive behavior in animals (Kasten-Jolly et al., 2012). It was found that the development of the righting reflex and eye-opening were delayed as a result of chronic low-level lead exposure. This suggested that chronic low-level lead exposure delayed the development of the nervous system. Future studies should examine what brain mechanisms are responsible for these delays in development. Additionally, increased aggressive behavior was detected towards litter mates as a result of chronic low-level lead exposure. This type of behavior is sub-served in part by the amygdala (Nelson & Chiavegatto, 2000). Future studies could examine whether this brain region is disrupted as a result of chronic low-level lead exposure in adult animals.

The above described studies are very valuable for understanding the effects of chronic low-level lead exposure in adult animals. However, none of these studies examined young animals (except for the Reiter et al. study which examined developmental milestones). In order to develop animal models that might be valuable for understanding effects in children, in our laboratory, the effects of chronic low-level lead exposure have been examined in young mice (PND 28) in three behavioral studies. These studies are described in detail below.

1.1.3 Studies examining the behavior of young animals. Our laboratory has investigated the sensitivity of several behavioral paradigms to the effects of early chronic lead exposure in pre-adolescent mice (PND 28). In one study (Flores-Montoya & Sobin, 2014), exploratory activity, exploratory ambulation, and gross motor coordination of chronic low-level lead exposed young mice were examined. Sixty-one C57BL/6J mice were exposed to one of three treatment conditions, including 0 ppm (controls; n = 16), 30 ppm (low-dose; n = 26) (BLLs ranged from 2 to 6 µg/dL), and 230 ppm (high-dose; n = 19) (BLLs ranged from 7 to 15 µg/dL) of lead acetate via dams' drinking water from PND 0 to 28. Mice were tested behaviorally at PND 28.

For examining exploratory activity, mice were tested in the nose poke (NP) task. Mice were placed in a square Plexiglas arena (16 in x 16 in x 16 in) that had an elevated platform with 16 evenly distributed 1 in diameter holes. Mice were initially placed in the right corner of the arena and were allowed to explore it freely for 3 min. A video-camera was mounted at the level of the arena and behavior was recorded. The outcome variable was the number of times that mice poked their noses into the holes.

For examining exploratory ambulation, mice were tested in the OF (open field) task. Mice were initially placed in the right corner of a square Plexiglas arena (16 in x 16 in x 16 in) that had four quadrants marked on the floor. Mice were allowed to explore the arena freely for 5 min. A video-camera was mounted at the level of the arena and behavior was recorded. The outcome variable was the number of times that mice crossed into each quadrant with all four paws.

For examining gross motor coordination, mice were tested with the RR (rotarod) task (described on page 12 above.) Mice were placed on the RR machine and were allowed to stabilize on it for 10 sec. After this, mice were tested in four trials with a duration of 300 sec per trial and an ITI of 3 min. During the 300 sec trial duration, the rotating barrel was accelerated from 3.5 rpm to 35 rpm. The outcome variable was the latency, in sec, that took the mice to fall from the rotating barrel. After all behavioral tests were completed, mice were sacrificed and heart blood was extracted for BLL analyses with ICP-MS (detailed description of ICP-MS is given on page 52.)

For the statistical analyses of each outcome variable, general linear mixed model (GLIMMIX) analyses with BLL and sex as fixed effects and litter as a random effect were used to examine possible linear associations. Significant effects were detected for the NP task. As BLL increased, exploration of the novel arena decreased for both males and females. On the other hand, for the OF and RR tasks, no significant effects of lead exposure were found. These results suggested that chronic low-level lead exposure reduced exploratory activity but not exploratory ambulation or gross motor coordination in young (pre-adolescent) C57BL/6J mice.

In a similar study (Flores-Montoya, Alvarez, & Sobin, 2015), the olfactory recognition memory of young mice exposed chronically to low-levels of lead was examined. Thirty-three C57BL/6J mice (13 females and 20 males) were exposed to one of three levels of lead acetate, including 0 ppm (control; n = 10), 30 ppm (low-dose; n = 10) (BLLs ranged from 3 to 5 µg/dL), and 330 ppm (high-dose; n = 13) (BLLs ranged from 10 to 20 µg/dL). Lead was administered via dams' drinking water from PND 0 to 28.

At PND 28, mice were tested on the novel odor recognition (NODR) task. This task included a habituation trial (10 min), a training trial (10 min), and a testing trial (5 min) with ITIs inter-trial intervals) of 5 min. For the habituation trial, mice were allowed to freely explore an empty square arena (8 in x 8 in x 24 in). For the training trial, mice were allowed to explore two identical odors placed in the upper right and upper left corners of the arena. For the testing trial, one odor was replaced by a novel odor (NO). If odor recognition memory was intact, mice were expected to spend more time exploring the NO as compared to the familiar odor (FO). Behavioral testing was video-taped and behavior was later rated by raters blind to the experimental condition. Immediately after behavioral testing was completed, animals were sacrificed and heart blood was extracted for analyses of BLL with ICP-MS.

For the statistical analyses, a DR (discrimination ratio) was calculated with the following formula = (time exploring NO / time exploring NO + time exploring FO). A DR greater than 0.50 meant that animals spent more time exploring the NO as compared to the FO. GLIMMIX analyses with BLL and sex as fixed effects and litter as a random effect were used to examine possible linear associations. It was found that as BLL increased, the DR decreased, regardless of sex. However when visually inspecting the graphs of DRs for males and females, a linear relationship between BLL and DR was evident in males but not in females. It could be that sex

differences were not statistically significant because unbalanced numbers of males (n = 20) and females (n = 13) were tested. Future studies should have a balanced number of males and females, or should examine separately effects on males and females in order to more thoroughly characterize these effects. To conclude, these results suggested that chronic low-level lead exposure diminished olfactory recognition memory in young C57BL/6J mice.

In another study (Sobin, Flores-Montoya, & Alvarez, 2017), spatial memory and object recognition memory in chronic low-level lead exposed mice as compared with controls was examined. Fifty-two C57BL/6J mice (28 males and 24 females) were exposed to one of three levels of lead acetate, including 0 ppm (control, n = 18), 30 ppm (low-dose, n = 16), and 430 ppm (high-dose, n = 18). Lead was administered via dams' drinking water from PND 0 to 28. And mice were tested behaviorally at PND 28.

For examining spatial recognition memory and object recognition memory, the object-in-place visual recognition (OIP) task was used. Mice were placed in the lower right quadrant of a clear Plexiglas circular arena (72 cm diameter) with a 38 cm x 30 cm white and black striped cardboard rectangle affixed to the upper left region as an orientation cue. The OIP task included a spatial memory challenge test and an object recognition memory challenge test. For the spatial challenge test, the memory of familiar locations vs. novel locations were examined. For the object recognition challenge test, memory for familiar objects vs. a novel object was examined.

The OIP task consisted of seven trials that lasted 4 min and had an ITI of 2 min. Trial 1 was the habituation trial. Animals were placed in an empty arena and were allowed to explore it freely. Trials 2, 3, and 4 were the learning trials for the spatial memory challenge test. During these trials mice were exposed to five objects. Trial 5 was the testing trial for the spatial memory

challenge. For this, one of the familiar objects (FOs) was displaced to a new familiar zone, and another FO was moved to a novel zone. These zones were then called the displaced object (DO) zones. During Trial 5, animals with an intact spatial memory were expected to spend more time exploring the DO zones as compared to the non-displaced object (NDO) zones. Trial 6 was the learning trial for the object recognition memory challenge test. During this trial, the objects remained in the same place as in Trial 5. Trial 7 was the testing trial for the object recognition memory challenge. For this, one of the FOs was replaced for a novel object (NO). During this trial, animals with an intact object recognition memory were expected to spend more time exploring the NO zone as compared to the FO zones.

The SMART software system (Harvard Pan Lab, Cambridge, MA) was used to demarcate the boundaries of the zones surrounding each of the objects (nine zones) and one zone demarcating the perimeter of the arena. Mouse behavior was recorded with a video camera placed above the arena. Behavior was later rated automatically by the SMART software system and processed for statistical analyses. Seven outcome variables were examined. Four outcome variables corresponded to behavior within the zones of interest and three outcome variables corresponded to behavior displayed in all zones within each trial. For the behaviors in the zones of interest, the outcome variables included number of entries in zones, total time in zones, immobile time in zones, and total rears (when the animals stood on their hind legs) in zones. For the behaviors in all zones, the outcome variables included total distance travelled, total rears, and total immobile time.

For the statistical analyses DRs (discrimination ratios) were calculated. For Trials 4 and 5, DRs for spatial memory were calculated with the following formula = (DO zones behavior / DO zones behavior + NDO zones behavior). For Trials 6 and 7, DRs for novel object recognition

memory were calculated with the following formula = (NO zone behavior / NO zone behavior + FO zones behavior). For spatial memory, DRs above 0.40 indicated more exploration of the DOs as compared to the NDOs. For novel object recognition memory, DRs above 0.20 indicated more exploration of the NO as compared to the FOs.

General linear mixed model analyses (GLIMMIX) with lead exposure and sex as fixed effects and litter as a random effect were used to examine possible effects of lead exposure treatment for all outcome variables. With regard to behavior in the zones of interest, it was found that the OIP task stimulated greater exploration of the DOs as compared to NDOs during the spatial memory challenge test, and that it stimulated exploration of the NO as compared to the FOs during the object recognition memory challenge test, however significant differences between treatment groups were not detected. On the other hand, with regard to behavior in all zones, it was found that low- and high-dose groups had increased global rearing behavior in zones of object memory retrieval and away from zones of spatial memory retrieval. These results suggested chronic low-level lead exposure altered the development of an exploratory behavior (rearing) that is critical for learning and memory. Furthermore, the results suggested that the OIP task is not sensitive to the effects of chronic low-level lead exposure in young mice however the task is a valid measure of spatial and object recognition memory in young C57BL/6J mice. Global alterations in rearing behavior, motor behavior, and exploratory ambulation must be thoroughly characterized in future studies to understand their contribution to performance on specific cognitive challenges.

1.1.4 Summary of behavioral studies (young animals). The three studies described above are the only studies to date that examined effects of early chronic low-level lead exposure in pre-adolescent animals and all three studies showed that early chronic exposure to low-level

lead altered behavior in young animals. Some of the findings were consistent between the studies and some were not.

Only one study examined exploratory activity (NP task). In this study, as BLL increased, exploratory activity decreased (Flores-Montoya et al., 2014). In this same study, effects on gross motor ambulation or exploratory ambulation were not observed. It was suggested that the reduced exploratory activity in the NP task was due to diminished "curiosity" and/or increased anxiety for exploring a novel environment. Brain areas associated with exploration of a novel environment and specifically nose-poke behavior include the hippocampus (Brodkin, 1999; Crusio, 1995) and the amygdala (Takeda, Tsuji, & Matsumilla, 1998).

Findings with regard to memory were specific to the type of memory tested. Deficits in olfactory recognition memory were observed (Flores-Montoya et al., 2015). No effects however were detected for object recognition memory or visuo-spatial memory, instead, increased global rearing behavior was detected during memory retrieval. These results suggested that chronic low-level lead exposure might alter visuo-perceptual orientation and perhaps learning in young mice (Sobin et al., 2017).

Differences in the type of memory affected may be an important clue regarding the brain areas most vulnerable to low-level lead exposure. Olfactory recognition memory, spatial and object recognition memory are sub-served, in part, by the hippocampus (Kee et al., 2007; Takeda et al., 2014), however, the pathways mediating each of these memories are distinct (Broadbent, Squire, & Clark, 2004). Olfactory recognition memory is sub-served by pathways linking the olfactory bulb, entorhinal cortex, and hippocampus (Brennan & Keverne, 1997). In contrast, spatial memory is subserved by the visual cortex, parietal lobe, and hippocampus; object recognition memory is subserved by the visual cortex, entorhinal cortex, and hippocampus

(Eichenbaum, Yonelinas, & Ranganath, 2007; Wang, Gao, & Burkhalter, 2011). Thus, it might be that in young animals, chronic low-level lead exposure specifically targets circuits associated with entorhinal cortex and hippocampus.

Results from adult studies reviewed above further suggested that as animals age into adulthood, the effects may expand to alteration of brain pathways also sub-serving spatial and object recognition memory (Azzaoui et al., 2009; Leasure et al., 2012). Several hypotheses are suggested by these findings and studies designed to specifically address the hypotheses are needed.

The findings suggested another possibility as well. Early chronic lead exposure appeared to globally increase rearing behavior. Thus another possibility is that excessive rearing behavior interrupted memory based exploration and in this way obscured the quantification of memory based exploration. In order to understand whether effects on rearing behavior are due to changes in visuo-perception, learning and/or motor function, a battery of tests that include both examination of cognition and simple tests of motor function must be conducted in order to more thoroughly characterize these effects.

To summarize, chronic low-level lead exposure results in a wide-variety of behavioral deficits in animals. In adult animals, deficits are evident in spatial memory and object recognition memory, as well as in exploratory ambulation. In younger animals (perhaps with high relevance to chronically exposed children), deficits in olfactory recognition memory and exploratory activity, and increases in rearing behavior during a cognitive challenge are evident. More behavioral studies examining the effects of chronic low-level lead exposure in young animals are needed. Future studies should replicate memory deficits in young mice using

olfactory recognition memory tasks and examine deficits in motor behavior using basic measures of motor function. Tables 1 and 2 below give a summary of the behavioral studies discussed.

Table 1. Summary of behavioral findings of chronic low-level lead exposed animals (behaviors measured in two or more studies).

Studies			**Behavioral Findings**			
Authors and year	*Species and Strain*	*PND*	*Memory*	*Gross Motor Coordination*	*Exploratory ambulation*	*Locomotion, d-amphetamine*
Reiter et al. (1975)	Sprague-Dawley rats	120-125	…	…	decreased*	decreased
Ferguson and Bowman (1990)	Rhesus monkeys	1,440	…	…	increased*	…
Leasure et al. (2008)	C57BL/6J mice	365	…	decreased	decreased	increased
Azzaoui et al. (2009)	Wistar rats	90-180	decreased*	…	increased	…
Kasten-Jolly et al. (2012)	Balbl/c mice	adulthood	decreased	*no effects*	decreased	…
Flores-Montoya et al. (2014)	C57BL/6J mice	28	…	*no effects*	*no effects*	…
Flores-Montoya et al. (2015)	C57BL/6J mice	28	decreased	…	…	…
Sobin et al. (2017)	C57BL/6J mice	28	*no effects*	…	increased	…

**Represents effects that were replicated in two or more studies.*

PND = post-natal day

Table 2. Summary of behavioral findings of chronic low-level lead exposed animals (behaviors measured in one study).

Studies			Behavioral Findings			
Authors and year	*Species and Strain*	*PND*	*Eye-opening*	*Righting reflex*	*Exploratory activity (nosepoke task)*	*Aggression*
Leasure et al. (2008)	C57BL/6J mice	365	delayed	delayed	…	…
Kasten-Jolly et al. (2012)	C57BL/6J mice	365	…	…	…	increased
Flores- Montoya et al. (2014)	C57BL/6J mice	28	…	…	decreased	…

Note. This table shows behavioral outcomes measured only in one study. *PND* = post-natal day.

1.2 Brain Mechanisms Underlying Disruptions of Behavior

The underlying brain mechanisms that drive behavioral deficits as a result of early chronic low-level lead exposure are currently not known. Decreases in memory in children and disruption of exploratory behavior and retrieval memory in young mice however suggest that hippocampus may be a target of effects. Also, studies at higher levels of exposure have provided evidence that interactions between the central nervous system and the neuroimmune system (e.g. astrocytes and microglial cells) might be, in part, responsible for behavioral disruptions and have suggested avenues of research for examining effects at lowest levels of exposure (e.g. Legare et al., 1998; Liu, M.-C., et al., 2012).

1.2.1 Pathways of lead neurotoxicity. Lead can be neurotoxic for the brain via a direct pathway and/or an indirect pathway. In the direct pathway, lead might cross the blood brain

barrier (tightly packed endothelial cells that separate circulating blood from extracellular fluids) and accumulate in astrocytes (Legare et al., 1998). Once lead is in astrocytes, it can bind to the molecular chaperone, glucose-regulated protein 78 (GPR78) (Qian, Harris, Zheng & Tiffany-Castiglioni, 2000) and lower the secretion of interlukin-6 (IL-6). Since IL-6 is neuroprotective, a decrease in this cytokine can increase the likelihood of neurotoxicity (White et al., 2007, review). In the indirect pathway, lead enters erythrocytes, binds with delta-aminolevulinic acid dehydratase δ-ALAD (the second enzyme in the heme biosynthesis pathway) and causes levels of its substrate, δ-ALA, to rise (Klaassen, 2006). Excessive δ-ALA in the brain blocks and reduces the number of glutamate receptors (Villayandre, Paniagua, Fernández-López, & Calvo, 2005). Additionally, δ-ALA can block glutamate transporters, i.e. GLT-1, and thus increase the levels of extracellular glutamate (Emanuelli, Pagel, Porciúncula, & Souza, 2003) which could induce chronic microglia activation and neurotoxicity (Murugan, Ling, & Kaur, 2013, review.)

Although lead exposure has the potential to alter a variety of brain mechanisms and brain regions via the indirect and/or direct pathways, because of the implications for learning, examining mechanisms that could disrupt hippocampal function are of critical importance. In fact, molecular studies have shown that the hippocampus is exquisitely sensitive to the effects of lead exposure (Du et al., 2015; Liu, Chen, Zhang, Kuang, & Chen, 2015; Selvin-Testa, Loidl, Lopez-Costa, Lopez, & Pecci-Saavedra, 1994).

1.2.2 The hippocampus as a memory center. The hippocampus is a brain structure that supports memory acquisition, storage, and retrieval. When new information is acquired by the brain, a "reverberating circuit" is activated. In this circuit, information travels first to the entorhinal cortex, then to the dentate gyrus (DG), then to the cornus ammonis (CA) 3, then to area CA1 of the hippocampus, and finally back to entorhinal cortex forming a loop (Brenan &

Keverne, 1997; Hebb, 1949). (The pathway is referred to as the "perforant pathway" because of its structure.) This circuit supports memory via activation of long-term potentiation (LTP) (Lynch, 2004). LTP refers to a process that strengthens electrical signals in specific pathways through repeated activation (Lynch, 2004). This process is a form of synaptic plasticity and is supported, initially, by the birth of new synapses (synaptogenesis) and later by the increase in the density of dendritic spines (protusions that are located on the dendrites of neurons, at the level of the synapse) (Engert & Bonhoeffer, 1994; Hasegawa, Sakuragi, Tominaga-Yoshino & Ogura, 2015.)

1.2.3 Effects of lead on the hippocampus. How chronic low-levels of lead alter the hippocampus is unknown. However *in vitro* and *in vivo* studies showed that a variety of mechanisms that support memory were altered at higher levels of lead exposure. For example, lead exposure has been shown to diminish synaptogenesis (Hu et al., 2014), decrease the density of dendritic spines (Ge et al., 2015), block glutamate receptors, i.e. N-methyl D aspartate receptors (NMDA receptors) (Neal, Worley & Guilarte, 2011), increase the spontaneous release of neurotransmitters (Braga, Pereira, Marchioro & Albuquerque, 1999), decrease the evoked release of neurotransmitters (Lasley & Gilbert, 1996), and reduce neurogenesis (Gilbert, Kelly, Samsan & Goodman, 2005) in the hippocampus. Within the hippocampus, microglia are often a focus of study because of their central role in pathway development and neurogenesis. At higher levels of exposure, lead has been shown to activate microglia in the hippocampus (Kumawat, Kaushik, Goswami, & Basu, 2014; Liu, M.-C., et al., 2012; Liu et al., 2015) and at lowest levels of exposure, in the DG (dentate gyrus) of the hippocampus, lead reduced the number of microglia cells while altering their morphological structure (Sobin et al., 2013). Microglia play multiple functions in the brain, and for this reason, changes in the number, morphology, and/or

function of microglia have profound implications for the early and perhaps life-long health of the brain. The multiple functions that microglia play in the brain are described below.

1.2.4 Functional roles of microglia in the brain. Microglial cells comprise the neuroimmune system and constitute from five to twelve percent of cells in the brain (Lawson, Perry, Dri, & Gordon, 1990). Microglial cells are the innate immune system of the brain and thus they are the first line of defense against an insult to the brain (Kreutzberg, 1995). These cells are phagocytes and can display antigens (molecules that can induce an immune response) on their surface (Magnus, Chan, Grauer, Toyka, & Gold, 2001). Microglial cells can be differentiated from other populations of innate immune system cells because they express high-levels of ionized calcium-binding adapter molecule (IBA) 1 (Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001), high levels of CD11b, and low-levels of CD45 (Derecki, Cronk, & Kipnis, 2012).

Microglial cells play different roles under non-pathological vs. pathological conditions (Prinz & Prieller, 2014). Under non-pathological conditions, microglial cells present a ramified form and are constantly surveilling the environment for a potential threat. On the other hand, under pathological conditions, microglial cells morph into an amoeboid form and travel to regions in the brain where a "threat" such as a toxin or a pathogen is detected. At the site of threat microglia release pro-inflammatory and/or anti-inflammatory cytokines which facilitate the removal of the detected threat and these might be beneficial or detrimental for the brain.

Microglial cells not only participate in neuroimmune processes, they also play an important role in brain development and memory. Studies have shown that throughout the life-span, microglial cells support synapse formation, brain pathway development, pruning, and neurogenesis (Paolicelli et al., 2011; Ransohoff & Cardona, 2010; Schafer et al., 2012).

Microglial cells are abundant in the DG (dentate gyrus) of the hippocampus (Sierra et al., 2010). The DG is one of three brain areas where neurogenesis occurs (Pignatelli & Belluzi, 2010) and as described earlier, the DG forms part of the circuit that supports memory and LTP (long-term potentiation) (Brenan & Keverne, 1997; Hebb, 1949).

Microglia cells are also known to support LTP (Justin et al., 2011), dendritic spine formation (Parkhurst et al., 2013), and the maturation of NMDA receptors at the postsynaptic terminals (Hoshiko, Arnoux, Avignone, Yamamoto & Audinat, 2012). Interestingly, studies have shown that microglial activation reduced LTP (Griffin et al., 2006; Cox, Carney, Miller & Lynch, 2012). Additionally, depleting the brain of microglia reduced the formation of postsynaptic dendritic spines and diminished memory (Parkhurst et al., 2013). Thus, via multiple processes, microglia contribute to brain development and synaptic plasticity, and thus memory.

1.2.5 Effects of lead on microglia. *In vitro* and *in vivo* studies showed that at highest levels of exposure, lead activated microglial cells and caused neuronal death in the hippocampus. For example, an *in vivo* study showed that in adult Wistar rats, chronic exposure to 100 ppm of lead increased microglial activation, reduced LTP, and increased neuronal death in the hippocampus (Liu, M.-C., et al., 2012). Similarly, an *in vitro* study showed that lead exposure activated microglial cells which resulted in neuronal death via activation of caspase 3 (Kumawat et al., 2014). Alterations in these mechanisms might be, in part, responsible for deficits in memory at high levels of lead exposure.

It is unknown however whether the above mentioned mechanisms are also altered at lowest levels of exposure. As stated previously, we must understand effects at lowest levels of exposure because child studies have repeatedly shown detrimental effects on memory at lowest

levels of exposure; and child exposure to chronic low-level lead is still likely to occur among children living in low-income neighborhoods (WHO, 2010).

Because of the sensitivity of microglial cells in the hippocampus to lead and because of the important role of microglia in neuroimmune processes and memory, in a recent study in our laboratory the effects of chronic low-level lead exposure were examined in microglial cells in the DG of the hippocampus of young C57BL/6J mice (Sobin et al., 2013). This is the only study that has exposed animals *in vivo* to chronic low-levels of lead (BLLs ranged from 2.66 to 20.31 µg/dL) and examined the number and volume of microglial cells in the DG (dentate gyrus) of the hippocampus of young mice. For this reason, and in order to understand effects in microglial cells at lower levels of exposure, the findings of this study guided the development of the hypotheses for this dissertation. This study is described in detail below.

In a study (Sobin et al., 2013), the number and volume of microglial cells in the DG of the hippocampus and expression of cytokines in whole brain of chronic low-level lead exposed and unexposed young mice were examined. Forty-six C57BL/6J mice were exposed to one of three levels of lead acetate, including 0 ppm (control; n = 10), 30 ppm (low-dose; n = 10) (mean BLL 2.64 µg/dL), and 330 ppm (high-dose; n = 10) (mean BLL 13.45 µg/dL). Lead was administered via dams' drinking water from PND 0 to 28. Heart blood was extracted for analyses of BLL with ICP-MS (method described on pages 52).

For microglial cell analyses, at PND 28, mice were sacrificed (n = 30), brains were perfused and harvested, and microglia were labeled using immunohistochemical methods (IBA-1). The number and volume of microglial cells were counted with a *Stereologer* ™ software (Stereology Resource Center, Chester, MD) and analyzed with two-way ANOVA (group x sex). When significant differences were detected, Tukey's post-hoc tests were used.

For cytokine expression analyses, at PND 28, mice were sacrificed (n = 16), brain tissue was harvested and frozen on dry-ice. At a later time, brain lysates were prepared with hippocampus and without hippocampus and were analyzed for neuroinflammatory markers including IL-6, IL-10, TNF-α, iNOS, HO-1, and GRP78. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to measure genetic markers of neuroinflammation. Data were analyzed with a three way (group x sex x brain section) ANOVA.

With regard to the number of microglial cells in DG, it was found that the low and high-dose groups had significantly fewer numbers of microglial cells as compared to controls, and that the high-dose group had fewer number of microglial cells in the DG as compared to the low-dose group and controls. With regard to the volume of microglial cells, it was found that the morphological structure as indicated by cell size was highly variable in the low-dose group as compared to cells in the control group; no significant difference between the control group and the high-dose group were found.

With regard to cytokine expression analyses, a significant decrease only in IL-6 was detected. Thus, these results did not support a general inflammatory model. Observed effects in gene expression studies however must be interpreted with caution. This is because a change in the level of a transcript does not necessarily result in a change in the level of the protein produced. In other words, transcripts can be degraded before they are even translated into a protein (Voge & Marcotte, 2012). Furthermore, transcript levels were averaged across different types of cells, thus it is not known which types of cells down-regulated IL-6.

To summarize, chronic low-level lead exposure reduced the number of microglia in the DG of the hippocampus and reduced IL-6 transcripts in whole brain. Because of the importance

of microglial cells in brain pathway development, pruning, and neurogenesis, a reduction in the number of these cells in the hippocampus are a plausible explanation for disruption of brain development and thus reduced memory function at lowest levels of early chronic lead exposure. Studies are needed to examine mechanisms that can explain why microglial cells were missing and morphologically altered in the DG of the hippocampus of chronic low-level lead exposed mice.

1.2.5.1 Why were the structure and number of microglia altered in the DG low-level lead exposed mice? An obvious explanation for results from the Sobin et al. (2013) study could be that microglial cells were missing in the DG because chronic low-level lead exposure resulted in microglial cell death and/or reduced proliferation. Given our current understanding however of microglial cell morphology (microglial cells become enlarged when they are activated), the ability of microglial cells to uptake lead particles readily and preserve their viability (Etemad, 2013), and increased proliferation of microglial cells when they are exposed to an environmental threat (Mander, Jekabsone & Brown, 2006), an alternative explanation might be more plausible. Recent findings by Iliff et al. (2012), Louveau et al. (2015), and Aspelund et al. (2015) that show the presence of a lymphatic system in the brain might suggest a means by which microglial cells are trafficked away from the DG in chronic low-level lead exposed animals and might explain why the number of microglial cells were reduced in the DG of the hippocampus.

1.3 The Lymph System

1.3.1 "Lymphatics." The lymph system is a critical component of the immune system. In the periphery, it includes lymphatic vessels and lymph nodes which are filled with lymph that is made up of interstitial fluid (ISF). ISF is fluid that exists throughout the extracellular environment and flows constantly between the tissues; it is collected into the lymph system through lymphatic capillaries. Lymph provides the means by which immune cells and solutes drain (Förster, Davalos-Misslitz & Rot, 2008, review).

When an immune cell from the innate immune system (e.g. a macrophage or a dendritic cell) detects a toxin or a pathogen, it phagocytoses (takes in) the toxin, presents an antigen on its own surface in the form of major histocompatibility complex (MHC) and upregulates the C-C chemokine receptor 7 (CCR7). Specifically, upregulation of CCR7 initiates migration of the macrophage and/or dendritic cell towards the chemokine ligands 19 and 21 (CCL19 and CCL21). These ligands are expressed by endothelial cells of lymphatic vessels and stimulate the migration of macrophages and/or dendritic cells into lymphatic vessels and lymph nodes. Once these cells enter the lymphatic vessels, they access the nearest lymph node and present MHC to T-lymphocytes (T-cells), thereby communicating that a tissue has sustained damage. T-cells can then be activated and might initiate an adaptive response (humoral or cell-mediated) that can be beneficial or detrimental for the tissue (Förster et al., 2008, review).

The lymphatic system in the periphery ("below the neck") has been well-characterized for hundreds of years and it has been assumed that the brain lacked classical lymphatic vessels (Torgan, 2015). However, recent studies have shown that cerebrospinal fluid (CSF) drains via the nasal mucosa and the dural sinuses into cervical lymph nodes (Liu, H., et al., 2012; Mathieu,

Gupta, Macdonald & Yücel, 2013). These studies raised questions regarding what the specific channels that drain CSF might be if in fact the brain lacked classical lymphatic vessels. Other studies began to characterize a "glymphatic system" in the brain (Iliff et al. 2012) and these important studies were followed by the astonishing recent discovery of the presence of meningeal lymphatic vessels by Louveau et al. (2015) and Aspelund et al. (2015). These findings are revolutionizing our understanding of a brain lymphatic system and provide entirely new means by which the immune system directly impacts the brain. These studies are described below.

1.3.2 The glymphatic system. In the absence of known lymphatic vessels in the brain Iliff et al. (2012) examined whether there is a system in the brain that could act similarly as lymphatic vessels in the periphery and clear solutes from the brain. Since it is known that the brain is encircled by CSF (meningeal fluid) and that ISF within the brain carries solutes and cells, the researchers investigated whether there was any channel that might drain these fluids and thus serve as lymphatic-like structures for the brain. In order to investigate this question, the direction of flow of CSF and ISF in the brain was examined.

To study CSF flow, in anesthetized mice, radio-labeled tracers were injected into the lateral ventricles or into the cisterna magna (located in the subarachnoid space of the meninges in the caudal region of the brain). After injections, the brains of the mice were retrieved, sliced with a vibratome, fixed, and examined with fluorescence imaging. It was found that 30 min after the injection into the lateral ventricles, the tracers localized in the zones surrounding the ventricles but not in distal ventricular zones or in the brain. On the other hand, it was found that 30 min after injection into the cisterna magna, the tracers moved ("flowed") into brain tissue via the spaces surrounding brain arteries (para-arterial spaces).

To confirm these findings, mice were examined *in vivo* with two-photon laser scanning microscopy to examine in real time the flow of CSF in cortical areas, and with an *ex vivo* model using double-transgenic mice (that allowed the visualization of arteries and veins) to examine the CSF flow within arteries and veins penetrating the brain.

The *in vivo* model showed that after injection of fluorescent tracers, the tracers localized in the para-arterial regions of the cortex. The *ex vivo* model showed that the tracers that were injected into the cisterna magna, moved almost immediately along para-arterial regions, starting by areas near the basal ganglia and thalamus. On the other hand, about one hour after injection, the tracers moved along surrounding veins (para-venous regions). Interestingly, it was found that the majority of the tracers exited the brain mainly along the outer sides of the medial internal cerebral veins and the lateral caudal ventral rhinal veins. ISF flow was also examined by injecting a tracer in the extracellular space of the brain, close to the basal ganglia and thalamus. Similar to the exit pathways of the CSF, it was found that the ISF exited via para-venous regions.

In summary, it was found that the para-arterial space, which is delimited by the end-feet of astrocytes, is filled with CSF. Arterial pulsations provide a pumping action on the outside of the artery that allows the CSF to recirculate throughout the brain (Hadazeck et al., 2006). On the other hand, the para-venous spaces also delimited by the end-feet of astrocytes serve as a drainage channel for both CSF and ISF and the researchers suggested that it drained ISF and CSF into cervical lymph nodes. Because this "system" is dependent on glial cells (astrocytes) and appears to serve the same function as that of the lymphatic system in the periphery (i.e. drainage of solutes, among other possible functions) the researchers named this system the "glymphatic system."

The findings by Iliff et al. (2012) were a great step towards understanding how the brain might drain solutes and perhaps immune cells into the periphery. The discovery of the glymphatic system stimulated other researchers to examine more thoroughly the vasculature of the brain in the meningeal spaces. This examination led to the historical discovery that lymphatic vessels in fact existed in the para-venous regions located in meninges. The meninges are protective tissue layers that lie around the brain and spinal cord. They comprise the outer dura mater, the arachnoid space, the sub-arachnoid space, filled with CSF, and the pia mater that is the inner layer of the meninges and is the layer most proximal to the brain (Carlson, 2010).

1.3.3 The meningeal (or dural) lymphatic system. Studies that discovered the existence of meningeal (or dural) lymphatics, published in June 2015, may be one of the most remarkable discoveries in neuroscience in the past century. Given the significance of these studies, they will be described in detail below.

In search for possible exit pathways of neuroimmune cells from the meningeal spaces into the periphery, C57BL/6J adult mice were sacrificed and their skulls and meninges were retrieved from the brain (Louveau et al., 2015). The meninges were then stained for markers of endothelial cells (CD31), T-cells (CD3e), and MHC II-expressing cells. The authors found that the majority of these cells were aligning along the dural sinuses (venous channels). These sinuses are known to drain blood from the external and internal veins of the brain into the internal jugular veins.

The authors then examined whether, along the dural sinuses, there was any structure that expressed classical lymphatic-vessel markers. The dural meninges were labeled with a lymphocytic endothelial cell marker, LVEY-1, and with the endothelial marker CD3. After labeling, a 2-photon microscope was used to examine the stained meninges. It was found that

some vessels were positive for these markers and were located in close proximity to the sinuses. In order to rule out the possibility that these vessels were simply venous vessels, before euthanasia, animals received an intravenous (i.v.) injection of DyLight 488 lectin. This injection revealed that the cells expressing LVEY-1 were not part of the vasculature. Additionally, the LVEY-1 vessels were examined for additional markers, including PROXY 1 (which is a transcription factor of lymphatic vessels), PDPN (a protein that is known to link lymphatic and blood vessels), the vascular endothelial growth factor receptor (VEGFR3), and CCL21 (a CCR7 chemokine ligand). Remarkably, these vessels were positive for all of the markers described above confirming that they were in fact lymphatic-like structures. The authors named these vessels "meningeal lymphatic" vessels. Visualization of these vessels showed that they were located throughout the transverse sinus, medial sagittal sinus, the olfactory bulb, and the eyes.

Given these findings, the functionalities of the meningeal lymphatic vessels were analyzed to examine whether they drained CSF and immune cells into the deep cervical lymph nodes (dCLNs). Adult mice were anesthetized and given an intravenous injection of fluorescein and an intracerebroventricular (i.c.v) injection of a fluorescent dye (QDot655). Thin skull sections were then examined visually with a multiphoton microscope and it was found that the meningeal lymphatic vessels were filled with QDot655 but not fluorescein. Later a simultaneous i.c.v. injection with QDot655 and Alexa 488 anti-LVEY1 showed that in fact, these vessels drained CSF.

Additionally, the presence of T-cells and MHC II-expressing cells were examined by labeling the cells with the antibodies described above. It was found that 25% of the T-cells and 14% of the MHC II-expressing cells located in the meningeal space were located within the meningeal lymphatic vessels. The meninges were also labeled for makers of CD11c (that stains

antigen presenting cells) and BB220 (a marker of B-cells). Cells expressing these markers were found in meningeal lymphatic vessels.

Finally, in order to test whether these vessels connected to the cervical lymph nodes and served as a drainage channel, i.c.v. injections of evans blue (a dye that is typically used to mark lymphatic vessels in the periphery) showed that within two hours post-injection, the dye stained the dCLNs (deep cervical lymph nodes) and a few hours later it stained the superficial cervical lymph nodes (sCLNs). Additionally, the dCLNs and sCLNs were resected or ligated. It was found that the resection of the dCLNs resulted in an accumulation of T-cells in the meningeal zone. Ligation of the dCLNs but not of the sCLNs resulted in an increase in the diameter of meningeal lymphatic vessels. These results suggested that the meningeal lymphatic vessels drained CSF and immune cells directly into the dCLNs but not the sCLNs. These findings in combination with previous findings from Iliff et al. (2012) (described above) for the first time provided nearly indisputable evidence that brain lymphatic-like structures exist.

In another study, the possible presence of brain lymphatics was examined in PROX1-GFP and *Vegfr3*$^{+/LacZ}$ reporter mice and wild-type (WT) mice (Aspelund et al., 2015). The skull, meninges, and brain were labeled with fluorescent antibodies including antibodies against LYVE1, PROX1, PDPN, CCL21, VEGFR3, and PECAM1 (a protein that is part of the junctions of the endothelial cells).

After staining, a network of lymphatic vessels was visualized in the meninges. These vessels expressed high levels of LYVE1, PROX1, PDPN, CCL21, VEGFR3, and low levels of PECAM1, which are markers that are characteristic of lymphatic vessels. In areas of the inner skull, these vessels were found to run along the transverse sinus, the retroglenoid vein, the rostral

rhinal vein, and the major branches of the medial and anterior meningeal arteries. In the superior outer skull, they were localized in the superior sagittal sinus, transverse sinus, rhinal veins, the transverse sinus, the rostral rhinal veins, and the middle meningeal artery. Other lymphatic vessels were observed running through the distal regions of cranial nerves. And in the dural lining of the cribriform plate and from the skull into the nasal mucosa. These results provided evidence that lymphatic vessels exist in the dura mater of the skull and drain outside of the skull through the foramina along the base of the skull, and surround the arteries, veins, and cranial nerves. The researchers named these vessels "dura mater" lymphatic vessels.

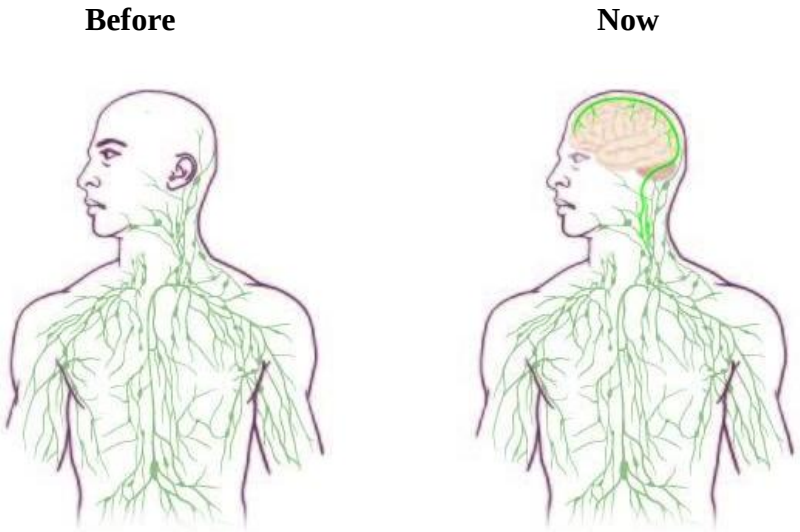
Once the researchers discovered the existence of these vessels, they investigated whether they drained to dCLNs and sCLNs, and if CSF and ISF could be transported through the dura mater lymphatic vessels. In order to examine this question, fluorescent tracers were injected in the brain parenchyma of PROX1-GFP mice. After two hours of the injection, it was found that these tracers entered through para-venous areas and into the CSF. Additionally, it was found that these tracers drained into the dCLNs but not the sCLNs. The tracer was followed upstream of the dCLNs and they appeared to drain from the base of the skull. Additionally, the efferent arm of the lymphatic vessels was ligated and it was found that the dura mater vessels had increased filling. The authors suggested that the dural lymphatic vessels absorb ISF and CSF and drain it to the dCLNs.

The *Vegfr3*$^{+/LacZ}$ reporter mice were also used to study what happens in the absence of a key molecule VEGFR3. They found that the lymphatic vessels on the superior and basal regions of the skull were missing but not in wild type (WT) mice. Also, contrary to what was expected mice did not have any sCLNs but they did have dCLNs. Additionally, whether the absence of these dura lymphatic vessels would have an effect on ISF pressure in the brain and water content

was examined. It was found that the absence of these vessels did not have any effect on this, which suggested that there might be other alternative routes of ISF efflux. Also, injection of a fluorescent dye in the cisterna magna revealed that transgenic (TG) mice had diminished draining in the dCLNs as compared to controls. The researchers concluded that the dura mater lymphatic vessels contribute to clear macromolecules from the brain.

To summarize, until June 2015, it was believed that there were no lymphatic vessels in the brain. These very recent discoveries have shown that these vessels exist in the dura mater of the meninges and are responsible for the drainage of cells into dCLNs, Figure 1 below.

Figure 1. Representation of the lymphatic system before and after the discovery of the meningeal lymphatic vessels.



Note. On the left (before) schematic of the long-held belief that lymphatic vessels do not exist in the brain. Recently, in June 2015, two independent laboratories showed that there are in fact lymphatic vessels in the brain. On the right (now) schematic of the meningeal or dural lymphatic vessels (Torgan, 2015). Image retrieved from: https://www.nih.gov/news-events/nih-research-matters/lymphatic-vessels-discovered-central-nervous-system

1.3.4 How are the "glymphatics" and "meningeal lymphatic vessels" related?

Details regarding how the "glymphatics" and "meningeal lymphatic system" are functionally related remains to be determined. Their anatomical proximity and similarity of carried fluids, namely CSF and ISF, suggest that these two systems are closely related and constantly exchange solutes and/or immune cells. It is logical to suggest that they serve functions very similar to that of peripheral lymphatic vessels, and thus drain immune cells into the dCLNs perhaps for antigen presentation (e.g. MHC II) to T-cells.

1.4 Can Microglia Traffic Towards Lymphatics?

Studies have shown that microglial cells upregulate proteins known to facilitate trafficking of cells in the peripheral immune system and in this way facilitate migration of these cells towards lymphatic vessels for antigen presentation to T-cells in adjacent lymph nodes. For example, similar to peripheral immune cells, microglial cells upregulated MHC II (major histocompatibility complex II) (Beyer, Gimsa, Eyüpoglu, Hailer, & Nitsch, 2000; Harms et al., 2013) and CCR7 (C-C chemokine receptor 7) in response to exposure to an antigen (e.g. liposaccharide) (Dijkstra, de Haas, Brouwer, Boddeke, & Biber, K., 2006). Also microglial cells expressing CCR7 migrate towards the ligand CCL21 (Takahashi, Rochford, & Neumann, 2005), which is expressed in meningeal (or dural) lymphatic vessels (Louveau et al., 2015; Aspelund et al., 2015) and in peripheral lymphatic vessels.

As described earlier, in the periphery, when a tissue is exposed to a toxin or a pathogen, innate immune cells (e.g. macrophages or dendritic cells) upregulate CCR7 and migrate towards its ligands CCL19 and/or CCL21 and access lymphatic vessels and lymph nodes. Therefore, it is plausible to hypothesize that after an insult to the brain (e.g. exposure to lead or increased brain

ALA), microglia upregulate MHC II and CCR7, migrate towards meningeal (or dural) lymphatic vessels and drain into dCLNs to communicate to T-cells that the brain tissue has sustained damage.

1.5 Lead-Exposed Immune Cells Might Traffic Towards Lymphatics

There are no studies examining whether chronic low-level lead exposure causes the upregulation of MHC II and CCR7 in microglia cells. Studies examining cells in the peripheral immune system however showed that at higher levels of lead exposure, macrophages (which are innate immune cells functionally similar to microglia) upregulated MHC II and primed T-cells towards a Th2 phenotype (humoral immunity, i.e. increased antibody production) (Dietert & Piepenbrink, 2006, review; Kasten-Jolly & Lawrence, 2014, review). This suggested that lead exposure caused the migration of macrophages towards lymphatic vessels and lymph nodes for antigen presentation of MHC II to T-cells. Since the peripheral immune system and neuroimmune system are more tightly linked than previously thought (Louveau, Harris, & Kipnis, 2015, review) it is plausible to suggest that chronic low-level lead-exposed microglial cells behave similarly as lead-exposed macrophages in the periphery.

1.6 Summary of Studies

Early chronic low-level lead exposure is still common in some lower-income neighborhoods throughout the U.S. and children are particularly vulnerable to its CNS effects. Memory deficits and effects on learning are perhaps of greatest concern and have been shown in both child and juvenile animal studies at lowest levels of lead exposure. It is necessary to develop new models for determining the mechanisms by which lowest level lead exposure alter the brain and specifically memory because no interventions are currently available for children

chronically exposed to low-level lead. Studies of both adult and young animals have shown that murine models are valuable for understanding memory effects in children at lowest levels of exposure.

Studies at higher levels of exposure have provided the groundwork for studies at lowest levels of exposure and have repeatedly shown the profound effects of lead on the CNS, the immune system and the neuroimmune system. Lead can be toxic for the brain via direct and indirect pathways. Lead can bind to the molecular chaperone GPR78 in astrocytes and reduce IL-6 (Qian et al. 2000); also lead can bind with δ-ALAD in erythrocytes and increase circulating and brain δ-ALA (Klaassen, 2006). Decreased IL-6 and increased δ-ALA are both potentially neurotoxic.

Understanding the specific effects of low-level lead in hippocampus is necessary because studies have shown that the hippocampus is exquisitely sensitive to the detrimental effects of lead exposure (Du et al., 2015) and memory is a foundational cognitive skill for early learning and for brain function throughout the lifespan. Studies have shown that lead altered a variety of mechanisms associated with memory formation in the hippocampus (Braga et al., 1999; Ge et al., 2015; Gilbert et al., 2005; Hu et al., 2014; Lasley & Gilbert, 1996; Neal et al., 2011). Studies have shown that lead exposure activated microglial cells which in turn reduced LTP (long-term potentiation), and caused neuronal death in the hippocampus (Liu et al., 2012; Kumawat et al., 2014). It is unknown however whether the above mentioned mechanisms are altered at lower levels of exposure and whether these mechanisms mediate memory deficits. Only one published study thus far has examined effects at lowest levels of exposure in microglial cells (Sobin et al., 2013) and showed abnormally reduced numbers of microglia in dentate gyrus. The specific mechanisms by which these effects occurred are unknown. Microglial cells are critical for

neural pathway development, neuroimmune protection of specific brain regions, and neurogenesis throughout the lifespan. Altering the number or distribution of these cells could have profound effects on brain development and neurocognitive function. Given that the CNS and neuroimmune system are now understood to be inter-dependent (Louveau et al., 2015, review) some of these deficits might be mediated by interactions between the CNS and the neuroimmune system (e.g. microglial cells).

The recent discoveries of the glymphatic system (Iliff et al., 2012) that drains CSF mainly along the medial internal cerebral veins and lateral caudal medial veins, and the discovery of the meningeal (or dural) lymphatic vessels by Louveau et al. (2015) and Aspleund et al. (2015) that are located in paravascular regions, provided the foundation for novel hypotheses regarding the trafficking and abnormal distribution of microglial cells following early chronic low-level lead exposure and the impact of these effects on memory. Whether microglial cells traffic to meningeal (or dural) lymphatic vessels and to dCLNs (deep cervical lymph nodes) as a result of chronic low-level lead exposure is not known. Studies examining effects of lead in peripheral immune cells showed that lead increased MHC (major histocompatibility complex) II in macrophages and predisposed T-cells towards a Th2 phenotype (Dietert & Piepenbrink, 2006, review; Kasten-Jolly & Lawrence, 2014, review). Thus in the periphery, macrophages migrate towards lymphatic vessels and lymph nodes for antigen presentation to T-cells. In the periphery, migration of macrophages to lymphatics occurs via the C-C chemokine receptor 7 (CCR7).

Given that microglial cells are functionally similar to macrophages, are capable of upregulating MHC II and CCR7 after they have been exposed to an antigen, and given that meningeal (or dural) lymphatic vessels lie in close proximity to the brain, it is plausible to suggest that as a result of chronic low-level lead exposure microglial cells upregulate MHC II

and CCR7 and migrate to meningeal (or dural) lymphatic vessels and to dCLNs for antigen presentation to T-cells. (If so, this could begin to suggest why microglial cells were missing in the DG of the hippocampus at lowest levels of exposure.)

Animal models of chronic low-level lead exposure are needed to further test memory and motor deficits and examine molecular mechanisms that could explain why microglia cells were reduced in the DG of young mice with early chronic low-level lead exposure.

1.7 Models and Hypotheses

The studies described in this project are interdisciplinary and translational, and include complementary behavioral and molecular studies.

1.7.1 Behavioral system model. For behavioral studies, thirty-six C57BL/6J male mice were exposed to one of three treatment levels including 0 ppm (control), 30 ppm (low-dose) and 430 ppm (high-dose) via dams' milk from PND 0 until PND 28. Mice were examined behaviorally at PNDs 7, 14, 21 and 28 for developmental milestones. Following on results from three previous studies conducted in our laboratory (described above on pages 16-21), at PND 28, olfactory memory and motor function were assessed. After behavioral testing animals were sacrificed and heart blood was retrieved for analyses of BLL with ICP-MS. It was hypothesized that chronic low-level lead exposure would delay the development of the nervous system, would reduce olfactory memory, and would disrupt basic motor functions in young C57BL/6J male mice.

1.7.2 Neuroimmune system model. To examine immune factors that could promote activation and trafficking of hippocampal microglial cells, thirty-six C57BL/6J mice were exposed chronically to low-levels of lead as described above. At PND 28, animals were

sacrificed, the cerebellum was removed, the two hemispheres were separated and left and right hippocampi were dissected. FACS (fluorescence-activated cell sorting) analyses were used to count the number of microglial cells in the hippocampus and examine the quantity of MHC II and CCR7 in these cells. It was hypothesized that MHC II and CCR7 in the hippocampal microglia of young C57BL/6J male mice would be upregulated.

CHAPTER 2

METHODS

2.1 Animals

The animal procedures used for this study were conducted in compliance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by the Institutional Animal Care and Use Committee (IACUC, Protocol #A201403-1). Mice of the strain C57BL/6J were purchased from Jackson Laboratories and housed and bred in the Bioscience Research Facility at the University of Texas at El Paso (UTEP). Male and female mice were group-housed by sex in ventilated cages (22.22 cm x 36.83 cm x 13.97 cm) and with *ad libitum* access to food and water; with a room temperature of 20º-26º C, relative humidity of 30 percent, and a 12 hour light-dark cycle.

At PND 60, 18 dams were mated with 9 sires (two females per male) by harem breeding. Pregnancy was determined by vaginal plug. When pregnancy was determined, females were removed to separate cages. Dams gave birth to litters of five to nine pups and culled to five pups per litter. For this study, males but not females were tested because past studies (Leasure et al., 2008; Flores-Montoya et al., 2015) showed that effects of chronic low-level lead exposure in males and females often differed. (Determining the mechanistic basis for these sex differences is of course important and future studies will examine effects in females.)

2.2 Lead Exposure

The methods for lead exposure that will be described have been previously shown by our laboratory to reliably produce low and high BLLs in the selected mouse strain and are published in peer reviewed journals (e.g. Flores-Montoya & Sobin, 2014; Flores-Montoya et al., 2015). From birth to PND 21, dams were exposed to one of three treatment conditions via drinking water. The control group was exposed to 0 ppm, the low-dose group was exposed to 30 ppm

(mean BLL 4 µg/dL), and the high-dose group was exposed to 430 ppm (mean BLL 21 µg/dL) of 99.4% pure lead acetate, Sigma Aldrich. Via this method, pups were exposed to lead via dams' milk. At PND 21, pups were weaned and placed in new cages (five mice per cage) and continued treatment exposure via water bottles until PND 28.

2.3 Behavioral Studies

Thirty-six mice were examined behaviorally, N = 36 (controls = 11, low-dose = 12, high-dose = 13). Specifically, they were observed for developmental milestones (eye-opening, body weight, and righting reflex) and were tested behaviorally with the inverted screen test (muscular strength), open field behavior test (exploratory behavior and anxiety), and odor habituation/dis-habituation test (olfactory memory). All mice completed behavioral testing for the open field task. Three mice however did not complete full testing for the rest of the tasks due to technical errors. The final groups included a minimum of 11 animals per experimental group per test (one more per group than numbers used in our previous studies showing significant group effects.)

At PND 28 and immediately after behavioral testing was completed, mice were anesthetized with Avertin (250 mg/kg) and heart blood was collected for analyses of BLLs with ICP-MS

2.3.1 Developmental milestones.

2.3.1.1 Eye-opening. C57BL/6J mice begin opening their eyes between PND 12 and PND14 (Sundberg, Smith, & John, 2002) with full eye-opening occurring PND 15-16. Mice were checked daily from PND 0 until they opened both eyes fully. The day of full eye opening was recorded for each mouse.

2.3.1.2 Body weight. Mice were weighed at PND 7, 14, 21, and 28 (day of behavioral testing).

2.3.1.3 Righting reflex test. This is a simple test of vestibular locomotor spatial displacement and overall body strength. Mice can be tested from PND 0 until they develop the righting reflex (DiDonato & Bogdanik, 2011). Because pups were exposed to lead via drinking dams' milk beginning at PND 0, the righting reflex test was conducted first at PND 7 to allow time for lead ingestion and distribution in experimental groups, and for three weeks thereafter at PND 14 and 21.

For testing righting reflex, each mouse was removed from its cage and body weight was measured and recorded. The mouse was then placed on its back and was lightly stabilized by the experimenters' index finger so that the mouse had its four paws up in the air. As the experimenter released the index finger hold, another experimenter, blind to the experimental condition, used a stopwatch to record the number of seconds required for the mouse to flip over onto its stomach with all four paws touching the base. Mice were allotted 60 sec to right themselves; all animals displayed a righting reflex within the allotted time.

2.3.2 Inverted screen test. This test was used to measure muscle strength. Mice have a natural tendency to grab with their four paws and to use their muscular strength to hold on to a screen that has been inverted. For this test, mice were placed individually on a $1/8^{th}$ inch metal mesh screen attached to a wooden frame box. The screen was then turned upside down and the latency to fall into an open field arena was recorded.

2.3.3 Open field test. This task was used to examine mouse exploratory activity and anxiety. In previous studies, our laboratory used a square arena with marked quadrants and observed no differences between experimental groups and controls. To explore the sensitivity of different approaches for these studies a circular Plexiglas arena (70 cm x 29 cm; area 3848 cm^2) was used and behaviors in the periphery and center of the arena were quantified. An

environmental cue consisting of a cardboard rectangle with vertical black and white stripes (38 x 30 cm) was placed on the lower left area of the arena. At PND 28, mice were placed in the circular arena and the SMART software system (Harvard PanLab Systems, Cambridge, MA) connected to a video camera and mounted on top of the arena was used to demarcate two circles on the floor of the arena, one in the periphery and one in the arena center. Mice were placed in the upper right area of the arena and were allowed to explore it for 5 min. Mice were then removed from the arena, placed in a holding cage and the floor of the arena was cleaned with 70% isopropyl alcohol to prepare for the next testing.

For measuring exploratory behavior and locomotor activity, the number of rears that mice displayed in the periphery (along the walls of the arena) and center of the arena; and the total distance travelled were quantified. For measuring anxiety, the distance travelled and time spent in the periphery (thigmotaxis) were quantified (Simon et al., 1994).

2.3.4 Odor habituation/dis-habituation test. This task was used for examination of olfactory memory. The methods used for this task followed published methods (Yang & Crawley, 2009). At PND 28, mice were removed from their cage, and placed individually in a new mouse cage scattered with home-cage bedding. The testing cage had a cotton-tip applicator attached to the dispenser hole of a water bottle. Animals were habituated to the new cage and the cotton-tip applicator for 30 min.

After habituation, animals underwent the testing trials. For these trials, mice were habituated and dishabituated to five odors consecutively. Three of these odors were non-social odors and two were social odors. Animals were exposed to the non-social odors via three cotton-tips that were dipped (for 2 sec) in distilled water, almond extract (McCormic® food-edible

product, 1:100 dilution), or orange extract (McCormic® food-edible product, 1:100 dilution). Animals were exposed sequentially to social odors via two cotton-tips that had been treated with two different unfamiliar social cage scents (social scent 1 and social scent 2). The social cage scents were obtained by dipping each cotton-tip (for 2 sec) in the bedding of one of two unfamiliar mouse cages.

Animals were tested in 15 consecutive trials (the complete test lasted 59 min per animal). Animals were first exposed for three consecutive trials, with an ITI (inter-trial interval) of 1 min, to a cotton-tip with distilled water (3 min, control exposure). Then they were exposed for three consecutive trails, with an ITI of 1 min, to a cotton-tip with almond, orange, unfamiliar social cage scent 1, and unfamiliar social cage scent 2 (3 min each for experimental exposures). After behavioral testing was completed for each mouse water bottles were cleaned with 70% isopropyl alcohol and fully dried.

A video camera was mounted at the level of the mouse cages and behavior was recorded. Odor exploration was measured by recording the number of seconds the animals had their noses within $\leq$ 2 cm of proximity to the cotton-tips. At a later time, video-taped behavior was examined by specially trained raters blind to experimental condition. Animals with an intact memory were expected to spend less time exploring the familiar odors (habituation) and they were expected to spend more time exploring a novel odor (dishabituation).

2.3.5 Blood collection. Immediately after behavioral testing, mice were weighed and anesthetized with Avertin (approximately 0.5 ml, depending on body weight). Once mice were unresponsive to corneal touch and paw pinch tests, 50 µL of heart blood per animal was retrieved via right auricle puncture. Blood samples were refrigerated for later ICP-MS analyses.

2.3.6 Inductively coupled plasma mass-spectrometry (ICP-MS) analyses of blood lead.

2.3.6.1 Instrumentation. For ICP-MS analyses an Agilent 7500ce ICP/MS equipped with an octople reaction system and a CETAX AS20 autosampler was used (Agilent, Santa Clara, CA). The samples were introduced into plasma using a Micro-Mist U-series nebulizer (Glass expansion, West Melbourne, Australia) and a double-pass quartz spray chamber. The parameters set for the instrument were carrier gas 0.78/1 min, make up gas 0.15 1/min, radiofrequency power 1420 W, and spray chamber temperature 2ºC.

2.3.6.2 Sample treatment analyses. Certified whole blood standards were analyzed (Le Centre du Toxicologie du Quebec, Canada) to establish the reproducibility of the instrument results. Sample blood standards were prepared as described (Agilent technical note no. 5988-0533 EN). A propylene tube was filled with 5.58 ml of water (18 M Ω DI: Labconco WaterPro PS Station, Kansas city, MO), 300 µl of whole blood, 60 µl of internal standard solution containing a 100 ppb each germanium, yttrium, and terbium in 5% nitric acid (Fisher Optima: ThermoFisher Scientific, Walthman, MA) and 60 µl aqueous 10 ppm gold in 3% hydrochloric acid solution (EMD, chemicals, Gibbstown, NJ). Internal standards were added to every sample to identify and correct instrument drift; internal standards were also used when building the calibration curve. The final internal solution was 20-fold, the final internal standard concentration was 1 ppb, and the final gold concentration was 100 ppb. A six-point external calibration curve was prepared from a lead concentration stock solution in 1% nitric acid. ICP-MS standard solutions containing the elements in 2% nitric acid were obtained from inorganic ventures (Christiansburg, VA). Samples were vortexed and centrifuged for 1 min at 2,000 rcf, and the supernatant was analyzed by ICP-MS.

2.3.7 Statistical analyses of behavioral data. All data was analyzed with SPSS *Version 23.0*. Data were entered, checked for accuracy, and examined for missing values, distribution properties, and outliers. Additionally, tests for homogeneity of variance and distribution properties were conducted in order to determine adequate statistical analyses for examining possible effects of lead exposure in observed differences and described in detail below in the relevant section.

2.3.7.1 Blood lead levels. One-way ANOVA was used to examine test differences between groups with regard to BLLs.

2.3.7.2 Developmental milestones.

Eye-opening. The day that mice opened their eyes was recorded and results were displayed as percentages. Chi-square test was conducted to test possible differences between groups.

Body weight (4 time points). Body weight was measured at four developmental time points including PNDs 7, 14, 21, and 28 (time of sacrifice). General linear model analyses with treatment group as the between subjects factor and PND (of body weight measurement) as the within subjects factor were used to examine possible significant main effects of treatment group and PND on body weight and the interaction (group x PND).

Righting reflex (3 time points). Righting reflex was measured at four developmental time points including PND 7, 14, and 21. General linear model analyses with treatment group as the between subjects factor and PND (of righting reflex measurement) as the within subjects factor were used to examine possible significant effects of treatment group and PND on righting reflex and the interaction (group x PND).

2.3.7.3 Inverted screen test (1 time point, PND 28). Muscle strength was measured at

PND 28. Independent samples median tests (non-parametric analyses) were used to examine possible differences between groups with regard to latency to fall from the inverted screen.

2.3.7.4 Open field test (1 time point, PND 28). Generalized linear model analyses were used to examine possible differences between groups with regard to outcome variables measured in open field. The Poisson distribution was specified for models predicting count data (number of rears). The Gaussian distribution was specified for models predicting continuous data (total distance travelled, distance travelled in the periphery, and time spent in the periphery).

2.3.7.5 Habituation/dishabituation test (1 time point, PND 28). General linear model analyses with treatment group as the between subjects factor and trial (of the odor) as the within subjects factor were used to examine possible significant effects of treatment group and trial on odor exploration and the interaction (group x trial).

2.4 Microglia Cell Study

Thirty-six male animals (controls n = 12, low-dose n = 12, and high-dose n = 12) were sacrificed at PND 28. Immediately after sacrifice, brains were retrieved and the left and right hippocampi were dissected. In order to ensure sufficient number of microglial cells for detecting possible significant differences between groups, the hippocampi and brains of three mice (from within each treatment group) were combined, yielding a total number of N = 12 samples (control samples n = 4, low-dose samples n = 4, high-dose samples n = 4). Hippocampal and brain microglial cells were examined with FACS (fluorescence activated cell sorting) analyses for possible differences between treatment groups with regard to percentage and number of cells and presence of MHC II and CCR7 on the cell membrane surface. Additionally, in order to better understand effects of lead on the neruoimmune system, exploratory analyses were conducted to

examine the same parameters described above in macrophages and percentages of immune cells (lymphocytes and monocytes) in hippocampus and brain.

2.4.1 Fluorescence-activated cell sorting (FACS) analyses

2.4.1.1 Apparatus. FACS is a technique used in flow cytometry that allows the sorting and counting of different types of cells by detecting the proteins that they express. Because cells within each animal are identical genetically but differ greatly with regard to the proteins that they express, cells can be sorted by anti-body labeling of the proteins/molecules that they uniquely express or express in higher or lower quantities as compared to other populations of cells.

FACS techniques can be used with cell lines or with primary cells extracted from whole organism tissue. Although much more challenging, for the purposes of these studies, we conducted the studies using primary cells from mouse hippocampus and brain. Briefly, the tissues of interest were first dissected, and the cells of the tissues were dissociated into singlets. After this, the proteins expressed in the cells of interest were labeled with primary antibodies that were directly conjugated to a fluorescent molecule (fluorophore). (Fluorophores of different colors must be carefully selected for labeling of different molecules of interest.) Labeled single cells were then run through a flow cytometer. One cell at a time entered a small nozzle with a laser beam passing perpendicularly through it. When a cell passed through the laser, it scattered light, and when a fluorophore passed through the laser, it excited the fluorophore's electrons. Once the electrons decayed to a ground state they emitted excess energy as a photon of light. The scattered light was collected by photomultiplier tubes and the data measuring the amount of light scattered and fluorescent properties were collected by a computer and displayed on a graph.

Using computer software for FACS, the cells were gated (selected) in the graph by demarcating the cells of interest and avoiding non-specific signals (that might be created simply by adding antibodies.) The graph then allowed the examination of the presence of the cells of interest by displaying the number of cells present, their size, and their internal complexity.

For the present study, the flow cytometer was adjusted to use a violet laser (excitation wavelength 405) with one detector; a blue laser (excitation wavelength of 488 nm) with two detectors; and a red laser (excitation wavelength 633 nm) with two detectors. The detector of the violet laser included zombie violet (peak wavelength of 423 nm); the blue laser included FITC (peak wavelength of 519 nm) and PE (peak wavelength of 612 nm). The detectors of the red laser included APC (peak wavelength of 660 nm) and Alexa Fluor 700 (peak wavelength of 719 nm). The adjustment of these parameters allowed the detection of five different fluorophores.

2.4.1.2 Procedure. The methods used for this study closely followed published methods (Derecki et al., 2012). At PND 28, each mouse was anesthetized and when the mouse was completely unresponsive, forceps were used to pull up the chest skin of the mouse and the skin was cut with sharp scissors exposing the chest. In order to eliminate blood contamination, mice were perfused transcardially with pH 7.4 0.1M ice cold PBS. After this, the brain and the hippocampus were dissected. The cells from the brain and hippocampus were then dissociated into singlets and were labeled with the antibodies of interest.

Brain extraction and hippocampal dissection. Briefly, the skull was stripped of soft tissues, the mandible and the skull were removed and the brain was immediately placed on a cold surface for dissection. The cerebellum was then separated from the brain and the two

hemispheres were separated using a scalpel. The left and right hippocampus were then visualized with a magnifying lamp and dissected with spatulas.

Single cell suspensions. Brain and hippocampi cells were gently dissociated in 6 ml of microglia media (1x PBS, 0.01 M HEPES, 3% FBS) by using the plunger of a 5 ml syringe and 70 micron screens. Cells from the brain and hippocampi were placed in separate 15 ml falcon tubes containing 3 ml of isotonic percoll and 1 ml of microglia media. Cells were then gently transferred using a Pasteur pipette into a 15 ml falcon tube containing 2 ml of 70% percoll and centrifuged at 500 g for 30 min (acceleration 9 with no break at room temperature). At the interface between isotonic percoll and 70% percoll the immune cells formed a visible a layer. The supernatant was then aspirated until 3 ml of liquid was left in the falcon tube. Using a 1 ml pipette cells were collected from the immune cell interphase layer and placed into FACS tubes. After this, percoll was washed from cells with 2 ml of microglia media and counted by hemocytometer.

Labeling of microglial cells with antibodies. Microglial cells were labeled with primary antibodies conjugated to a fluorophore. The selection of the antibodies for identifying microglial cells were determined following published methods (Cronk et al., 2015; Derecki et al., 2012; Louveau et al., 2015). Cells were labeled with a cocktail of antibodies that labeled dead cells and markers expressed by microglial cells (and macrophages only.) These antibodies were zombie dye (for labeling of dead cells) (1 µl), anti-mouse CD11b conjugated with PE (0.5 µl); anti-mouse CD45 conjugated with Alexa 700 (2.5 µl); anti-mouse MHC II conjugated with FITC (0.5 µl); and anti-mouse CCR7 (4B12) conjugated with APC (0.5 µl). The antibody concentrations were determined by titrating the antibodies on brain and hippocampus tissue.

Three controls were used to ensure appropriate detection of the cells of interest and markers of interest. The controls included an unstained sample of cells (used to determine whether the labeled cells emit higher fluorescence than the auto-fluorescence emitted by unstained cells) (see Appendix B), fluorescence minus one procedures (FMOs) to identify the gates for CD45, MHC II and CCR7 (see Appendix B), and an fc blocker was used to avoid non-specific binding of antibodies.

For establishing the unstained sample of cells, single cell preparations of the brain and hippocampi were run in FACS without prior manipulation (cells were not labeled with antibodies.) For establishing FMOs, single cell preparations from the brain were used. One FMO included PE CD11b, APC CCR7 (AB12), and FITC MHC II, but excluded Alexa 700 CD45 (this was used to establish the gate for microglial cells and macrophages). Another FMO included PE CD11b, Alexa 700 CD45, and FITC MHC II, but excluded APC CCR7 (4B12) (this was used to establish the gate for microglial cells and macrophages expressing CCR7); another FMO included PE CD11b, Alexa 700 CD45, APC CCR7 (4B12), but excluded FITC MHC II (this was used to establish the gate for microglial cells and macrophages for MHC II.)

Cells were first counted by hemocytometer and were adjusted to ≤ 200,000 cells/100µl per sample. Cells were then blocked for 15 min with fc blocker. After this, cells were labeled with the appropriate cocktail of antibodies for 30 minutes (at 4ºC) and were protected from light. Cells were then placed in FACS tubes and analyzed. All samples were run simultaneously in the flow cytometer (Gallios, Beckman Coulter Lifesciences, Indianapolis, IN) in the core facility of the Biosciences Research Building, UTEP.

In order to identify the cells of interest, cells were first gated into singlets and then immune cells of interest were detected and gated. After this, microglia and macrophages were identified via CD11b and CD45 positive markers; and then MHC II and CCR7 markers were gated (detailed explanation of gating procedures and figures is given below). Flow Jo *Version 10.30* was used to gate the cells of interest.

Gating strategy for single cells. Immune cells can form doublets and/or cluster, and this in turn can confound effects when examining cells in FACS. For this reason, doublets were identified and eliminated from the analyses. The strategy used for the present study for selecting single cells and eliminating doublets consisted of plotting the cells on the *x-axis* for forward scatter area (displaying the size and the area of the cells) and the *y-axis* for forward scatter height (displaying the size and the height of the cells). See Figure 2 below for a representative image of the gating strategy.

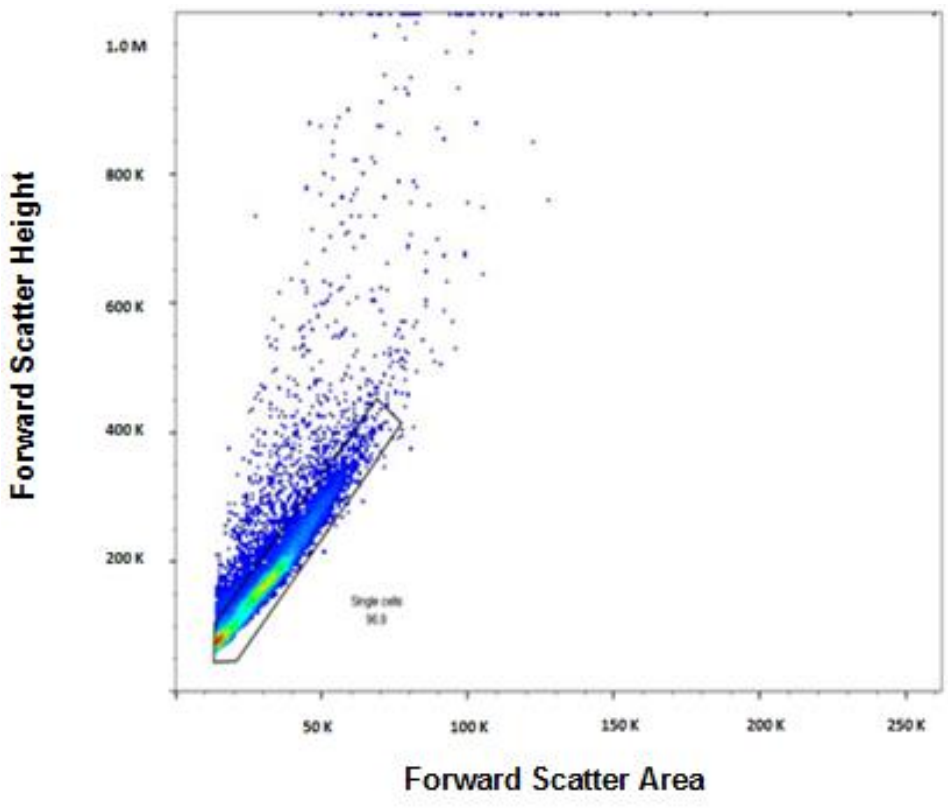


Figure 2. Representative image of gating strategy of single cells. Cells were plotted with regard to their area (*x-axis*) and height (*y-axis*). Single cells were selected based on the shape of the distribution. Cells that were closer together and formed an angle of approximately 45 degrees were selected. The rest of the cells (outliers) were not included in further gating strategies and analyses because they were most likely to represent doublets or clusters of cells.

Gating strategy for immune cells. Once single cells were selected, they were plotted on the *x-axis* for forward scatter area (representing the size and area of the cells) and on the *y-axis* for side scatter area (representing the internal complexity of the cells). See Figure 3 below for a representative image of the gating strategy of immune cells.

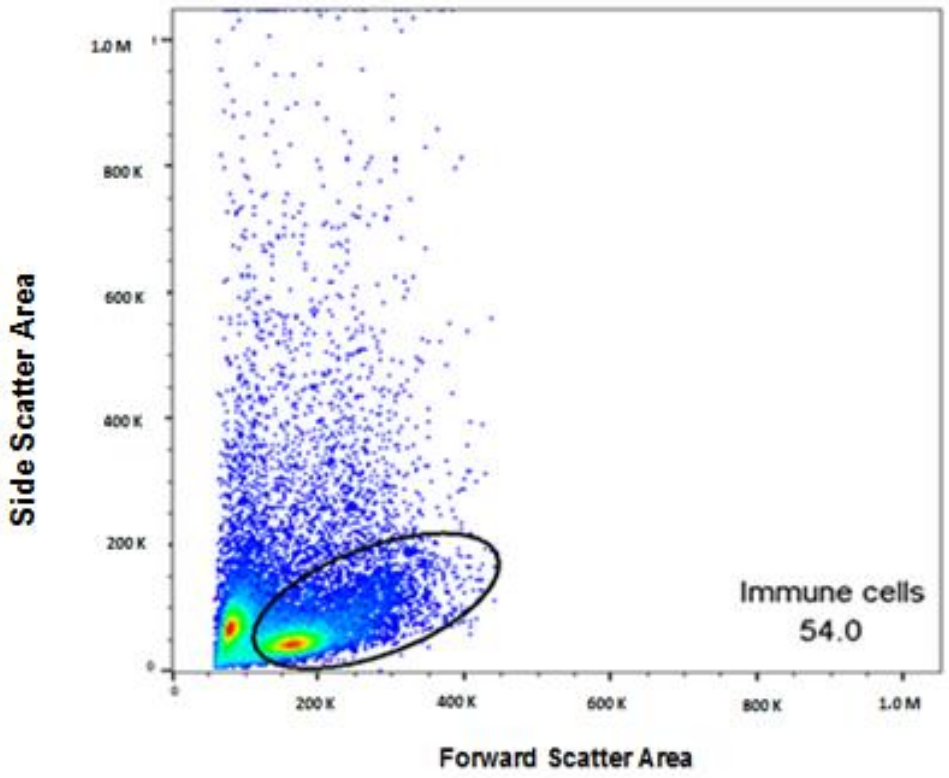


Figure 3. Representative image of gating strategy of immune cells. Cells were plotted for forward scatter area (*x-axis*) which represents the size of the cells and for side scatter area (*y-axis*) which represents the internal complexity of the cells. Cells that were not gated included dead cells (cells that were very small and/or had very high internal complexity) and granulocytes (cells that had large internal complexity). (Dead cells were excluded using this gating strategy instead of gating with zombie dye because zombie dye results suggested that the dye did not readily detect dead cells.)

Gating strategy for microglia and macrophages. Microglial cells can be distinguished from other immune cells because they have a high expression of CD11b (PE fluorophore) and low expression of CD45 (Alexa 700 fluorophore). On the other hand, macrophages have a high expression of CD11b and high expression of CD45 (Derecki et al., 2016). For this reason, macrophages and microglial cells can be identified by plotting CD11b and CD45 in the *x* and *y-axis*. For this study, CD11b expression was plotted on the *x-axis* and CD45 expression was plotted on the *y-axis*. Microglia and macrophages were then selected as described above. See Figure 4 below for a representative image of the gating strategy for microglia and macrophages.

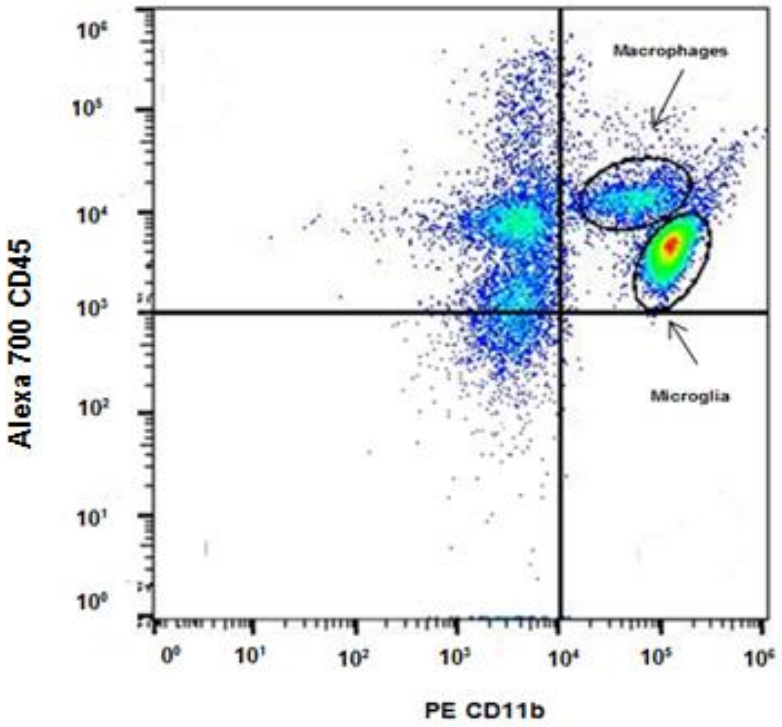


Figure 4. Representative image of gating strategy for identifying microglia and macrophages. Immune cells were plotted on the *x-axis* for PE CD11b and on the *y-axis* for Alexa 700 CD45. The images shown are highly similar to others in the literature illustrating the distributions of these cells (Wlodarczyk et al., 2015). Gated cells that were high in CD11b and lower in CD45 were selected as microglia. Gated cells that were high in CD11b and higher in CD45 were selected as macropahges.

Gating strategy for MHC II and CCR7 "single positives" and MHCII/CCR7 "double positives" in microglia and macrophages. Once microglia and macrophages were gated, each type of cell was plotted on the *x-axis* for CCR7 expression (APC fluorophore) and on the *y-axis* for MHC II expression (FITC fluorophore). See Figure 5 below for a representative image of the gating strategy for microglia MHC II and CCR7 and Figure 6 for a representative image of the gating strategy of MHC II and CCR7 on macrophages.

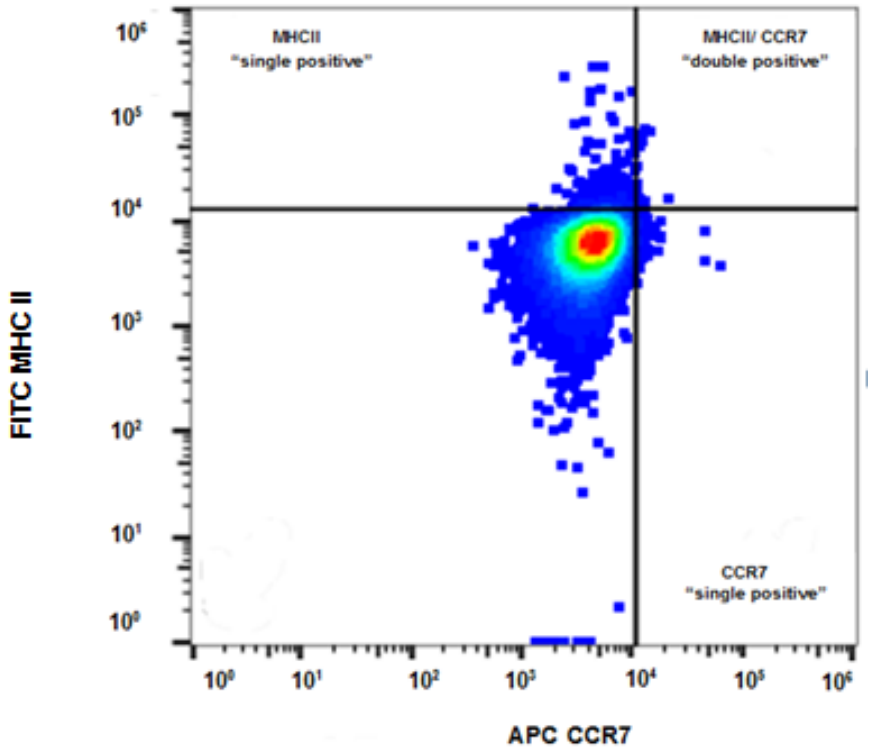


Figure 5. Representative image of gating strategy for identifying MHC II "single positve", CCR7 "single positive", and "double positive" cells (MHCII/CCR7) in microglia. Microglial cells were plotted on the *x-axis* for APC CCR7 expression and on the *y-axis* for FITC MHC II expression. The CCR7 "single positive" quadrant represents cells that express CCR7 but not MHC II. The MHC II "single positive" quadrant represents cells that express MHC II but not CCR7. The MHCII/CCR7 "double positive" quadrant represents microglial cells that simultaneously express MHCII and CCR7.

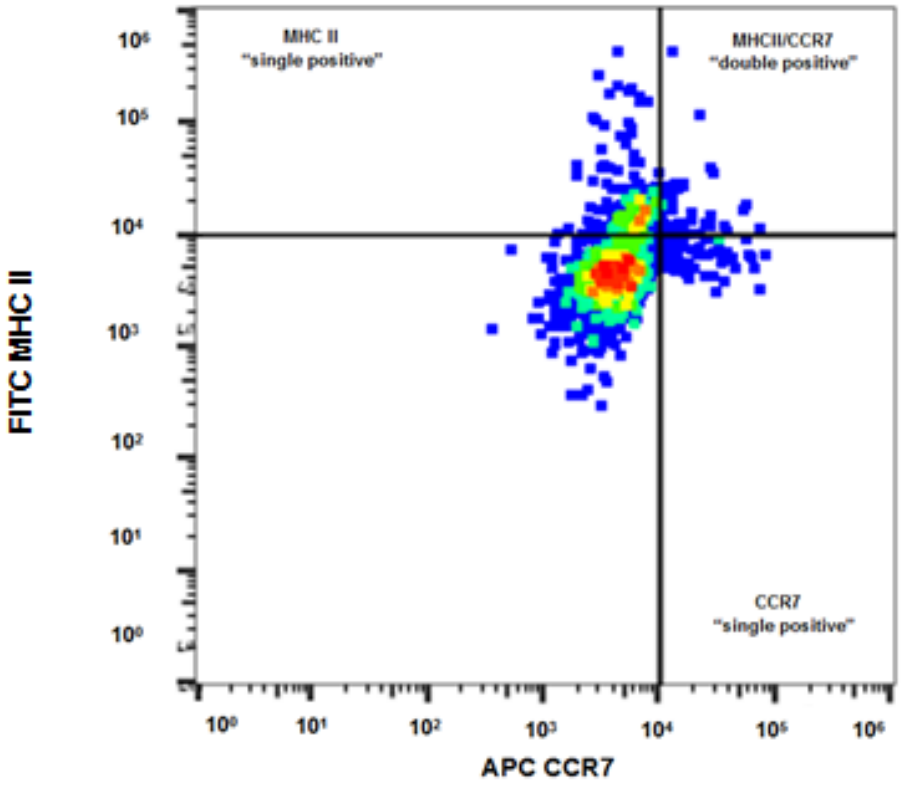


Figure 6. Representative image of gating strategy for identifying MHC II "single positve", CCR7 "single positive", and "double positive" cells (MHCII/CCR7) in macrophages. Macrophages were plotted on the *x-axis* for APC CCR7 expression, and on the *y-axis* for FITC MHC II expression. The CCR7 "single positive" quadrant represents macrophages that express CCR7 but not MHC II. The MHC II "single positive" quadrant represents macrophages that express MHC II but not CCR7. The MHCII/CCR7 "double positive" quadrant represents macrophages that simultaneously express MHCII and CCR7.

2.4.2 Outcome variables measured. It was hypothesized that low-level lead exposure would cause the up-regulation of MHC II and CCR7 in hippocampal microglia of pre-adolescent mice. The outcome variables measured for the primary hypotheses and also exploratory analyses examining possible effects of chronic low-level lead exposure on macrophages and immune cells (lymphocytes and monocytes) are described below.

2.4.2.1 Microglia in hippocampus and brain. Thirteen outcome variables were measured for microglia in hippocampus and brain. These variables included microglia percentage of parent population (number of microglial cells gated relative to total number of immune cells gated), microglia number, microglia MHC II and CCR7 mean fluorescence intensity (MFI, measured as geometric mean); "single positive" MHC II percentage of parent population, number, and MFI; "single positive" CCR7 percentage of parent population, number, and MFI; and "double positive" (MHC II/CCR7) percentage, number, and MFI (see Figure 5).

2.4.2.2 Macrophages in hippocampus and brain. Thirteen outcome variables were measured for macrophages in hippocampus and brain. These variables included macrophage percentage of parent population (number of macrophages gated relative to total number of immune cells gated), macrophage number, macrophage MHC II and CCR7 MFI; "single positive" MHC II percentage of parent population, number, and MFI; "single positive" CCR7, percentage of parent population, number (count), and MFI; and "double positive" (MHCII/CCR7) percentage, number, and MFI (see Figure 6).

2.4.2.3 Immune cells in hippocampus and brain. Two outcome variables were measured for immune cells in hippocampus and brain. These variables included immune cell percentage of parent population (number of immune cells gated relative to total number of cells in the sample) and immune cell number (see Figure 3).

2.4.3 Statistical analyses of FACs data. After gating the cells of interest, the percentage, count, MFI, and coefficient of variation for each of the outcome variables described above were obtained with Flow Jo *Version 10.30*. Data were entered, checked for accuracy, and examined for missing values, distribution properties, and outliers and analyzed with SPSS *Version 23.0*. Tests of homogeneity of variance and normality distribution were conducted (see Appendix A) and guided the selection of the statistical analyses for each outcome variable.

As is common for FACS data, tests of homogeneity of variance and distribution properties revealed that data was highly variable and non-normally distributed. For this reason, non-parametric tests were used to examine possible statistical significant differences between groups. For examining possible differences in percentage of parent populations, chi-square tests were run. When significant effects were detected in chi-square tests, independent samples median tests were run to determine which comparisons accounted significant model effects. For examining outcome variables other than percentages, independent samples median tests were run to examine possible significant differences between groups.

CHAPTER 3

RESULTS

3.1 Behavioral Studies Results

Results of first set of behavioral studies examining effects of early chronic low-level lead exposure in pre-adolescent mice, and providing the foundation for the current work, were described in the introduction and served as the basis for the additional behavioral studies here described. To summarize, in previous published studies, early chronic low-level lead exposure was associated with decreased exploratory activity measured on the unbaited nosepoke task (Flores-Montoya & Sobin, 2014) and decreased novel odor recognition in a two-choice paradigm (Flores-Montoya et al., 2015). In addition, an object in place task revealed significant differences in rearing during memory retrieval suggesting memory retrieval disruption in young mice (Sobin et al., 2017) (see Appendix C).

The purpose of the second set of behavioral studies here described was to explore whether any other paradigms, or modifications of our previous paradigms, would increase sensitivity to effects at preadolescence of early chronic low-level lead exposure. For these studies, we tested whether developmental milestones (body weight, eye-opening, and righting reflex), muscle strength measured with the inverted screen test, open field behavior in a large circular (rather than smaller square) arena, and/or odor habituation/dishabituation at pre-adolescence (PND 28) in C57BL6/J mice would improve detection of outcomes following early chronic low-level lead exposure. It was hypothesized that lead exposure would delay the development of the nervous system, reduce olfactory memory, and disrupt basic motor functions in young male C57BL/6J mice.

3.1.1 Descriptive statistics for blood lead levels (BLLs) and behavioral outcomes.

Below is a summary of descriptive statistics for BLLs and behavioral outcomes in young C57BL/6J mice with and without early lead exposure. (Statistical tests of observed differences are given in the following section.) Data were examined for homogeneity of variance and distribution properties. The selection of tests for evaluating statistical significance in observed differences was based on the variability and distribution properties of the outcomes measured. (See Appendix A, Tables 25-29 for detailed results from tests of homogeneity of variance and distribution properties.)

3.1.1.1 BLLs. As shown in Table 3 below, the low-dose and high-dose groups had higher mean BLLs as compared to controls. Similarly, the high-dose group had higher mean BLLs as compared to the low-dose group.

Table 3. BLLs means and standard deviations (SDs) in young C57BL/6J mice with and without early lead exposure.

Lead exposure group	Blood lead levels (µg/dL)
0 ppm	0.556 ± 0.4980
30 ppm	4.24 ± 0.61
430 pm	21.30 ± 7.03

3.1.1.2 Developmental milestones.

Eye-opening. As shown in Table 4, 27% of animals in the low-dose and control groups opened their eyes at PND 15 and 73% of animals in each of these groups opened their eyes at PND 16. Among high-dose animals, 100% opened their eyes at PND 16. (Chi-square test was not statistically significant, χ^2 (df = 2) = 3.67, p > 0.05).

Table 4. Eye-opening in C57BL/6J mice with and without early lead exposure.

Eye-opening day	0 ppm n = 11	30 ppm n = 11	430 ppm n = 11
PND 15	27%	27%	0%
PND 16	73%	73%	100%

Body weight. As shown in Table 5 below, for PND 7 and 14, groups had very similar mean body weights. For PND 21 and 28 however the low-dose group appeared to have higher mean body weight as compared to the control and high-dose groups. At PND 28, the high-dose group appeared to have decreased mean body weight as compared to the control group.

Table 5. Body weight means and SDs of young C57BL/6J mice with and without early lead exposure.

	0 ppm n = 11	30 ppm n = 11	430 ppm n = 11
Body Weight			
PND 7	3.44 ± 0.90	3.97 ± 0.29	3.79 ± 0.43
PND 14	7.78 ± 0.64	7.56 ± 0.74	7.39 ± 1.42
PND 21	9.03 ± 0.63	10.23 ± 0.49	9.32 ± 1.59
PND 28	14.75 ± 1.81	16.37 ± 0.69	13.68 ± 1.64

Righting reflex. As shown in Table 6, at PND 7, 14, and 21 lead-exposed mice appeared to be faster in displaying the righting reflex as compared to controls. This apparent difference was especially pronounced at PND 7, and less apparent as the animals aged.

Table 6. Means and SDs for righting reflex in young C57BL/6J mice with and without early lead exposure.

	0 ppm n = 11	30 ppm n = 11	430 ppm n = 11
Righting reflex test			
PND 7	9.28 ± 12.14	2.77 ± 2.48	3.74 ± 3.30
PND 14	0.79 ± 0.30	0.64 ± 0.16	0.43 ± 0.18
PND 21	0.26 ± 0.05	0.19 ± 0.04	0.22 ± 0.07

3.1.1.3 Inverted screen test. As shown in Table 7 below, at PND 28 mice in the low-dose group appeared to have the shortest latency to fall (fell most quickly) from the inverted screen as compared to controls and high-dose mice. Additionally, high-dose animals appeared to have a shorter latency to fall from the inverted screen as compared to controls.

Table 7. Means and SDs for latency to fall from inverted screen test in young C57BL/6J mice with and without early lead exposure.

	0 ppm n = 11	30 ppm n = 11	430 ppm n = 11
Latency to fall	69.54 ± 59.03	22.04 ± 18.84	41.16 ± 44.85

3.1.1.4 Open field

Rearing in periphery. As shown in Table 8, low-dose animals displayed approximately 32% fewer rears as compared to controls; and 23% fewer rears as compared to high-dose animals, in the periphery of the arena. Similarly, high-dose animals displayed approximately 12% fewer rears as compared to controls.

Rearing in center. As shown in Table 8, low-dose mice reared approximately 58% less as compared to controls, and 63% more as compared to high-dose animals in the center of the arena. Also, high-dose mice reared approximately 75% less as compared to controls.

Total distance travelled. As shown in Table 8 below, low-dose animals traveled approximately 1% more in the open field arena as compared to controls and approximately 4% less as compared to high-dose animals. High-dose animals traveled approximately 11% more in the periphery as compared to controls.

Distance in periphery. As shown in Table 8 below, low-dose animals traveled approximately 4% more as compared to controls whereas low-dose mice travelled approximately 7% less as compared to high-dose animals.

Time in periphery. As shown in Table 8 below, low-dose animals spent approximately 5% more time in the periphery as compared to controls and 3% less time as compared to high-dose animals. High-dose animals spent approximately 8% more time in the periphery as compared to controls.

Table 8. Means and SDs for open field behaviors in young C57BL6/J mice with and without early lead exposure.

	0 ppm n = 11	**30 ppm** n = 12	**430 ppm** n = 13
Rear number			
Periphery	34.18 ± 1.76	23.08 ± 1.38	29.92 ± 1.52
Center	1.82 ± 0.41	0.75 ± 0.25	0.46 ± 0.188
Distance Traveled			
Total	4092.13 ± 157.69	4132.29 ± 150.98	4309.85 ± 145.06
Periphery	3034.5 ± 118.7	3188.09 ± 113.68	3401.73 ± 109.22
Time in Periphery	0.7716 ± 0.018	0.8118 ± 0.018	0.8343 ± 0.017

3.1.1.5 Odor habituation/dishabituation task.

Habituation. The habituation trials of this task tested how quickly animals habituated to an odor presented to them in three sequential trials. Decreased exploration time of a familiar odor indicated habituation.

Non-social odors. As shown in Table 9, all mice decreased exploration of the cotton swab from trial 1 to trial 3 for water and almond. Specifically, for water, exploration time decreased by about 82% for control mice, 77% for high-dose mice, and 63% for low-dose mice. For the almond scent, exploration time decreased approximately 70% for low-dose mice, 63% for control mice, and 36% for high-dose mice. For the orange scent, only high-dose animals decreased their exploration time (approximately 30%) whereas low-dose and control animals increased their exploration time by about 1%.

Social odors. As shown in Table 9, all mice decreased exploration of the non-social odors from trial 1 to trial 3, with the exception of the control animals which had increased exploration of social scent 2. For social scent 1, exploration time decreased approximately 77% for low-dose mice, 75% for high-dose mice, and 61% for control mice. For social scent 2, exploration decreased by about 45% for high-dose mice and 25% for low-dose mice, while exploration increased by approximately 57% for control mice.

Dishabituation. These dishabituation trials tested whether mice differentiated between a habituated odor (Trial 3 of a habituated/familiar odor) and a new (Trial 1 of the changed scent). Increased exploration time of the new odor meant recognition of novelty and thus odor dishabituation.

As shown in Table 9, control animals appeared to increase their exploration by approximately 75% from water Trial 3 to almond Trial 1 and high-dose animals increased their exploration by about 25%. On the other hand, exploration time decreased by approximately 56%

in low-dose animals. From almond Trial 3 to orange Trial 1 control and low-dose animals had a small increase in exploration whereas high-dose animals had a decrease in exploration. For orange Trial 3 to social scent 1 Trial 1, exploration time increased approximately 10-fold in high-dose animals, 2-fold in control animals whereas low-dose animals only increased exploration by about 44%. With regard to exploration from social scent 1 Trial 3 to social scent 2 Trial 1, an increase of approximately 5-fold was observed for low-dose animals and approximately 5% for high-dose animals. On the other hand, control animals had a decrease in exploration of about 17%.

Table 9. Means and SDs for odor exploration time in habituation/dishabituation task in young C57BL/6J mice with and without early lead exposure.

Group		0 ppm n = 11	30 ppm n = 11	430 ppm n = 11
Water	Trial 1	15.42 ± 10.93	19.69 ± 26.61	16.66±22.58
	Trial 2	6.64 ± 4.28	7.79 ± 5.87	8.44±9.49
	Trial 3	2.80 ± 3.09	7.31 ± 8.42	3.77±4.56
Almond	Trial 1	4.92 ± 5.16	3.22 ± 1.88	4.71±6.36
	Trial 2	1.35 ± 2.16	2.95 ± 2.29	1.94±2.14
	Trial 3	1.83 ± 3.03	0.95 ± 1.45	3.03±3.34
Orange	Trial 1	2.29±4.09	2.03±2.55	1.24±1.98
	Trial 2	0.61±1.31	0.46±0.63	0.90±1.51
	Trial 3	2.32±3.54	2.23±6.65	0.88±2.45
Social Scent 1	Trial 1	7.87±12.73	3.21±10.33	10.93±16.61
	Trial 2	3.97±9.18	4.56±12.08	3.80±5.92
	Trial 3	3.05±6.23	0.71±2.12	2.77±4.73
Social Scent 2	Trial 1	2.52±8.37	5.56±8.52	2.91±4.39
	Trial 2	2.63±5.69	3.20±6.44	2.83±4.99
	Trial 3	3.97±11.19	3.17±5.55	2.17±4.7

3.1.2 Tests of homogeneity of variance and distribution properties and selection of inferential statistics.

The descriptive statistics above illustrate high variability and non-normal distributions for many of the behavioral outcome variables. Tests examining the assumptions of homogeneity of variance and distribution properties were conducted in order to determine the appropriate statistical tests (parametric vs. non-parametric) to use for testing observed differences. Specifically, for tests examining possible between group differences measured at one time point (i.e., PND 28) including the screen strength test and open field behavior, Levene's test statistic was used to examine the assumption of homogeneity of variances and Kolmogorov Smirnov (KS) test statistic was used to examine the assumption of normality of distribution. Additionally, for tests examining possible effects of lead exposure measured at multiple developmental time points (i.e., body weight, righting reflex, and odor habituation/dishabituation task) Mauchly's test of sphericity was used to examine the assumption of homogeneity of variance. This tested whether the variances across time points (within subjects) were equal; and KS tests were calculated to examine the assumption of normality for these outcomes (see Appendix Tables 25-29.) In some instances, when the assumption of homogeneity of variance was met but assumptions of normality were not met, general linear model was selected because it tends to be robust for violations of assumptions of normality.

3.1.2.1 Developmental milestones

Body weight. Mauchly's test of sphericity did not suggest unequal variances. Similarly, KS test statistic did not reveal significant deviations from normality, with the exception of PND 14 (low-dose group, see Appendix A, Table 27). General linear model analyses examining main effects of group and PND on body weight and the interaction (group x PND) were used to examine possible effects of treatment group and PND on body weight.

Righting reflex. Mauchly's test of sphericity revealed unequal variances. For this reason, the Greenhouse-Geisser correction was used for tests examining possible significant effects of treatment group and PND on righting reflex. Also, the KS test statistic did not reveal deviations from normality at PND 14, however it revealed deviations from normality at PND 7 and PND 21 (see Appendix A, Table 27). General linear model analyses examining main effects of group and day of testing and the interaction (group x PND) were run to examine possible effects of lead exposure on righting reflex.

3.1.2.2 Inverted screen. Levene's test indicated unequal variances, F (df = 2) = 4.77, $p < 0.05$. Additionally, KS test revealed that data in the low-dose group did not follow a normal distribution, F (df =11) = 0.325, $p < 0.01$. For this reason, a non-parametric test, the independent samples median test, was used to examine possible differences between treatment groups with regard to muscular strength.

3.1.2.3 Open field. The KS test did not reveal significant differences between groups with regard to normality of the distribution for continuous data variables (distance travelled and time). As expected, for count data variables (number of rears in the center of the open field), KS revealed that data were not normally distributed (see Appendix, Table 27). For this reason, a generalized linear model with Poisson distribution was used to examine possible differences between groups for count data (rears); a Gaussian distribution was used to examine possible differences between groups for continuous data (distance travelled and time.)

3.1.2.4 Odor habituation/dishabituation task. Mauchly's tests of sphericity revealed that sphericity was violated for all trials with the exception of social scent 2. For this reason, the Greenhouse-Geisser correction was used in tests of possible significant effects. The KS tests revealed that some variables were normally distributed and others were not. Specifically,

deviations from normality were obvious for the social odor task (see Appendix A, Tables 28 and 29). Possible significant differences between groups and within groups in odor exploration during habituation and dishabituation trials were examined with general linear model analyses with group and trial as main effects and the interaction (group x trial).

3.1.3 Results of inferential tests of behavioral outcomes.

3.1.3.1 Blood lead levels: demonstration of the exposure model. One-way ANOVA detected statistically significant differences between groups in BLLs at time of sacrifice (PND 28), F (df = 2, 36) = 82.99, $p < 0.01$. Post-hoc analyses with LSD revealed that the low-dose group had significantly higher BLLs as compared to the control group, (*M diff* = 3.68, *SEM* = 1.78, $p < 0.05$), and significantly lower BLLs as compared to the high-dose group, (*M diff* = 17.06, *SEM* = 1.71, $p < 0.01$). Similarly, the high-dose group had significantly higher BLLs as compared to the control group (*M diff* = 20.75, *SEM* = 1.74, $p < 0.01$) (see Figure 7).

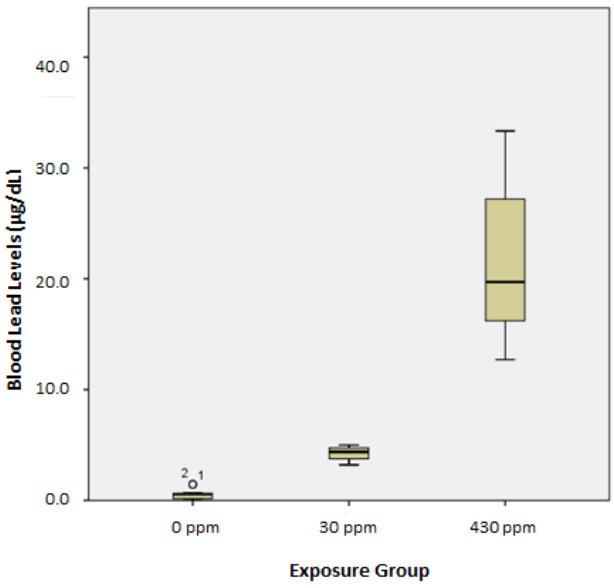


Figure 7. Blood lead levels measured in µg/dL in C57BL/6J young mice with and without early chronic lead exposure.

3.1.3.2 Developmental milestones.

Body weight. General linear model analyses revealed a significant main effect of group on body weight, F (df = 2, 30) = 9.48, $p < 0.01$, a significant main effect of PND on body weight, F (df = 2, 60) = 347.32, $p < 0.01$, and the interaction (group x PND), F (df = 4, 60) = 4.05, $p < 0.01$. For main effect of PND on body weight, it was found that mice weighed more as they aged, from PND 14 to PND 21 (*M diff* = 0.87, *SEM* = 0.29), $p < 0.06$, and weighed more from PND 21 to PND 28 (*M diff* = 0.824, *SEM* = 0.25), $p < 0.03$. For the main effect of group on body weight, mice in the low-dose group weighed significantly more as compared to controls (*M diff* = 0.86, *SEM* = 0.29), $p < 0.01$, and as compared to high-dose mice (*M diff* = 1.25, *SEM* = 0.29), $p < 0.01$. No significant difference was detected between the high-dose and control groups. With regard to the interaction, it was found that mice in the low-dose group gained significantly more weight than mice in the control group and high-dose group from PND 21 to PND 28, F (df = 2, 60) = 5.37, $p < 0.01$. (See Figure 8, below and Appendix A Table 1).

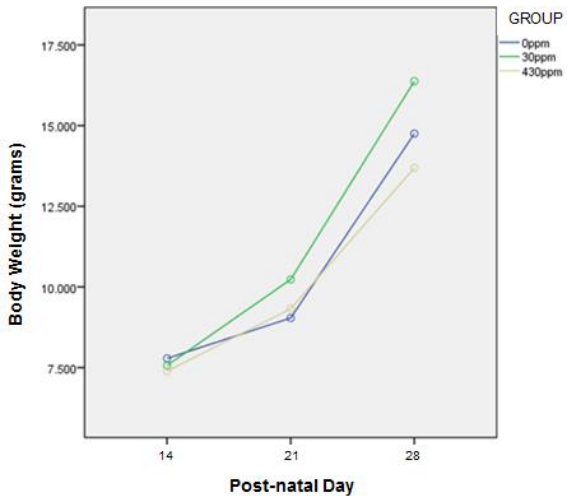


Figure 8. Body weights at PNDs 14, 21, and 28 measured in young mice with and without early lead exposure.

Righting reflex. General linear model analyses detected a significant main effect of PND on righting reflex, F (df = 2, 60) = 14.31, $p < 0.01$. Specifically, it was found that all mice gained speed in accomplishing the righting reflex from PND 7 (M = 5.27, SEM = 1.28) to PND 14 (M = 0.622, SEM = 0.039), and from PND 14 to PND 21 (M = 0.22, SEM = 0.01). No significant main effects however were detected for group, or for the interaction (treatment x PND) (see Figure 9 below and Appendix A Table 2).

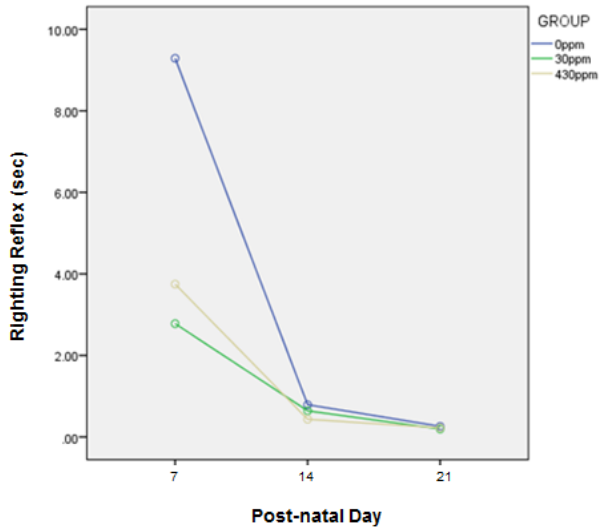


Figure 9. Righting reflex measured at PNDs 14, 21, and 28 in young mice with and without early lead exposure.

3.1.3.3 Inverted screen test. Independent sample median test did not detect significant differences between groups.

3.1.3.4 Open field test.

Rearing in periphery. Generalized linear model analyses revealed significant differences between groups with regard to rearing number in the periphery of the open field, X^2 (df = 2) = 25.58, $p < 0.01$ (controls M = 34.18, SEM =1.76; low-dose M = *23.08*, SEM = *1.38*; high-dose M = 29.92, SEM = 1.51). Post-hoc analyses revealed that the low-dose group displayed significantly less rearing in the periphery as compared to the control group (M *diff* = 11.10, SEM

= 2.24), $p < 0.01$, and as compared to the high-dose group (*M diff* = 6.84, *SEM* = 2.05) $p < 0.01$ (see Figure 10 below and Appendix A Table 3). No significant difference was detected between the high-dose and control group.

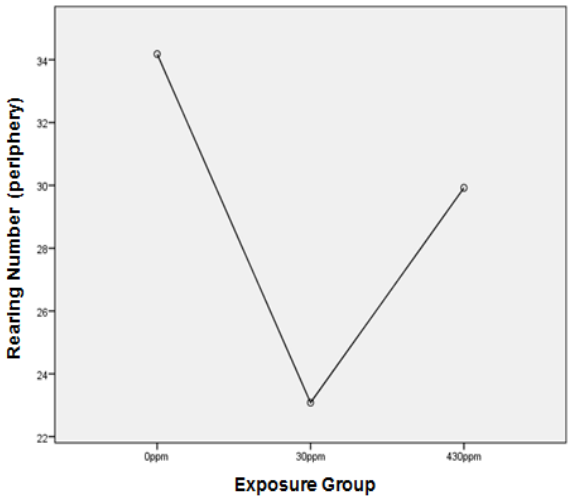


Figure 10. Rearing number in the periphery of open field arena in young C57BL/6J mice with and without early chronic lead exposure.

Rearing in center. Generalized linear model analyses controlling for litter revealed significant differences between groups with regard to rearing number in center of the open field, X^2 (df = 2) = 11.43, $p < 0.01$ (controls *M* = 1.82, *SEM* = 0.41; low-dose *M* = 0.75, *SEM* = 0.25; high-dose *M* = 0.46, *SEM* = 0.18). Post-hoc analyses revealed that the low-dose group displayed significantly less rearing in the center of the arena as compared to the control group (*M diff* = 1.07, *SEM* = 0.477), $p < 0.05$. Similarly, the high-dose group displayed significantly less rearing in the center of the arena as compared to the control group (*M diff* = 0.29, *SEM* = 3.13), $p < 0.01$. (see Figure 11 below and Appendix A Table 3.) No significant difference was detected between the low-dose and the high-dose group.

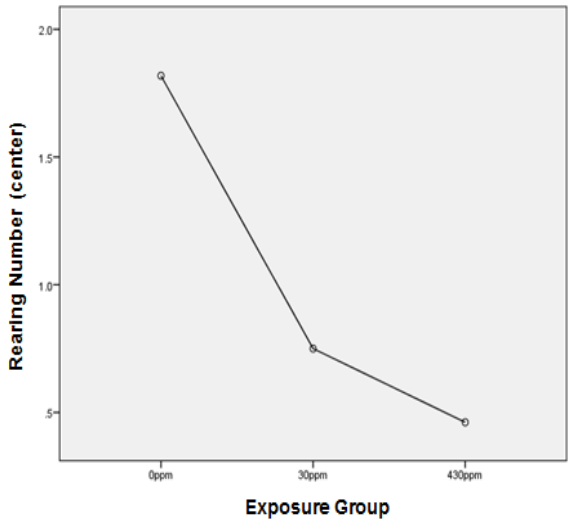


Figure 11. Rearing number in the center of open field arena in young C57BL/6J mice with and without early chronic lead exposure.

Distance travelled in periphery. Generalized linear model analyses of group differences were not significant with regard to total distance travelled in the periphery of the open field.

Total Distance travelled. Generalized linear model analyses of group differences were not significant with regard to total distance travelled in the center of the open field.

Time in periphery. Generalized linear model analyses of group differences were not significant with regard to time spent in the periphery of the open field.

3.1.3.5 Odor habituation/dishabituation test.

Habituation to water. General linear model analyses detected a significant main effect for trial, F (df = 2, 60) = 13.03, $p < 0.01$ (trial 1 M = 17.26, SEM = 3.67; trial 2 M = 7.62, SEM = 1.20; trial 3 M = 4.62, SEM = 1.01). Post-hoc analyses using least significant difference (LSD) pairwise comparisons revealed that all mice independent of treatment group spent significantly less time exploring the cotton swab with water from Trial 1 to Trial 2, ($M\ diff$ = 9.63, SEM =

4.14 , $p < 0.01$), and from Trial 1 to Trial 3 (*M diff* = 12.63, SEM = 3.03), $p < 0.01$. Similarly, mice spent significantly less time exploring the cotton swab from trial 2 to trial 3 (*M diff* = 2.99, *SEM* = 3.03), $p < 0.01$ (see Figure 12 below and Appendix A Table 4). Thus all mice habituated to the cotton swab with water from Trial 1 to Trial 3. No significant main effects for group or the interaction (Trial x Group) were detected.

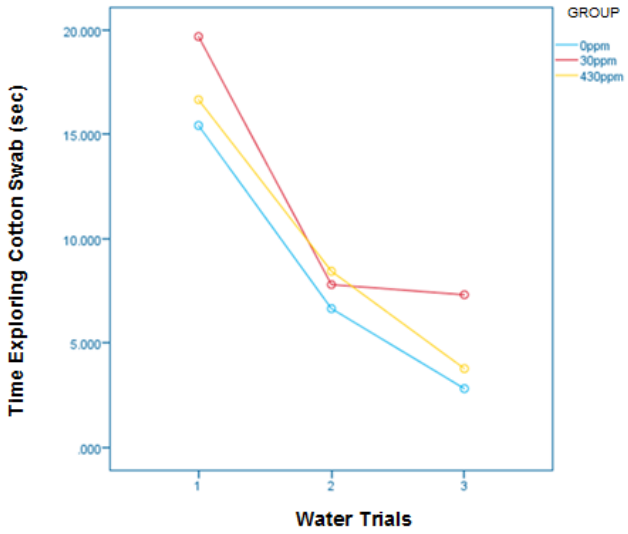


Figure 12. Time exploring a cotton swab with water during the habituation phase of the odor habituation/dishabituation task in young C57BL/6J mice with and without early chronic lead exposure.

Habituation to almond. General linear model analyses detected a significant main effect for Trial, F (df = 2, 60) = 6.83, $p < 0.05$ (Trial 1 *M* = 4.28, *SEM* = 0.84; Trial 2 *M* = 2.08, *SEM* = 0.38; Trial 3 *M* = 1.94, *SEM* = 0.47). Post-hoc analyses with LSD revealed that all mice independent of treatment group spent significantly less time exploring the cotton swab with almond from Trial 1 to Trial 2, (*M diff* = 2.19, *SEM* = 0.83), $p < 0.05$, and from Trial 1 to Trial 3 (*M diff* = 2.34, *SEM* = 0.73), $p < 0.05$ (see Figure 13 below and Appendix A Table 4). On the other hand, no significant difference was detected with regard to time exploring the cotton swab

from Trial 2 and Trial 3. No significant main effects were detected for group or the interaction (Trial x Group). Thus all mice habituated to the cotton swab with almond from Trial 1 to Trial 2.

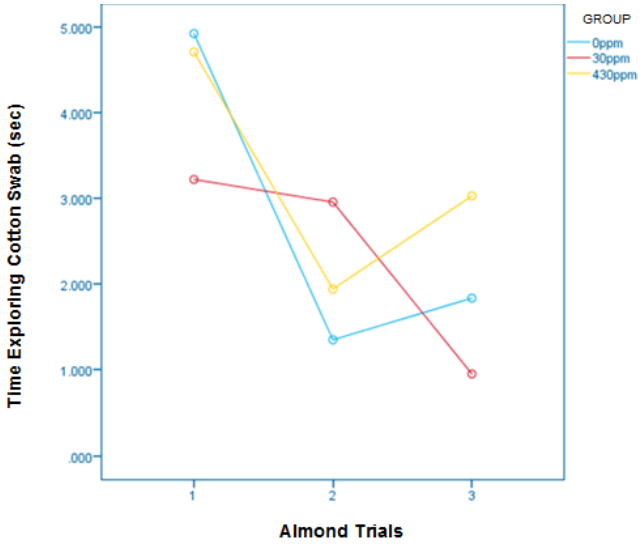


Figure 13. Time exploring a cotton swab with almond during the habituation phase of the odor habituation/dishabituation task in young C57BL/6J mice with and without early chronic lead exposure.

Habituation to orange. General linear model analyses did not detect significant differences between groups with regard to the time spent exploring the cotton swab with orange.

Habituation to social scent 1. No significant main effects of trial or the interaction (Trial x Group) were detected with regard to the time spent exploring the cotton swab with social scent 1 (scent from a non-familiar mouse cage).

Habituation to social scent 2. No significant main effects of trial or the interaction (Trial x Group) were detected with regard to the time spent exploring the cotton swab with social scent 2 (scent from a different non-familiar mouse cage).

Dishabituation, water Trial 3/almond Trial 1. No significant main effects of trial or the interaction (Trial x Group) were detected with regard to the time spent exploring the cotton swabs.

Dishabituation, almond Trial 3/orange Trial 1. No significant main effects of trial or the interaction (Trial x Group) were detected between groups with regard to the time spent exploring the cotton swabs.

Dishabituation, orange Trial 3/social scent 1 Trial 1. General linear model analyses revealed a significant main effect with regard to the time spent exploring the odors from orange Trial 3 to social scent 1 Trial 1, F (df = 1, 30) = 7.37, $p < 0.05$ (orange Trial 3 M = 1.81, SEM = 0.80; social scent 1 Trial 1 M = 7.34, SEM = 2.35). Post-hoc analyses with LSD revealed that all mice, regardless of treatment group, had increased time exploring the odor from orange Trial 3 to social scent 1 Trial 1 ($M\ diff$ = 5.53, SEM = 2.04), $p < 0.05$. No significant main effects however were detected for group or the interaction (Trial x Group). The result indicated that all mice recognized social scent 1 as a novel stimulus.

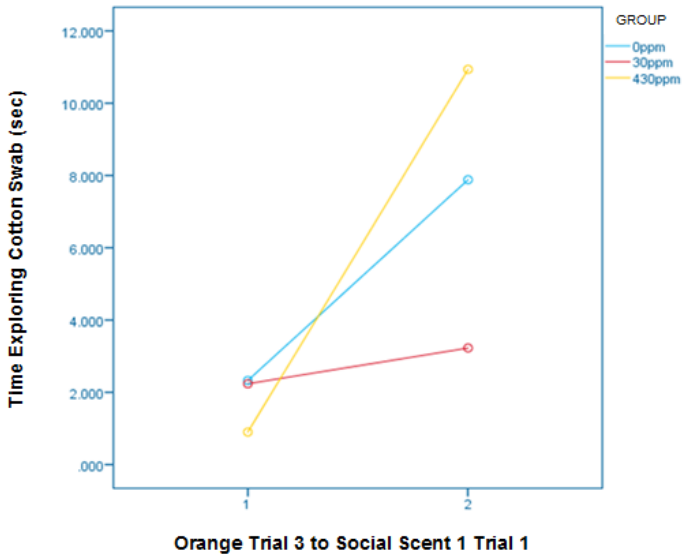


Figure 14. Time exploring a cotton swab with orange during Trial 3 and with social scent 1 Trial 1 during the dishabituation phase of the task in young C57BL/6J mice with and without early chronic lead exposure.

Dishabituation, social scent 1 Trial 3/social scent 2 Trial 1. No significant main effects of Trial or the interaction (Trial x Group) were detected with regard to the time spent exploring the cotton swab with social scent 2 (that had a scent from a non-familiar mouse cage).

3.2 Summary of Behavioral Studies Results

Overall, effects of early lead exposure were observed for body weight at PND 28 and rearing in the periphery and center of the open field arena measured at PND 28. With regard to odor memory, animals in all groups habituated and dishabituated to some or most of the odors presented however inconsistent results for habituation and dishabituation across odors among controls suggested that the task may have been too long and/or too challenging for pre-adolescent mice. (See Appendix A Tables 4-6.) This will be considered in Discussion below.

3.2.1 Developmental milestones.

3.2.1.1 Eye-opening. Daily observations revealed that 27% of mice in the control and low-dose groups opened fully their eyes at PND 15, whereas 73% of the mice opened their eyes at PND 16. On the other hand, 100% of the high-dose mice opened their eyes at PND 16. The differences were not statistically significant but observed delays in the high-dose group might suggest that other tests of neurological development should be tried.

3.2.1.2 Body weight. A significant main effect of group and PND on body weight and the interaction (group x PND) were found. Specifically, low-dose mice had increased body weight as compared to controls and high-dose groups, and significant weight gain in low dose-mice occurred from PND 21 to PND 28. No significant difference however between the high-dose group and control group was detected.

3.2.1.3 Righting reflex. A significant main effect of PND was found. That is, all mice were faster at displaying a righting reflex from PND 7 to PND 21. No significant main effect of group nor the interaction (group x PND) were detected.

3.2.2 Inverted screen test. Independent sample median test did not reveal significant differences between groups with regard to mouse motor persistence (strength) despite the large observed difference in faster fall time for low-dose animals.

3.2.3 Open field test.

3.2.3.1 Rearing in periphery. Mice in the low-dose group reared significantly less in the periphery of the open field arena as compared to the control and high-dose group. No significant difference was detected between the high-dose group and the control group.

3.2.3.2 Rearing in center. Mice in the low-dose and high-dose groups had significantly less rearing in the center of the arena as compared to controls. No significant difference however was detected between the high-dose group and control group. The actual difference observed however was quite small and its "clinical" significance is questionable.

3.2.3.3 Total Distance travelled. No significant differences between groups were found with regard to the total distance travelled in the open field.

3.2.3.4 Distance travelled in periphery. No significant differences between groups were found with regard to the distance travelled in the periphery of the open field.

3.2.3.5 Time in periphery. No significant differences between groups were found with regard to the time spent in the periphery of the open field.

3.2.4 Odor habituation/dishabituation test.

3.2.4.1 Habituation. A significant main effect for trial during habituation to the cotton swab with water and the cotton swab with almond was found. No significant main effect however was detected for group or the interaction. Similarly, no significant main effects or the interaction (Trial x Group) were detected for the habituation phase with orange, social scent 1 or social scent 2.

3.2.4.2 Dishabituation. A significant main effect for trial during the dishabituation phase of the task for time spent exploring orange Trial 3 to time exploring social scent 1 Trial 1 was detected. No significant main effect however was detected for group or the interaction. This suggested that animals detected that that there was a novel odor however the test was not sensitive to the effects of lead exposure. No significant main effects or the interaction were detected for dis-habituation from almond Trial 3 to orange Trial 1, or for social scent 1 Trial 3 to

social scent 2 Trial 1. Overall, the findings for the odor task suggested that the task was likely too long, or too challenging for PND 28 mice. This will be considered in detail in Discussion below.

Table 10. Summary of significant effects in BLLS and behavior in young C57BL6/J mice with and without chronic lead exposure.

	30ppm v 0ppm	430ppm v 0ppm	430ppm v 30ppm
BLLs	↑	↑	↑
Developmental Milestones			
Eye-opening			
Body weight			
PND 7			
PND 14			
PND 21			
PND 28	↑		
Righting reflex			
PND 7			
PND 14			
PND 21			
Inverted screen test			
Open Field			
Rearing			
Periphery	↓		
Center	↓	↓	
Distance travelled			
Total			
Periphery			
Time in Periphery			

Table 11. Summary of significant main effects and the interaction for habituation dishabituation task (group x trial) in young C57BL6/J mice with and without chronic lead exposure.

	Group	Trial	Group x Trial
Odor habituation/dishabituation			
Habituation			
Water		↓	
Almond		↓	
Orange			
Social Scent 1			
Social scent 3			
Dishabituation			
Water T3 to Almond T1			
Almond T3 to Orange T1			
Orange T3 to Social Scent 1 T1		↑	
Social scent 1 T3 to Social scent 2 T1			
Social scent 1 T3 to Social scent T1			

T. refers to trial.

3.3 Neuroimmune System Studies Results

Effects of early lead exposure were predicted for MHC II and CCR7 in hippocampal microglia. It was hypothesized that chronic low-level lead exposure would cause the upregulation of MHC II and CCR7 in hippocampal microglia of young C57BL/6J male mice. With regard to microglia in brain (all other regions except hippocampus and excluding cerebellum) effects on microglia were measured but no a priori hypotheses were proposed. The results for hippocampal and brain microglia are shown below. In addition, exploratory analyses of macrophages in hippocampus and brain were conducted, and exploratory analyses of immune cells (lymphocytes and monocytes) in brain and hippocampus were conducted to better understand effects of early lead exposure on microglial cells and the early neuroimmune system.

3.3.1 Descriptive statistics for flow cytometry data. To ensure sufficient numbers of hippocampal cells, brain tissue from 3 subjects were combined, creating 4 samples per group, N=12, generated from 36 animals. (See Appendix A Tables 7-11 for descriptive statistics of flow cytometry data.) All analyses were calculated with N=12. It is also important to note that flow cytometry data including forward and side scatter, and mean fluorescence intensity (MFI) are dependent on the numbers of cells counted, and these vary according to the amount of sample run. Thus flow cytometry does not yield meaningful estimates of absolute cell density. Moreover, several outcome variables violated the assumptions of homogeneity of variance and/or distribution normality (see Appendix A Tables 30-47). For this reason, non-parametric tests were selected to examine possible differences between groups.

Chi-square analyses are commonly used in flow cytometry studies. Because of the large numbers of parent population cells counted however, chi-square analyses are extremely liberal and commonly all analyses are “statistically significant.” For this reason, additional median

comparison analyses were used for count and percentage data, and median comparisons were also used for analyses of geometric mean fluorescence intensity (MFI, measured on a log scale). Group differences for count data were not interpreted unless both chi-square and median comparison analyses were statistically significant. The drawback of this approach however was that because subjects had to be combined into groups of 3, median comparison tests and associated post-hoc comparisons were extremely conservative. Nonetheless, all main analyses were calculated using N=12.

As will be described below, the most robust findings (using N=12) were not with regard to microglia, but with regard to macrophages. For exploratory purposes, and for only hippocampal macrophage results, simulated data distributions were generated randomly (SPSS) using minimum and maximum values from each sample by variable by group, to create an additional 8 subject data lines per group, producing a simulated dataset including 12 animals per group within the minimum and maximum variable values produced by 12 animals group. Median comparisons with post-hoc tests were then calculated again using this simulated sample. The simulation results are interpreted and discussed separately below and clearly indicated in relevant tables.

3.3.2 Inferential statistics for flow cytometry data. (see Appendix A Tables 12-24 for inferential statistics).

3.3.2.1 Hippocampal microglia.

Microglia percentage of parent population. Chi-square test revealed a significant difference between groups with regard to the percentage of microglial cells in hippocampus, X^2 (df = 2, N = 1421.166) = 1437.33, $p < 0.01$, (controls = 58.1%; low-dose = 45.0%; high-dose =

70.9%) and suggested fewer microglia in the low-dose exposure group. Independent samples median test did not detect significant differences between groups.

Microglia cell number. Independent samples median test revealed significant differences between groups with regard to the number of microglial cells in hippocampus, *MEDCOMP test-statistic* (df = 2) = 8.00, $p < 0.05$. Post-hoc analyses examining differences between groups indicated that the high-dose group (*Mdn* = 12883) had an increased number of microglial cells in hippocampus as compared to the low-dose group (*Mdn* = 1846), *MEDCOMP test statistic* = 8.00, $p < 0.014$. Post-hoc tests showed no significant difference between the high-dose group and control group (*Mdn* = 4656), or between the low-dose group and control group.

Overall analyses of MHC II and CCR7 in hippocampal microglia.

Microglia MHC II mean fluorescence intensity. Independent samples median test did not detect significant differences between groups with regard to MFI of MHC II in microglial cells.

Microglia CCR7 mean fluorescence intensity. Independent samples median test showed significant differences between groups with regard to MFI of CCR7 in hippocampal microglia, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Contrary to what was hypothesized, post-hoc analyses indicated that the low-dose group (*Mdn* = 4656) had decreased MFI of CCR7 in microglial cells as compared to the control group (*Mdn* = 5604), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant difference was detected between the high-dose and control group, or between the high-dose and low-dose group (see Figure 15).

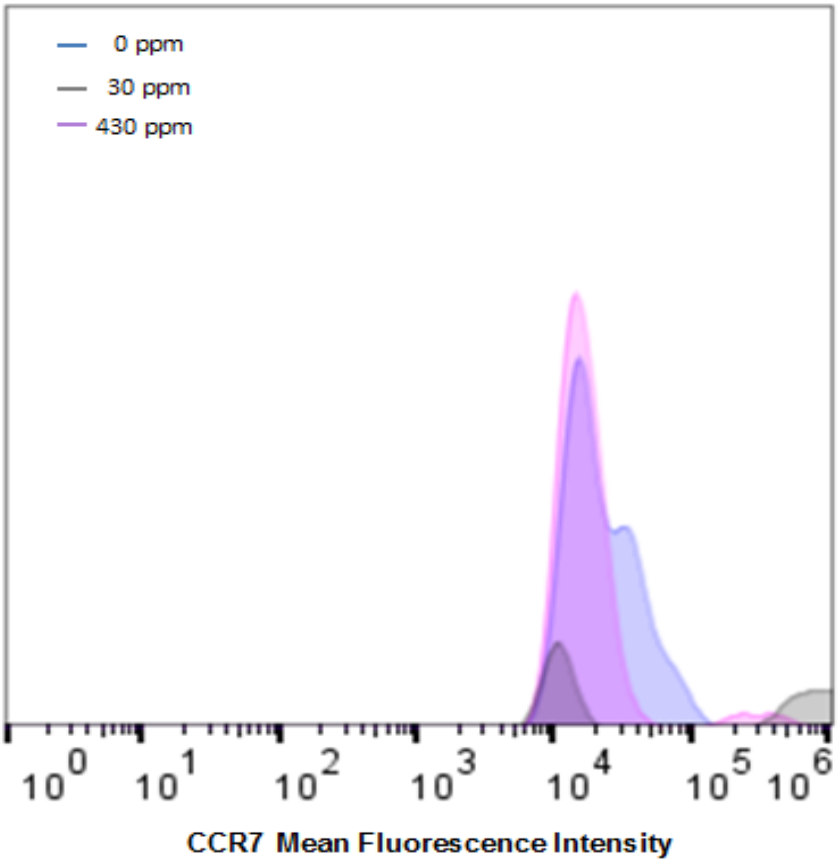


Figure 15. Representative histograms of effects of low-level lead exposure on hippocampal microglia on mean fluorescence intensity of CCR7 (overall analyses). Histogram shifts towards the right show higher mean fluorescence intensity and thus increased CCR7. Low-level lead exposed animals had decreased number of CCR7 (measured as mean fluorescence intensity) on their hippocampal microglial membrane surface as compared to controls. As can be noted however low-dose groups had an outlier expressing high levels of CCR7.

Quadrant-specific analyses of "single positive" hippocampal microglia.

Microglia MHC II percentage of parent population, single positive. Chi-square tests revealed significant differences between groups with regard to percentage of MHC II in hippocampal microglial cells, X^2 (df = 2, N = 36052) = 6.494, $p < 0.05$ (controls = 0.4%; low-dose = 0.2%; high-dose = 0.5%). Independent samples median test however did not show statistically significant differences between groups.

Microglia MHC II cell number, single positive. Independent samples median test indicated significant differences between groups, *MEDCOMP test-statistic* (2) = 8.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group (*Mdn* = 83) had increased number of MHC II positive microglial cells in hippocampus as compared to the low-dose group (*Mdn* = 15), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No statistically significant difference was detected however between the high-dose and control group, or between the low-dose and control group.

Microglia CCR7 percentage of parent population, single positive. Chi-square tests showed a significant difference between groups with regard to percentage of CCR7 positive hippocampal microglial cells, X^2 (df = 2, N = 36154) = 46.801, $p < 0.01$ (controls = 0.2%; low dose = 0.2%; high dose = 0.6%). Independent samples median test however did not show a statistically significant difference between groups.

Microglia CCR7 cell number, single positive. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$ (controls *Mdn* = 25 ; low-dose *Mdn* = 9; high-dose *Mdn* = 45). Post-hoc analyses however did not reveal statistically significant differences between groups. The decreased cell number in the low-dose group was noted however.

Microglia CCR7 mean fluorescence intensity, single positive. Independent samples median tests did not reveal statistically significant differences between groups.

Quadrant-specific analyses of "double positive" hippocampal microglia.

Microglia percentage of parent population, double positive. Chi-square tests did not reveal statistically significant differences between groups with regard to the percentage of microglial cells that simultaneously express membrane MHC II and CCR7.

Microglia MHC II mean fluorescence intensity, double positive. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the low-dose group (*Mdn* = 13532) had decreased MFI of MHC II in hippocampal microglial cells as compared to the control group (*Mdn* = 18395), *MEDCOMP test statistic* = 8.00, $p < 0.05$. No statistically significant difference was detected between the high-dose and control group and between the high-dose and low-dose group (See Figure 16).

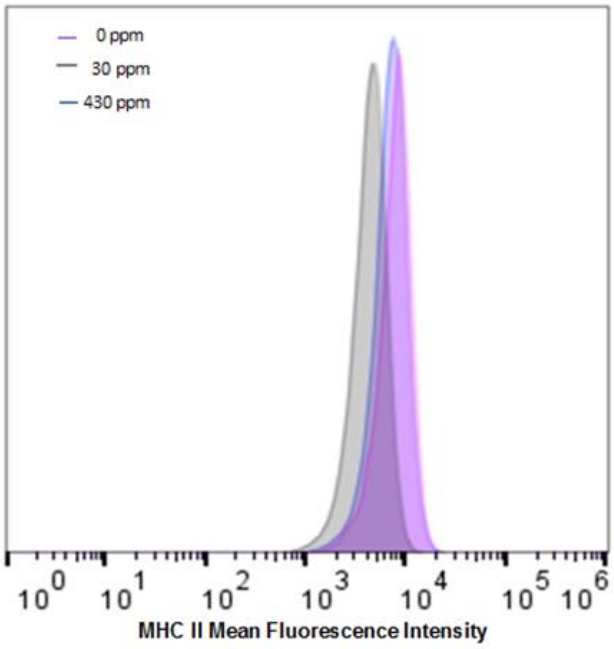


Figure 16. Representative histograms of effects of low-level lead exposure on hippocampal microglia mean fluorescence intensity of MHC II (double positive cells). Histogram shifts towards the right show higher mean fluorescence intensity and thus increased MHC II. Low-level lead exposed animals had decreased number of MHC II on their hippocampal microglia membrane surface as compared to controls in double positive cells.

Microglia CCR7 mean fluorescence intensity, double positive. Independent samples median test did not reveal significant differences between groups.

3.3.2.2 Brain microglia.

Microglia percentage of parent population. Chi-square test revealed a significant difference between groups with regard to percentage of microglial cells in brain, X^2 (df = 2, N =165507) = 879.41, $p < 0.01$ (controls = 79.2%; low-dose = 84.7%; high-dose = 77.7%). Independent samples median test however did not show significant differences between groups.

Microglia cell number. Independent samples median test did not show significant differences between groups.

Overall analyses of MHC II and CCR7 in brain microglia.

Microglia MHC II mean fluorescence intensity. Independent samples median test did not show significant differences between groups.

Microglia CCR7 mean fluorescence intensity. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "single positive" brain microglia.

Microglia MHC II percentage of parent population, single positive. Chi-square tests showed a significant difference between groups with regard to the percentage of MHC II positive microglial cells in hippocampus, X^2 (df = 2, N =165508) = 859.48, $p < 0.01$ (controls = 0.3%; low-dose = 0.3%; high-dose = 1.8%). Independent samples median test however did not reveal significant differences between groups.

Microglia MHC II cell number, single positive. Independent samples median test did not show significant differences between groups.

Microglia MHC II mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

Microglia CCR7 percentage of parent population, single positive. Chi-square tests revealed a significant difference between groups with regard to percentage of CCR7 positive microglial cells in brain, X^2 (df = 2, N = 165507) = 40.01, $p < 0.01$ (controls =1.2%; low-dose = 0.8%; high-dose = 0.9%). Independent samples median test showed significant differences between groups (controls *Mdn* = 0.18%; low-dose *Mdn* = 0.22%; high-dose *Mdn* = 0.23%). Post-hoc analyses however did not reveal significant differences between groups.

Microglia CCR7 cell number, single positive. Independent samples median test did not show significant differences between groups.

Microglia CCR7 mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "double positive" brain microglia.

Microglia percentage of parent population, double positive. Chi-square tests showed a significant difference between groups with regard to percentage of double positive (MHC II/CCR7) microglial cells in brain, X^2 (df = 2, N = 165508) =19.84, $p < 0.01$ (controls = 0.1%; low-dose = 0.001%; high-dose = 0.1%). However, independent samples median test did not show significant differences between groups.

Microglia cell number, double positive. Independent samples median test did not show significant differences between groups.

Microglia MHC II mean fluorescence intensity, double positive. Independent samples median test did not show significant differences between groups.

Microglia CCR7 mean fluorescence intensity, double positive. Independent samples median test did not show significant differences between groups.

3.3.2.3 Hippocampal macrophages.

Macrophages percentage of parent population. Chi-square test showed a significant difference between groups with regard to percentage of macrophages in hippocampus, X^2 (df = 2, N = 36072) = 215.97, $p < 0.01$ (controls = 6.5%; low-dose = 3.7%; high-dose = 3.1%). Independent samples median tests showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, p < 0.05. Post-hoc analyses however were not significant.

Macrophages cell number. Independent samples median tests showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group (*Mdn* = 520) had an increased number of macrophages in hippocampus as compared to the low-dose group (*Mdn* = 210), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant difference however was detected between the high-dose and control group, or between the low-dose and control group. (The simulated dataset however suggested that with additional subjects, the low-dose group differed from the control group *MEDCOMPS* test-statistic (df = 2) = 6.00, $p < 0.05$.)

Overall analyses of MHC II and CCR7 in hippocampal macrophages.

Macrophages MHC II mean fluorescence intensity. Independent samples median test did not show a significant difference between groups.

Macrophages CCR7 mean fluorescence intensity. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "single positive" hippocampal macrophages.

Macrophages MHC II percentage of parent population, single positive. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages that express MHC II in hippocampus, X^2 (df = 2, N = 36034) = 14.07, $p < 0.01$ (controls = 1.8%; low-dose = 1.2%; high-dose = 1.3%). Independent samples median test however did not show significant differences between groups.

Macrophages MHC II cell number, single positive. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group had increased number of macrophages expressing MHC II in hippocampus (*Mdn* = 262) as compared to the low-dose group (*Mdn* = 86), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant difference was detected between the high-dose and control group or between the low-dose and control group.

Macrophages MHC II mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

***Macrophages CCR7 percentage of parent population, single positive*.** Chi-square test showed a significant difference between groups with regard to the percentage of CCR7 positive macrophages in hippocampus, X^2 (df = 2, N = 36075) = 74.66, $p < 0.01$ (controls = 0.3%; low-dose = 0.6%; high-dose = 0.001%).

Independent samples median test showed significant differences between the groups, *MEDCOMPS test-statistic* (df = 2) = 8.00, $p < 0.05$. Post-hoc analyses indicated that the high-

dose group (*Mdn* = 0.32%) had decreased percentage of macrophages expressing CCR7 in the hippocampus as compared to the control group (*Mdn* = 5.24 %), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant difference however was detected between the low-dose group (*Mdn* = 7.46%) and control group, or between the low-dose group and high-dose group (see Figure 17, below). Simulated data however revealed that the high-dose group differed from the low-dose group *MEDCOMP test-statistic* (df = 2) = 24.00, $p < 0.01$.

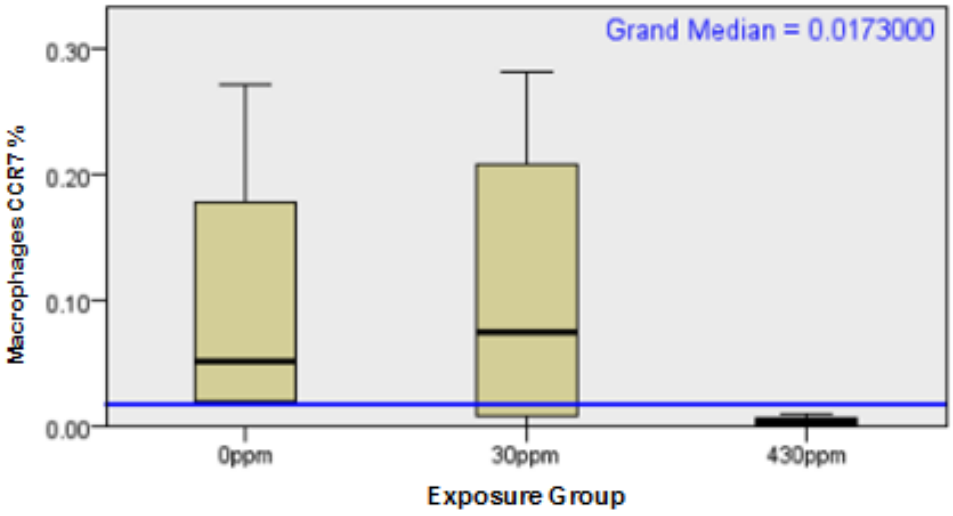


Figure 17. Hippocampal macrophages CCR7 "single positive" percentage of parent population in young C57BL6/J mice, with and without early chronic lead exposure.

Macrophages CCR7 cell number, single positive. Independent samples median test did not show significant differences between groups.

Macrophages CCR7mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "double positive" hippocampal macrophages.

Macrophages percentage of parent population, double positive. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages expressing MHC II and CCR7 in brain, X^2 (df = 2, N = 33126) = 434.93, $p < 0.01$ (controls = 27%; low-dose = 14%; high-dose = 21%). Independent samples median test however did not show significant differences between groups.

Macrophages MHC II mean fluorescence intensity, double positive. Independent samples median tests showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group (*Mdn* = 29264) had increased MFI of MHC II in hippocampal macrophages as compared to the control group (*Mdn* = 21371), *MEDCOMP test-statistic* = 8.00, $p < 0.014$ (see Figure 18). No significant differences were detected between the high-dose and the low-dose group (*Mdn* = 21720), or between the low-dose and control group. (The simulated dataset suggested that with increased sample size, the high-dose group would differ from the low-dose group *MEDCOMPS test-statistic* = 24.00, $p < 0.01$.)

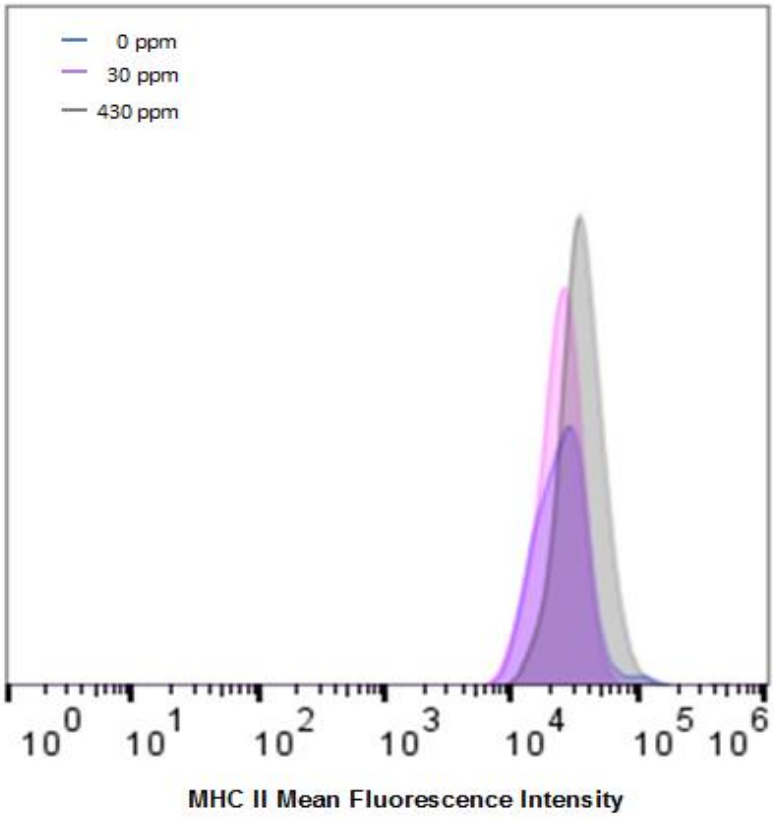


Figure 18. Representative histograms of effects of high-level lead exposure on hippocampal macrophages on mean fluorescence intensity of MHC II (in double positive cells). Histogram shifts towards the right show higher mean fluorescence intensity and thus increased MHC II. High-level lead exposed animals had increased number of MHC II on their hippocampal macrophage membrane surface as compared to controls and low-dose groups.

3.3.2.4 Brain macrophages.

Macrophages percentage of parent population. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages in brain, X^2 (df = 2, N = 165508) = 215.97, $p < 0.01$(controls = 5.1%; low-dose = 2.5%; high-dose = 3.6%). Independent samples median test however did not show significant differences between groups.

Macrophages cell number. Independent samples median test did not show significant differences between groups.

Overall analyses of MHC II and CCR7 in brain.

Macrophages MHC II mean fluorescence intensity. Independent samples median test did not show significant differences between groups.

Macrophages CCR7 mean fluorescence intensity. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "single positive" brain macrophages.

Macrophages MHC II percentage of parent population, single positive. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages expressing MHC II in brain, X^2 (df = 2, N = 36034) = 14.07, $p < 0.01$ (controls = 1.2%; low-dose = 0.8%; high-dose = 0.9%). Independent samples median test did not show significant differences between groups.

Macrophages MHC II cell number, single positive. Independent samples median test did not show significant differences between groups.

Macrophages MHC II mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

Macrophages CCR7 percentage of parent population, single positive. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages expressing CCR7 in brain, X^2 (df = 2, N =165508) = 74.66, $p < 0.01$ (controls = 0.1%; low-dose = 0.1%; high-dose = 0.01%). Independent samples median did not reveal significant differences between groups.

Macrophages CCR7 number, single positive. Independent samples median test did not show significant differences between groups.

Macrophages CCR7 mean fluorescence intensity, single positive. Independent samples median test did not show significant differences between groups.

Quadrant-specific analyses of "double positive" brain macrophages.

Macrophages percentage of parent population, double positive. Chi-square test showed a significant difference between groups with regard to the percentage of macrophages that simultaneously express membrane MHC II and CCR7 in brain, X^2 (df = 2, N = 165507) = 56.27, $p < 0.01$ (controls = 1.4%; low dose = 0.6%; high dose = 0.6%). Independent samples median test did not show significant differences between groups.

Macrophages cell number, double positive. Independent samples median test did not show significant differences between groups.

Macrophages MHC II mean fluorescence intensity, double positive. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group had increased MFI (*Mdn* = 25588) in macrophages expressing MHC II in brain as compared to the low-dose group (*Mdn* = 18752), *MEDCOMP test-statistic* = -7.25, $p < 0.05$ (see Figure 19, below). No significant effects however were detected between the low-dose and control group (*Mdn* = 24117), or between the high-dose and control group.

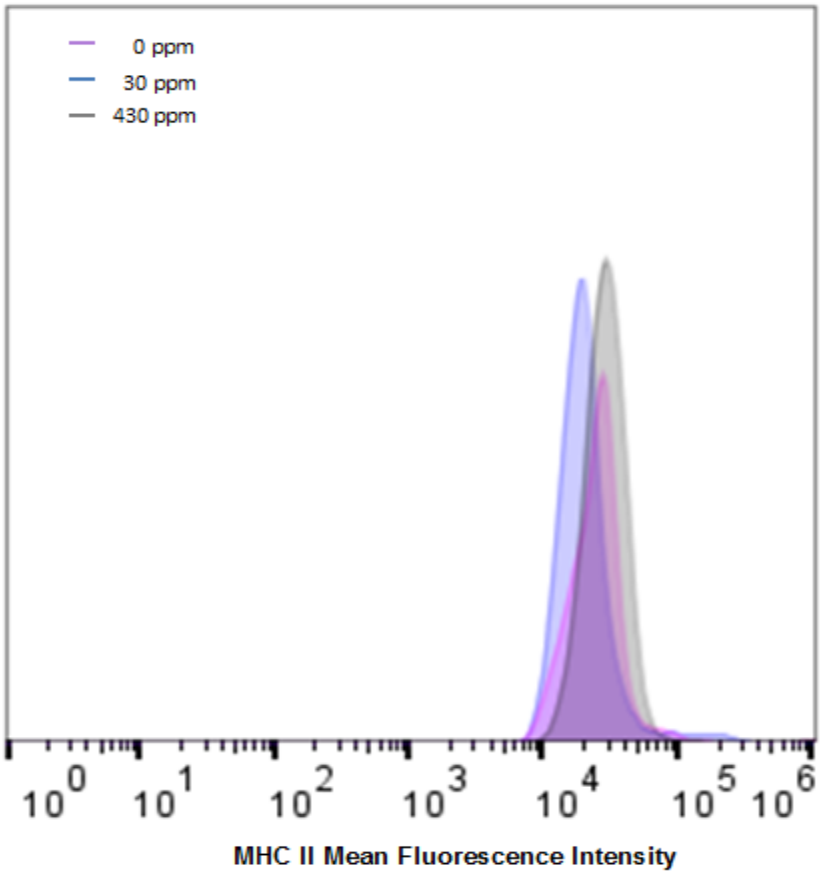


Figure 19. Representative histograms of effects of high-level lead exposure on brain macrophages on mean fluorescence intensity of MHC II (in double positive cells). Histogram shifts towards the right show higher mean fluorescence intensity and thus increased MHC II. High-level lead exposed animals had increased number of MHC II on the surface of macrophages in brain as compared to the low-dose groups in double positive cells.

Macrophages CCR7 mean fluorescence intensity, double positive. Independent samples median test did not show significant differences between groups.

3.3.2.5 Hippocampal immune cells.

Immune cell percentage of parent population. Chi-square test showed a significant difference between groups with regard to the percentage of immune cells in hippocampus, X^2 (df = 2, N = 79040) = 2585.21, $p < 0.01$ (controls = 50.3%; low-dose = 35.4%; high-dose = 60.5%).

Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 8.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group (*Mdn* = 56.65%) had an increased percentage of immune cells in hippocampus as compared to the low-dose group (*Mdn* = 33.30%), *MEDCOMP test-statistic* = 8.00, $p < 0.05$ (see Figure 20 below). No significant differences were detected between the high-dose and control group (*Mdn* = 46.40%), or between the low-dose group and the control group.

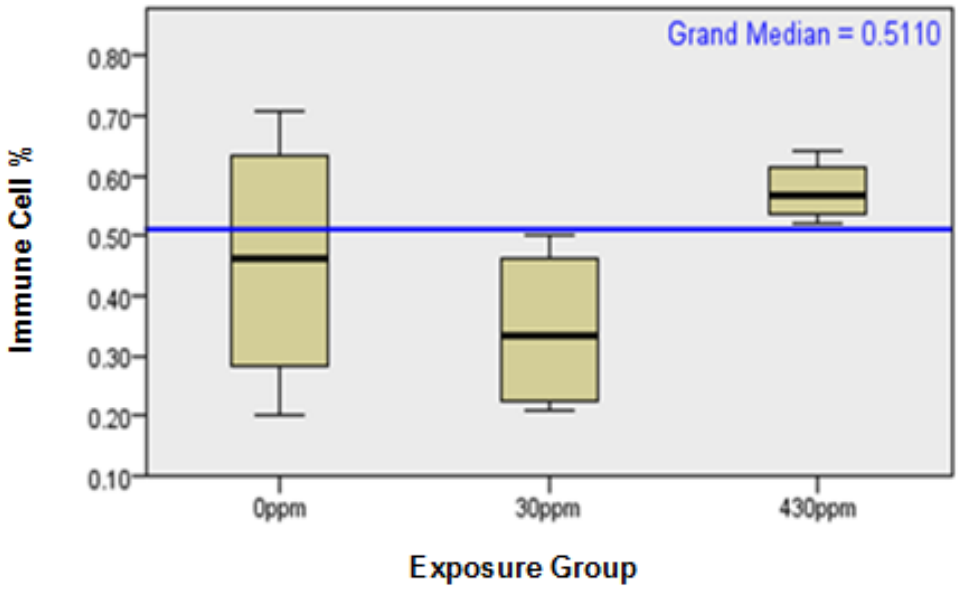


Figure 20. Hippocampal immune cell percentage of parent population in young C57BL6/J mice with and without early lead exposure.

Immune cell number. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 8.00, $p < 0.05$. Post-hoc analyses indicated that the low-dose group (*Mdn* = 4742) had a decreased number of immune cells in hippocampus as compared to the high-dose group (*Mdn* = 21934), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant differences however were detected between low-dose and control group, or between the high-dose group and control group (*Mdn* = 10882).

3.3.2.6 Brain immune cells.

Immune cell percentage of parent population. Chi-square test showed a significant difference between groups with regard to the percentage of immune cells in brain, X^2 (df = 2, N = 226980) = 2876.67, $p < 0.01$ (controls = 65.7%; low-dose = 74.1%; high-dose = 77.4%). Independent samples median test however did not reveal significant differences between groups.

Immune cell number. Independent samples median test showed significant differences between groups, *MEDCOMP test-statistic* (df = 2) = 6.00, $p < 0.05$. Post-hoc analyses indicated that the high-dose group had increased number of immune cells (*Mdn* = 80001) as compared to the control group (*Mdn* = 53304), *MEDCOMP test-statistic* = 8.00, $p < 0.05$. No significant differences were detected between the high-dose and low-dose group, or between the low-dose and control group.

3.4 Summary of Neuroimmune System Studies Results

Overall, significant group effects were observed primarily in hippocampus and not in brain (see summary Table 12).

3.4.1 Hippocampal microglia. Hippocampal microglial cells in low-dose mice, as compared to the control group, had decreased MFI of MHC II (in double positive cells) (see Figure 16), and decreased MFI of CCR7 (overall analyses) (see Figure 15). In other words, low-level lead exposure reduced the quantity of MHC II and CCR7 expressed on the surface of hippocampal microglial cells. No other significant differences were detected between groups with regard to percentage of parent population or MFI of the measured parameters.

3.4.2 Hippocampal macrophages. Hippocampal macrophages in high-dose exposed animals had an increased MFI of MHC II (in double positive cells), as compared to controls (see

Figure 18). At the same time, hippocampal macrophages in high-dose exposed animals had decreased percentage of CCR7 (single positive cells), as compared to controls (see Figure 17). Thus higher-level lead exposure reduced the number of hippocampal macrophages that expressed CCR7 on their membrane surface, but increased the quantity of MHC II present on the surface of hippocampal macrophages. No other significant effects were detected between groups with regard to percentage of parent population or MFI in the measured parameters.

3.4.3 Hippocampal immune cells. Independent samples median test revealed that higher-dose exposed animals had an increased percentage and number of immune cells in hippocampus as compared to the low-dose group (see Figure 20). Thus higher-level lead exposure caused an increase in the number of immune cells in the hippocampus. No other effects were detected.

3.4.4 Brain microglia. Independent samples median test did not detect any significant differences between groups with regard to the measured parameters in brain microglia.

3.4.5 Brain macrophages. Independent samples median test revealed that high-dose animals as compared to the low-dose animals had increased MFI of MHCII in macrophages (double positive cells) (see Figure 19). Thus high-level lead exposure increased the quantity of MHC II in macrophages in brain regions other than hippocampus. No other significant effects were detected between groups with regard to percentage of parent population or MFI for the measured parameters.

3.4.6 Brain immune Cells. Independent samples median test revealed that the high-dose group had increased number of immune cells in brain as compared to the control group.

Table 12. Summary of significant effects in hippocampal and brain cells in young C57BL6/J mice with and without chronic lead exposure.

	30ppm v 0ppm		430ppm v 0ppm		430ppm v 30ppm	
	%	MFI	%	MFI	%	MFI
HIPPOCAMPUS						
Microglia						
CCR7		↓				
MHCII						
Q1 MHCII						
Q2 CCR7+MHCII[1]		↓				
Q3 CCR7						
Macrophages						
CCR7						
MHCII						
Q1 MHCII						
Q2 CCR7+MHCII				↑		↑
Q3 CCR7			↓		↓	
Immune Cells					↑	
CCR7						
MHCII						
Q1 MHCII						
Q2 CCR7+MHCII						
Q3 CCR7						
BRAIN						
Microglia						
CCR7						
MHCII						
Q1 MHCII						
Q2 CCR7+MHCII						
Q3 CCR7						
Macrophages						
CCR7						
MHCII						
Q1 MHCII						
Q2 CCR7+MHCII						↑
Q3 CCR7						
Immune Cells						

[1] *Indicates significant results for MHC II in double positive quadrant (MHC II/CCR7).*

CHAPTER 4

DISCUSSION

The present studies build on previous studies conducted in our laboratory (see Introduction). These studies were designed to examine whether additional behavioral tests would be sensitive to the effects of early chronic low-level lead exposure in young C57BL/6J male mice; and to examine the presence of biological markers in microglial cells that could stimulate their trafficking away from brain perhaps via meningeal and/or para-vascular brain lymphatics. In order to examine these questions behavioral studies and a neuroimmune system study were conducted.

4.1 Behavioral System Studies

Animals chronically dosed with lead from PND 0 to PND 28 were developmentally monitored at 7-days intervals (body weight, eye-opening, and righting reflex) and tested behaviorally at PND 28 with regard to exploratory ambulation, rearing, anxiety, and olfactory memory. It was hypothesized that lead-exposed mice would have a delay in achieving developmental milestones and would have disrupted motor function and reduced olfactory memory. The BLLs induced were within expected ranges and clearly differentiated experimental groups from controls. It was found that early chronic low-level lead exposure increased body weight and decreased exploratory (rearing) behavior in the open field. The odor habituation/dishabituation task was effective at detecting habituation for two of five scents and dishabituation for one of four scent transitions, however the test was not sensitive to the effects of early chronic low-level lead exposure and may have been too difficult for pre-adolescent animals.

4.1.1 Blood lead levels. The BLLs of children in the U.S. are usually reported in relation to a current threshold of concern, or a level considered "elevated." Nationwide it is estimated that 97.5% of children have blood lead levels below 5 µg/dL and for this reason, as of January 2012, 5 µg/dL became the current level for "elevated" child blood lead. It is broadly accepted however that no level of lead exposure is safe in children and yet the numbers of children with blood lead below this current threshold are not known. Studies in our laboratory have shown that 50% of children living in lower income neighborhoods of downtown El Paso have BLLs that range between approximately 2.5 to 7.0 µg/dL (Sobin et al., 2015). Importantly, at these low BLLs, deficits in cognition and fine-motor dexterity have been repeatedly shown (e.g. Lanphear et al., 2005; Sobin, Flores-Montoya, Gutierrez, Parisi, & Schaub, 2015).

In order to understand effects at lowest levels of lead exposure, in past studies in our laboratory, we have used murine models and successfully induced BLLs and ranges similar to those measured in 50% of children from downtown El Paso (Flores-Montoya & Sobin, 2014; Flores-Montoya et al., 2015; Sobin et al., 2013; Basgen & Sobin, 2014). (See Table 13 below for a summary of BLLs averaged across four previous studies, Sobin et al., 2017.)

Table 13. Means and standard deviations (SDs) for BLLs measured with ICP-MS in young C57BL/6J mice with and without early chronic lead exposure.*

Lead exposure group	Blood lead levels (µg/dL)
Negative controls n = 51 (0 ppm)	0.2 (0.1)
Low-dose n = 51 (30 ppm)	3.3 (0.7)
High-dose n = 26 (230 ppm)	12.3 (2.6)
High-dose n = 25 (330 pm)	14.4 (2.6)

**Mean BLLs of male and female mice were averaged across four studies (see citations in text above) (N = 159, males n = 91, females n = 68)*

In the present study, C57BL/6J male mice were exposed chronically to one of three treatment levels, 0 ppm (controls), 30 ppm (low-dose), or 430 ppm (high-dose) of lead acetate

via dams' milk from birth until weaning (PND 21) (day at which dams were removed from the litter). From weaning until PND 28 (time of sacrifice) mice ingested lead directly from water bottles.

The present study was designed to test male mice only because past studies in our laboratory have shown that typically C57BL/6J dams produce an unbalanced number of males and females per litter (Flores-Montoya & Sobin, 2014, Flores-Montoya et al., 2015, Sobin et al., 2017) and achieving balanced numbers of males and females simultaneously requires very large breeding schemes. More importantly, studies have suggested that chronic low-level lead exposure, while detrimental to both sexes, might have different mechanisms of action for disrupting brain and behavior in males as compared to females (e.g. Leasure et al., 2008, Kasten-Jolly et al., 2012). Thus, the present study was designed to more thoroughly characterize effects of early chronic low-level lead exposure in pre-adolescent male mice. Future studies will need to examine the same effects in females.

At PND 7, litters were culled to five pups per litter (C57BL/6J litter sizes typically range from five to nine pups). In instances when litters had less than five male pups, female pups were kept with the litter in order to achieve equal litter sizes. This was done in order to reduce possible variability in BLLs that could be induced from factors such as possible maternal neglect (as shown in larger litters) (Agnish & Keller, 1997) and restricted access to dams' milk and/or water due to animal competition for resources (Hager, Cheverud, & Wolf, 2012).

At PND 28 (the last day of behavioral testing and sacrifice) BLLs were measured in 36 C57BL/6J male mice with ICP-MS. It is important to note that ICP-MS is the most precise method thus far developed for detecting lowest levels of lead in blood (Sobin, Parisi, Schaub, & de La Riva, 2011). One-way ANOVA was used to examine possible differences between groups

and revealed that the low-dose group had higher mean BLLs (M = 4.24, SD = 0.61) as compared to the control group (M = 0.55, SD = 0.49) and the high-dose group had higher mean BLLs (M = *21.30*, SD = 7.03) as compared to the low-dose group and as compared to controls.

The mean BLLs induced in low-dose mice in the present study approximated those induced in 30 ppm exposed mice in five previously published studies from our laboratory. Very few laboratories have attempted to replicate low child-relevant blood lead levels in animals, and very few have demonstrated the achievement of these levels using precise measurement (ICP-MS) of lead in blood. Additionally, as of 2014, there were no published studies reporting BLLs of young (preadolescent) mice exposed chronically to low-levels of lead. In this way, these studies have been among the first to model lead exposure in an animal model with improved clinical relevance to effects in children. Our consistent replication of low child-relevant BLLs in these studies suggested that for future studies chronic exposure to 30 ppm in C57BL/6J mice is a valuable approach for understanding effects of early chronic low-level lead exposure in children.

With regard to the higher-dose lead-exposed animals, our laboratory has used 230, 330 and in the present study 430 ppm. In the present study, BLLs measured with ICP-MS in higher level lead exposed mice (430 ppm) revealed that mice had mean BLLs that ranged from 12.71 to 33.32 µg/dL. In this study and others (summarized in Table 13 above), the high-dose group BLLs were more variable. There are several possibilities as to why this might have occurred. Even though attempts were made to fully dissolve lead particles in water (using glacial acetic acid) it could be that some lead particles were not fully dissolved causing some animals to ingest higher concentrations of lead than others. In this way, uneven suspension of higher level lead in the drinking water could have resulted in greater differences in both dam exposure, absorption and delivery to pups. Another possibility is that some mice ingested more milk that others

during lactation. Another possibility is that individual pup differences in absorption rates reflect true variability and could be important for understanding absorption differences in children. Future studies should examine whether at preadolescence higher levels of exposure alter absorption rates.

To date, circulating blood lead measured in whole blood is the most widely accepted biological marker for assessing chronic child lead toxicity (Barbosa, Tanus-Santos, Gerlach, & Parsons, 2005). Even though this is the most common method used, it is not yet understood whether circulating blood lead is the most accurate representation of child lead exposure. Until novel assessments of child lead exposure are developed BLL remains the gold standard for evaluating effects of low-level lead in both child studies and animal studies.

4.1.2 Body weight. In the present study, body weight was increased in low-dose mice as compared to high-dose and control mice. Our laboratory has observed this effect in previous studies. Specifically, in this study at PND 28 low-dose mice weighed 11% more as compared to controls and 20% more as compared to high-dose mice. On the other hand, high-dose mice weighed 7% less as compared to controls. Similarly, other studies have shown that adult male (but not female) mice exposed chronically to low-levels of lead (27-32 ppm) during development had increased body weight as compared to controls (Leasure et al., 2008; Wu et al., 2016; Faulk et al., 2013).

It is not yet known why chronic low-level lead exposure altered body weight in male mice. Interestingly however a few studies have provided some clues. Specifically, two studies have provided evidence that changes in gut microbiota, that is a collection of microorganisms that reside in the gut (Shreiner, Kao, and Yung, 2016), and altered metabolism were associated

with increased body weight in chronic low-level lead exposed male mice (Wu et al., 2016; Gao et al., 2017).

4.1.2.1 Low-level lead exposure and the gut microbiota. Particular attention has been paid to the gut microbiota over the past few years because studies have shown that it plays an important role in health and disease (Shreiner, Kao, & Yung, 2016). Among some of its functions, the gut microbiota aids in metabolic processes and thus the regulation of body weight.

In a recent study, A^{vy} wild type non-agouti mice were exposed to low-levels of lead during gestation and lactation (32 ppm) until PND 21 (weaning) (N = 28) (Wu et al., 2016). In order to investigate long-lasting effects of lead after exposure, from PND 21 until the day of sacrifice, all mice were given distilled water only. Animals were weighed in weekly intervals from PND 21(week 3) until week 33 and sacrificed at week 40 at which point their colons were harvested. The tissues were homogenized and bacteria colonies were plated (on agar nutrient plates) and incubated for later DNA sequencing analyses of bacterial 16S ribosomal RNA genes (genes commonly sequenced to characterize different types of bacteria).

It was found that low-level lead exposed male but not female mice had increased body weight as compared to controls. Additionally, it was found that lead-exposed male and female mice had changes in the quantity of gut microbiota. Specifically, an increased number of bacterial anaerobes (not dependent on oxygen) and a decreased number of bacterial aerobes (consumers of oxygen), and a smaller proportion of bacteroidetes as compared to firmicutes (bacterias commonly found in gut) were found in low-level lead exposed male mice as compared to controls. Studies have shown that changes in the proportion of gut microbiota are associated with obesity (e.g. Turnbaugh et al., 2009). The study suggested that even after exposure to lead

has ended, low-level lead can have long-lasting effects on gut microbiota composition and thus body weight.

In a similar study, C57BL/6J mice were exposed to low-levels of lead (10 ppm) via drinking water starting at 8 weeks after birth, and until 21 weeks (Gao et al., 2017) (n = 5) (BLLs were not reported). In order to retrieve gut microbiota, fecal pellets were obtained before exposure and at 4 and 13 weeks post-exposure. At a later time, fecal pellets were homogenized for DNA sequencing of the 16S ribosomal genes, whole genome sequencing, and examination of metabolites via gas chromatography spectrometry. It was found that low-level lead exposure altered the development of the gut microbiome, disrupted the diversity of gut bacteria, and altered several metabolic pathways including vitamin E, bile acids, nitrogen metabolism, energy metabolism, oxidative stress, and defense/detoxification mechanisms.

The results of the studies described above suggested that low-level lead might alter body weight in part through disruption of gut microbiota and of metabolic pathways in male mice. Other mechanisms could also contribute to increased body weight in low-level lead exposed male mice. For example, it could be that low-level lead exposure alters feeding behavior in preadolescent male mice and thus increases body weight.

4.1.2.2 Is feeding behavior associated with chronic low-level lead exposure? No studies thus far have examined whether chronic low-level lead exposure alters feeding behavior in preadolescent C57BL/6J mice. It could be important to examine whether feeding behavior and/or preference for a low-fat vs. high-fat diet is altered as a result of chronic low-level lead exposure. If studies reveal significant effects, it could suggest alterations in brain areas associated with turning “on” and “off” satiety signals (e.g. in the hypothalamus) and/or changes

in brain areas associated with the rewarding effects of feeding behavior (e.g. dopaminergic regions) (Volkvow, Wang, and, Baler, 2011, review).

4.1.2.3 Future directions. Thus far, changes in the gut microbiome have been the primary focus of research for understanding changes in body weight as a result of chronic low-level lead exposure. These results are compelling and suggested that chronic low-level lead exposure results in metabolic disruptions and could predispose organisms to obesity. More studies however should attempt to replicate these findings and expand on implications of metabolic changes for the long-term health of exposed organisms and whether these changes are associated with metabolic diseases such as diabetes. Additionally, future studies should examine whether and how chronic low-level lead exposure alters feeding behavior (and/or other goal-directed behaviors) in preadolescent mice. This could in turn provide a better understanding of whether increased body weight in early chronic low-level lead exposure occurs from metabolic disruption, disruptions in feeding brain centers, or a complex combination of mechanisms acting at different times during development.

4.1.3 Open field. The open field test is traditionally used for assessing spontaneous exploratory ambulation and activity in rodents (Tanaka et al., 2012). In previous studies, our laboratory has used the open field test in a square arena and has not found group differences. In the present study, in an attempt to improve detection of effects of chronic low-level lead exposure in preadolescent male mice, a larger round arena was used and several behaviors were measured. These behaviors included rearing (standing up on hind legs), total distance travelled rather than quadrants crossed (exploratory ambulation), and time and distance travelled in the periphery (used to assess thigmotaxis, i.e. anxiety) during a 5 minute testing trial. It was found

that chronic low-level lead exposure was associated with reduced rearing behavior in the periphery and center of the arena.

For rearing in the periphery, low-level lead exposed male mice had approximately 32% fewer rearing events; and high-dose male mice had approximately 23% fewer rearing events as compared to controls. No significant difference however was detected between the high-dose and low-dose group. For rearing in the center of the open field arena, low-dose mice had approximately 50% fewer rears; and high-dose mice had approximately 75% fewer rears as compared to controls. The clinical significance of center rearing however are questionable because the number of rears displayed in the center of the arena was extremely low for all animals. The possible significance of changes in rearing behavior will be considered below.

4.1.3.1 Rearing in open field. Rearing behavior is a common complex exploratory rodent behavior that is understood to facilitate learning in a novel environment (Alves et al., 2012). When animals rear, they change their body position from a horizontal (monocular 2D vision) to a vertical (binocular 3D vision) and the elevated position which allows acquisition of additional information about a novel environment through increased visual and olfactory cues.

Rearing is facilitated by several brain areas. These include but are not limited to areas associated with memory including frontal cortex and hippocampus (Lever at al., 2006), motor function including cortico-basal ganglia thalamic pathways (Kim, Ling, and Kang, 2016) and affective states including amygdala (Bailey & Crawley, 2009). In order to understand specific effects and brain areas associated with a decrease in rearing behavior observed in chronic low-level lead mice, and what the current results might suggest, it is important to consider results from previous and current findings relevant to exploration in low-level lead exposed mice.

Similarity of current findings to previous studies in low-level lead exposed mice of exploratory activity. Reduced exploratory behavior as suggested by reduced rearing in an open field is consistent with one previous study conducted in our laboratory. In that study exploratory activity in a novel environment was measured using the unbaited nose poke task (Flores-Montoya & Sobin, 2014). Briefly, using the same lead exposure protocol as that described for the current study, lead-exposed and control mice were allowed to explore an elevated platform with 16 equally spaced holes for a period of 3 min. It was found that as BLL increased, exploratory activity in the nosepoke task decreased. Also similar to the current study in which no differences were observed in total distance traveled, in the previous study gross motor function measured with the rotarod task and distance traveled measured as total quadrants crossed with four paws, revealed no significant effects. These results suggested that reduced exploratory activity in the nosepoke task was not due to changes in ambulation or gross motor function and instead suggested that animals had either decreased "curiosity" or increased anxiety for exploring a novel environment.

Thus, the results of the study described above follow a pattern similar to results from our previous studies. Both studies revealed that chronic low-level lead exposure decreased exploratory activity in a novel environment. Additionally in the present study, no significant effects were detected for differences in exploratory ambulation or motor function; and no effects were found suggesting increased anxiety (i.e. thigmotaxis). In this way, the current results suggested that a more complex cognitive function such as "curiosity" and not motor function or anxiety, might have been primarily responsible for the decreased exploratory activity observed in mice.

Rearing behavior as an index of "curiosity." "Curiosity" has been defined as a complex form of cognitive function that precedes learning (Litman et al., 2005). Despite its importance for understanding rodent learning, very few studies have attempted to examine this complex cognitive function. One such recent study showed that mouse nicotinic receptors in the ventral tegmental area expressing the β2 subunit were responsible for curiosity, and as the authors suggested "uncertainty seeking" when exploring a novel environment (Naude et al., 2016). Additional studies are needed to understand which factors drive "curiosity" in mice, to what extent rearing behavior is associated with the construct of "curiosity," and whether chronic low-level lead exposure alters this cognitive function.

Rearing behavior as an index of memory. Many studies have suggested that rearing behavior is closely linked to hippocampal function (Lever at al., 2006). For example, one study showed that lesions to the hippocampus CA3 (cornus ammonis 3) and hippocampus DG (dentate gyrus) resulted in decreased rearing behavior (Hunsaker, Rosenberg, & Kesner, 2005). It would be very interesting to examine whether these hippocampal regions are selectively vulnerable to early chronic low-level lead exposure particularly with regard to down-regulation of cellular function or cell loss. It is also important to note that studies frequently examine rearing behavior primarily as a measure of anxiety or motor function rather than a measure of hippocampal function. Future studies are needed to better characterize types of rearing behavior and associations with disruption of hippocampal regions with regard to early chronic low-level lead exposure.

Differences between studies in measures of exploratory activity. In past studies in our laboratory, we have found specific effects on rearing behavior in chronic low-level lead exposed mice during memory retrieval in the object in place visual recognition paradigm (OIP) (Sobin et

al., 2017). In this study, it was found that low-dose and high-dose exposed mice as compared to controls had markedly increased rearing during retrieval memory trials. Rearing behavior during the retrieval memory differs fundamentally from rearing behavior in the open field task because in the open field task no cognitive challenge other than self-initiated exploration is involved. With regard to the effects of early chronic low-level lead exposure, there are several ways to explain increased rearing during retrieval memory and decreased rearing in an open field. One explanation is that visuo-perceptual impairments and/or perceptual disorientation were responsible for an increase in rearing behavior observed in the OIP task. On the other hand, and as discussed above, decreased rearing in open field and decreased exploration in the nose poke tasks might have been mediated by brain areas associated with native "curiosity" and memory in a novel environment. The results from tests of memory in mice with early chronic low-level lead exposure are discussed next.

4.1.4 Memory. Past studies in our laboratory and others have shown that chronic low-level lead exposure disrupts memory in rodents. Specifically, studies examining rodents exposed to low-levels of lead and tested during adulthood have shown that low-level lead alters spatial recognition memory (Kasten-Jolly et al., 2012) and object recognition memory (Azzaoui et al., 2009). Perhaps with greater relevance to child chronic low-level lead exposure, a previous study completed in our laboratory showed that olfactory memory measured with a two-choice paradigm was disrupted in preadolescent mice (Flores-Montoya et al., 2015). At preadolescence however no effects were detected in young mice on a spatial and object recognition memory task (OIP task) (Sobin et al., 2017). These results suggested that olfactory memory tests might be more sensitive to the effects of chronic low-level lead exposure at preadolescence. For this reason, in the present study, another type of olfactory memory task was tried in an attempt to

broaden the total number of tests determined to be sensitive to early chronic low-level lead exposure in young mice.

4.1.4.1 Odor habituation/dishabituation task. In the present study, an odor habituation/dishabituation task was used. Briefly, the task used treated cotton swabs affixed to the ends of water bottle feeding tubes to present odors to the young mice. For the acclimation phase, the cotton swab carried only plain water; in the first testing phase non-social (almond and orange) scents were presented; in the second testing phase, social odors (social scent 1 and a different social scent 2) were used. Each phase included three consecutive trials of the same scent. Habituation to odors and thus intact memory was demonstrated when mice gradually decreased the time spent exploring the odors from Trial 1 to Trial 3. Dishabituation from odors and thus intact discrimination was demonstrated when mice increased the time spent exploring a new odor beginning the next testing phase.

This study is the first to attempt this task with pre-adolescent mice and overall, the findings suggested that the task may have been too challenging for young mice. With regard to habituation, it was found that all mice regardless of treatment group demonstrated habituation to only two of five stimuli presented. Specifically, they habituated to the water-treated cotton swab and the almond-treated cotton swab (the first non-social odor presented). No habituation was apparent for orange, social scent 1, or social scent 2.

With regard to dishabituation, it was found that all mice, regardless of treatment group demonstrated dishabituation to only one (orange) of four stimuli presented. (As compared to Trial 3 of the orange scent, exploration times for all mice were significantly greater when the social scent 1 was presented indicating that mice recognized social scent 1 as a novel odor.) No dishabituation was detected for water, almond, or social scent 1. Thus the task was successful at

inducing habituation in mice only at the beginning of the task, within the first 20 min of testing; and the task successfully induced detection of novelty (of a social odor) only when mice had been exposed previously (and dishabituated) to a non-social scent.

It might be important to note that significant effects might not have been detected because of high variability within groups, perhaps an effect of individual differences in memory development. In fact, although low-dose mice did not differ significantly from controls or high-dose mice with regard to habituation or dishabituation, low-dose mice seemed to follow a different pattern in exploration time. As compared to controls and high-dose mice, it appeared that low-dose mice took longer to habituate to the odors and had little recognition of social scent 1 as a novel odor.

The development of olfactory memory in young mice has not been well-studied. These findings bring into question the possibility that at preadolescence the brains of mice have not matured fully enough to complete a relatively complex behavioral olfactory task. In fact, a study showed that major maturational events within the hippocampus occur between embryonic day 16 to PND 30 (Mody et al., 2001). The variability of performance on this task suggested that developmental studies of olfactory memory are needed before a task such as this can be used to distinguish experimental groups from controls.

4.1.4.2 Future directions. The odor habituation/dishabituation test has been shown to measure habituation/dishabituation in adult mice, typically tested at six weeks of age (PND 42) or older (Yang & Crawley, 2009). For pre-adolescent mice, a different test design might be needed to capture differences between groups. There are several possibilities regarding how to adapt the odor habituation/dishabituation task for younger animals. First of all, the task used in this study involved a 30 min acclimation period to a dry cotton swab and after acclimation, full

testing lasted one additional hour. That duration is probably too long for PND 28 mice. Thus for future studies, it will be important to simplify and shorten the task so that animals are tested for habitation and dishabituation of perhaps two odors only. In one of our past studies, a simple test of non-social odor olfactory memory that lasted only 8 min was successful at detecting effects of chronic low-level lead exposure in preadolescent mice (Flores-Montoya et al., 2015). Thus, a shorter testing period may be more effective at detecting effects of lead in olfactory memory in young mice.

It might also be important to capitalize on the fact that in the present study mice seemed to recognize a social novel odor more readily than a non-social novel odor. Thus, future studies could examine whether simpler tests of olfaction using a neutral stimulus (e.g. cotton swab with water only) for habituation and a social odor for dishabituation, could better detect effects of chronic low-level lead measured at preadolescence. This in turn could reveal whether chronic low-level lead exposure alters hippocampal regions shown to mediate social memory such as CA2 (cornus ammonis 2) (Hitti & Siegelbaum, 2014). Additionally, testing a larger sample size of young mice would improve statistical power to detect group differences when individual differences in memory development produce broad within group variability.

4.1.4.3 Summary of behavioral findings and possible mechanism of action. Studies examining chronic low-level lead exposed young mice have shown that chronic low-level lead alters body weight, exploratory activity, and olfactory memory in young mice (as shown in a past study of a two-choice paradigm). The specific mechanisms by which these effects occur are not known. Studies in the literature have shown that body weight of low-level lead exposed mice is altered via disruptions of gut microbiota. It would be important to examine whether feeding behavior might also contribute to these effects. The results also suggested that chronic low-level

lead exposure disrupts rearing behavior and that alterations in hippocampus might be responsible for these effects.

In an attempt to understand the underlying mechanisms of behavioral disruptions caused by chronic low-level lead exposure, and since past studies have shown disruption in microglial cells in hippocampus which are responsible for the construction neural pathways during development, the present study also examined whether specific biological markers were altered in hippocampal neuroimmune cells. These findings are discussed next.

4.2 Neuroimmune System Studies

After the discovery in 2015 that lymphatic vessels exist in the meninges and paravascular regions in brain, new hypotheses developed with regard to how the CNS and neuroimmune system interact (Aspeleund et al., 2015; Louveau et al., 2015). Importantly, these studies revealed that immune cells in brain accessed brain lymphatics and that CSF (cerebrospinal fluid) and ISF (interstitial fluid) drained into deep cervical lymph nodes, perhaps allowing the passage of immune cells into the periphery (below the neck). This could in turn allow immune cells from brain to exchange information with peripheral immune cells and perhaps exacerbate or otherwise modify immune responses.

Previous neurostereological studies in our laboratory showed that in low-level lead exposed mice, microglia cell density in hippocampus/dentate gyrus was less than that of controls (Sobin et al., 2013) and less than high-dose (330ppm) animals. Since those findings, our laboratory has been considering different possible explanations for why and how early chronic low-level lead exposure reduced hippocampal microglia. The present study was designed to examine whether neuroimmune mechanisms, which could cause microglial cells to exit the brain,

were upregulated in hippocampal microglial cells of chronic low-level lead exposed young mice as compared to controls and high-dose mice.

Thirty-six mice were exposed to one of three treatment levels including 0 ppm (controls), 30 ppm (low-dose), and 430 ppm (high-dose). At PND 28, mice were sacrificed and their hippocampus and brains were retrieved. Immune cells were isolated and FACS (fluorescence activated cell sorting) analyses were used to examine the possible presence of MHC II and CCR7 in cells. It was hypothesized that as compared to controls, low-dose mice would have upregulation of MHC II and CCR7 in hippocampal microglia. Additional exploratory analyses were conducted to examine these same effects in brain microglia, and in hippocampal and brain macrophages and immune cells.

4.2.1 Microglial cells.

4.2.1.1 Hippocampal microglia had decreased MHC II and CCR7. Contrary to our hypotheses, hippocampal microglial cells in chronic low-level lead exposed mice expressed reduced quantity of MHC II on the membrane surface of double positive cells (*Mdn* = 13532) as compared to controls (*Mdn* = 18395). Similarly, it was found that low-level lead exposed mice expressed reduced quantity of CCR7 on the membrane surface of single positive cells (*Mdn* = 4656) as compared to controls (*Mdn* = 5604). No significant effects however were detected for microglia in whole brain regions without hippocampus. There are at least three possible explanations for these findings. It could be that by PND 28 microglial cells with upregulated MHC II and CCR7 had already exited the hippocampus of low-level lead exposed mice. Another plausible explanation could be that chronic low-level lead exposure somehow diminished levels of MHC II and CCR7 in hippocampal microglia. It is also possible that early

chronic low-level lead exposure diminishes mechanisms responsible for the expression of MHC II and CCR7 in hippocampal microglia. Further studies are needed to explore each of these possibilities. These possible explanations will be considered in greater detail below.

Studies in our laboratory showed that chronic low-level lead exposure reduced the number of microglial cells (measured with unbiased stereology methods) in the dentate gyrus of the hippocampus (Sobin et al., 2013). These studies were replicated and expanded on a more recent neurostereological study in which young mice with early chronic low-level lead exposure were shown to have reduced numbers of microglial cells in the entire hippocampus (Dominguez et al., 2017, unpublished data, manuscript in preparation). In the present study, chi-square tests revealed a similar pattern of findings to what was described above. Specifically, as compared to controls and high-dose animals, low-dose mice had a reduced percentage of parent population microglial cells (the number of microglial cells relative to the total number of immune cells examined in the sample). Specifically, the mean percentage of hippocampal microglial cells was 45% in low-dose, 58% in controls, and 71% in high-dose mice. While independent sample median tests did not detect significant differences between groups, it is possible that significant effects were not detected because the power to detect effects was diminished by using nonparametric tests for N = 12 (hippocampi of mice combined in samples of 3 to ensure adequate cell populations). Also it should be noted that FACS was not designed to provide unbiased cell counts. Cell counts in FACS are relative to the amount of sample tested. Neurostereology is one of very few methods that provide unbiased cell count estimates in three-dimensional space. Nonetheless, the fact that the FACS cell counts replicate findings of our neurostereology studies is interesting and corroborative. A recent study providing evidence that hippocampal immune cells exit brain via CCR7 may give some clues as to additional

mechanisms by which chronic low low-level reduces numbers of microglial cells in hippocampus at pre-adolescence.

Study suggesting that CCR7 is responsible for the exiting of immune cells from hippocampus into cervical lymph nodes. In a very recent study, C57BL/6J mice were injected with bone marrow derived dendritic cells and LPS (liposaccharide) in their right frontal lobe in order to elicit upregulation of CCR7 in dendritic cells (Clarkson et al., 2016). Some animals were injected with dendritic cells that were CCR7 wild type ($CCR7^{+/+}$) and other cells that were homozygously deleted in the CCR7 locus ($CCR7^{-/-}$). Flow cytometry, confocal microscopy, and immunohistochemistry were used to investigate the fate of these cells. Perhaps of particular relevance to the present study, it was found that $CCR7^{-/-}$ dendritic cells accumulated more readily in hippocampus and brains of mice as compared to $CCR7^{+/+}$ dendritic cells. Interestingly, $CCR7^{+/+}$ dendritic cells were found in cervical lymph nodes but $CCR7^{-/-}$ dendritic cells were not. Additionally, it was found that some $CCR7^{+/+}$ dendritic cells seemed to accumulate along areas in close proximity to DG (dentate gyrus) and CA3 (cornus ammonis 3) of hippocampus suggesting that dendritic cells may have exited the brain along the DG and hippocampus CA3.

These results suggested that CCR7 is one mechanism that promotes exiting of hippocampal dendritic cells from brain, and that these cells then move into cervical lymph nodes. Importantly, dendritic cells have a similar phenotype and share several functional characteristics with microglial cells and macrophages. For example, all three cell types express CD45, MHC II, and CCR7, and are also responsible for antigen presentation to cells of the adaptive immune system (Clarkson et al., 2016; Guilliams et al., 2014; Derecki, Cronk, and Kipnis, 2012; Merad et al., 2013). Thus, it is plausible to suggest that by PND 28 microglial cells of chronic low-level

lead exposed mice could have upregulated MHC II and CCR7 and exited the hippocampus. This could explain why there was a lower quantity of microglial cells with MHC II and CCR7 in hippocampus. It is acknowledged however that this model for microglial cells remains speculative. Future studies should examine whether hippocampal microglia of chronic low-level lead exposed mice access brain lymphatics and cervical lymph nodes.

Chronic low-level lead exposure might reduce MHC II and CCR7. Another possibility is that chronic low-level lead exposure reduced the levels of MHC II and CCR7 in hippocampal microglial cells. The effects of low-level lead on these molecules could range from changes at the level of gene expression to changes for example in the folding of MHC II and CCR7.

A recent study showed that chronic low-levels of lead altered the transcription of several genes (measured with reverse transcriptase PCR) that are associated with neuroimmune system function in whole brain of BALB/c mice (Kasten-Jolly et al., 2012). Interestingly, it was found that the expression of MHC II, chemokine receptors, and chemokine ligands was altered. It is important to mention however that effects were measured in full brain and not specifically in hippocampus. Future studies should examine whether changes in MHC II and CCR7 transcripts occur in the hippocampus. Another possibility is that effects occurred at a higher level of molecular processing such as disruptions in folding of MHC II and CCR7. In fact, *in vitro* studies have shown that at higher levels of exposure lead binds to the chaperone protein GRP78 (e.g. Qian et al., 2005). This might in turn result in disruption of the otherwise proper folding of proteins and thus proper function. Future studies should examine whether chronic low-level lead exposure alters MHC II and CCR7 at the level of gene expression and/or protein level in hippocampus.

It is important to note that the effects observed were based on relatively low numbers of hippocampal microglia cells expressing MHC II and CCR7 across all conditions. Future studies are needed that use methods specifically designed to amplify the total amounts of MHC II and CCR7 detected. For example, experimental methods that trigger high MHC II and CCR7 expression could be used in lead exposed animals to answer questions regarding the "health" of mechanisms regulating MHC II and CCR7 expression. It is also possible that mechanisms other than MHC II and CCR7 might have been responsible for the reduced number of microglial cells in hippocampus. Future studies need to examine additional neuroimmune factors that might be altered as a result of chronic low-level lead exposure.

4.2.2 Macrophages and immune cells.

4.2.2.1 Macrophages had a decreased percentage of cells with CCR7 but increased quantity of MHC II in double positive cells. Similar to effects in hippocampal microglia in the low-dose group, hippocampal macrophages in the high-dose lead exposed group had a decreased percentage of macrophages expressing membrane surface CCR7. Unlike hippocampal microglia however, hippocampal macrophages had an increased quantity of membrane surface MHC II on double positive cells.

In high-dose lead exposed animals, a dramatic decrease in the percentage of hippocampal macrophages with membrane surface CCR7 was detected (*Mdn* = 0.32%; *MFI Mdn* = 19903) as compared to controls (*Mdn* = 5.24%; *MFI Mdn* = 20778) and as compared to low-dose (*Mdn* = 7.46%; *MFI Mdn* = 45687) while at the same time, an increased quantity of MHC II (measured as mean fluorescence intensity) was found in double positive cells. Moreover, in high-dose animals an increased quantity of MHC II in double positive brain macrophages was detected.

These results suggested that similar mechanisms might have mediated results in microglia and macrophages. That is, it could be that hippocampal macrophages exited the brain to reach cervical lymph nodes while (fewer) MHC II-expressing macrophages remained in hippocampus and brain to continue neuroimmune surveillance. Alternatively, it could be that MHC II and CCR7 were directly reduced as a result of high-level lead exposure. The former possibility seems more plausible however because the decrease in percentage of CCR7 positive hippocampal macrophages was concomitant with an increase in the percentage of immune cells (lymphocytes and monocytes) in hippocampus. Thus, hippocampal macrophages might have exited brain and reached cervical lymph nodes to communicate to other immune cells that there was damage or threat to brain tissue, which in turn would promote increased trafficking of immune cells into hippocampus. Future studies should investigate whether T-cells and/or B-cells for example are present in higher than expected quantities in hippocampus of higher-level lead exposed young mice.

The findings of increased number of immune cells in high-level lead exposed animals is also consistent with findings at higher levels of lead exposure that revealed that macrophages upregulated MHC II and primed T-cells towards a Th2 phenotype (humoral responses, i.e., antibody mediated immunity), and suggested that macrophages accessed lymph nodes (Dietert & Piepenbrink, 2006, review; Kasten-Jolly & Lawrence, 2014, review).

4.2.3 Strengths of the neuroimmune studies. This is the first whole organism study to examine neuroimmune mechanism effects of chronic low-level lead exposure on primary hippocampal cells in young mice. Moreover, it is one of very few studies that have examined factors associated with the trafficking of immune cells out of hippocampus and brain (and

perhaps into deep cervical lymph nodes). Additionally, the present study is translational and might increase the relevance of animal models for understanding effects in children.

4.2.4 Limitations. Since this is the first study to examine effects of chronic low-level lead exposure in MHC II and CCR7 in neuroimmune cells the interpretation of the results is conservative. Additionally, it is acknowledged that a wealth of basic research needs to be conducted in order to better understand whether microglial cells and macrophages exit brain via CCR7 and reach cervical lymph nodes to present MHC II under threatening conditions such as exposure to environmental toxins and/or pathogens.

4.2.5 Conclusions. Overall, significant effects were observed in hippocampus and not in brain suggesting that the hippocampus is a critical structure to continue studying to understand effects of lead exposure in the developing brain. Contrary to our hypotheses, at pre-adolescence, low-dose lead exposed animals had down-regulated MHC II and CCR7 as compared with controls and higher-dose animals. Studies are needed to determine whether early chronic low-level lead exposure directly altered (the structure of) MHC II and CCR7, altered mechanisms responsible for regulation of MHC II and CCR7, or whether apparent down-regulation of these is in fact a secondary effect of changes in some as yet unknown altered immune factor. Patterns of percentages of cells detected appeared to corroborate previous findings from our laboratory showing that microglia cell density is significantly reduced in low-level lead exposed animals. Finally, interesting and unexpected findings with regard to effects in macrophages (but not microglia) in higher-dose animals suggested that early chronic low-level exposure promotes primary changes in hippocampal microglia, while early chronic higher-level exposure promotes primary changes in hippocampal macrophages. The findings suggest several avenues for future research.

4.3 Is Behavior Associated with Changes in the Neuroimmune System in Chronic Low-Level Lead Exposed Mice?

Because this is our first attempt to measure MHC II and CCR7 in hippocampus of chronic low-level lead exposed young mice and because behavior within treatment groups was highly variable there was no attempt to predict effects of CCR7 on behavior (using statistical models). Future studies however should predict effects of CCR7 on behavior and possible interactions between CCR7 and low-level lead on behavior. Interestingly, in the literature some studies have examined the association between CCR7 and behavior and between reduced number of immune cells in brain and behavior. The findings of these studies are discussed below.

4.3.1 Mouse studies showing a link between CCR7 and behavior. In a study CCR7 $^{-/-}$ and CCR7 $^{+/+}$ C57BL/6J mice were tested on a variety of behavioral tasks at 2-4 months in intervals of one week and body weight was measured at 3 months (N = 56) (Jaehne & Baune, 2013). The behaviors examined included exploratory ambulation (open field test), exploratory activity (nose poke task), object recognition memory (novel odor recognition task), spatial learning and recognition memory (barns maze and Y-maze), anxiety (elevated zero maze), depression-like behavior (forced swim test and saccharin preference test), and social behavior (sociability test). Additionally, the cytokines TNF α, IL-1β, and IL-6 were measured in prefrontal cortex and hippocampi of mice and examined with ELISA (enzyme-linked immunosorbent assay.)

It was found that CCR7 $^{-/-}$ mice displayed spatial learning and memory deficits measured with barns and Y-maze, increased anxiety measured with elevated zero maze, and increased anhedonia (i.e. lack of pleasure) measured with saccharine preference test as compared to

controls. No significant effects were detected for the other behavioral outcomes measured or for cytokine analyses. Interestingly it was also found that at 3 months of age CCR7 $^{-/-}$ male mice weighed less as compared to controls. These results suggested that in the healthy mouse CCR7 plays a role in regulating spatial learning and memory, anxiety, pleasure, and body weight.

In a similar study, the effects of CCR7 and the interaction between CCR7 and maternal separation were measured in CCR7 $^{-/-}$ and CCR7 $^{+/+}$ C57BL/6J mice (N = 50) (Harrison et al., 2014). At PND 0 mice were randomly assigned to be reared under normal conditions (non-maternal separation protocol, naïve mice) or they were separated from their dams 3 hours daily from PND 1 to PND 14 (maternal separation protocol). At 10 weeks of age several tests were performed to examine the effects of CCR7 in naïve mice and the effects of maternal separation of CCR7 on behavior. These tests included measures of depression-like behavior (saccharine preference test), exploratory ambulation and anxiety (open field test), anxiety (elevated zero maze), and social behavior (sociability test). Additionally, corticosterone levels were measured with ELISA to examine neuroendocrine function.

It was found that CCR7 $^{-/-}$ naïve mice had increased anxiety as compared to controls. It was also found that maternally separated CCR7 $^{-/-}$ mice had reduced interest in social novelty but increased corticosterone levels as compared to CCR7 $^{-/-}$ naïve mice and controls. These results replicated the findings described above and suggested that CCR7 plays a role in anxiety. Additionally, it was suggested that under circumstances of high stress such as maternal separation CCR7 plays a role in social behavior and these effects might be influenced by changes in corticosterone levels and thus alterations of the hypothalamic-pituitary-adrenal (HPA) axis.

Future studies should examine whether CCR7 plays a role in regulating other types of behaviors and cognitive functions including olfactory memory during both health and disease. Additionally, future studies should examine whether and how CCR7 influences the development and/or proper function of brain areas such as frontal cortex, hippocampus, and amygdala, and the HPA axis. The lack of significant differences in the cytokines described above (TNF α, IL-1β, and IL-6) suggested that when the animal is healthy CCR7 does not influence the levels of these pro-inflammatory cytokines in hippocampus or prefrontal cortex. It would be important however to examine whether CCR7 influences the levels of pro- and anti-inflammatory cytokines in hippocampus and prefrontal cortex after an insult to the brain such as exposure to low-levels of lead and its effects on behavior. Additionally, studies examining the relationship between CCR7 and body weight and lead exposure are needed.

4.3.2 How could CCR7 modulate behavior? It could be that when the animal is healthy the normal circulation of immune cells via CCR7 in brain lymphatics and drainage into cervical lymph nodes is necessary to support normal behavior. Although studies have not examined directly this question yet, some studies have shown that reduced levels of immune cells in brain caused changes on behavior and that these changes might be associated with normal circulation of cells perhaps via CCR7. These studies are discussed next.

4.3.3 Does reduced number of immune cells in brain alter behavior? A recent study showed that depletion of microglial cells in hippocampus resulted in deficits in object recognition memory measured with the NOR task (novel object recognition task) and that these deficits were associated with a reduction of BDNF (brain derived neurotrophic factor) (Parkhust et al., 2013). These results suggested that microglia plays an important role in modulating object recognition memory via BDNF. Future studies should examine whether the reduction of

microglial cells in hippocampus of chronic low-level lead mice is associated with reduced BDNF and whether these changes mediate deficits in memory and exploratory activity.

Interestingly, in another study it was found that MHC II $^{-/-}$ mice had decreased number of CD4+ T cells in the meninges and that this reduction was associated with impaired spatial memory measured with the MWM (morris water maze) (Radjavi, Smirnof, and Kipnis, 2014). In a similar study it was found that reduced number of T-cells in meninges resulted in reduced IL-4 levels and reduced spatial memory (Derecki et al., 2010). Additionally, studies have shown that removal of deep cervical lymph nodes results in increased numbers of CD4+ T cells in meningeal spaces and reduced spatial memory measured in the MWM (Radjavi et al., 2013). These results suggested that CD4+ T cells and perhaps the proper trafficking of these cells via CCR7 into deep cervical lymph nodes is necessary for maintaining spatial memory. Additionally, these results suggested that proper communication between CD4+ T cells and cells from the innate immune system such as microglia, macrophages, and/or dendritic cells via MHC II is needed for the recruitment of CD4+ T cells into meningeal spaces and thus modulation of spatial memory. Future studies should examine whether interactions between microglia, macrophages, and or dendritic cells via MHC II result in differential recruitment of CD4+ T cells and changes on behavior after exposure to chronic low-levels of lead.

4.3.4 Conclusions. A small but growing body of literature is starting to reveal a strong relationship between the CNS and the immune system in regulating behavior both during health and disease. The studies described above and the recent findings of lymphatic vessels in brain suggested that the pathway for communication between cells from the innate immune system that reside in brain (e.g. microglia, macrophages, and/or dendritic cells) and the adaptive immune system (e.g. T-cells) might be through trafficking of immune cells through brain lymphatics via

CCR7 and drainage into deep cervical lymph nodes for antigen presentation of MHC II. Also, these results suggested that during both health and disease this communication might regulate behavior via changes in cytokine levels such as IL-4 and other proteins such as BNDF. It will be important to examine whether these interactions are associated with behavior following exposure to environmental toxins such as low-levels of lead.

4.4 Final Remarks

The present study is one of few that have examined effects of chronic low-level lead exposure on behavior and the neuroimmune system in C57BL/6J young mice, perhaps with greater relevance for understanding effects in exposed children. Past studies in our laboratory have shown that chronic low-level lead exposure altered olfactory memory, exploratory activity, and rearing behavior during a retrieval memory task in young mice (Flores-Montoya & Sobin, 2014; Flores-Montoya et al., 2015; Sobin et al., 2017). Additionally, two studies in our laboratory have shown that low-level lead exposed mice have reduced microglial cell density in DG (dentate gyrus) and whole hippocampus (Sobin et al., 2013; Dominguez et al., 2017, manuscript in preparation). The present study showed that chronic low-level lead exposure decreased exploratory activity and increased body weight in young mice. It was also found that the percentage of microglial cells were reduced in hippocampus of low-level lead exposed mice and that MHC II and CCR7 were reduced. Additional exploratory analyses showed that high-level lead exposed mice had reduced macrophages expressing CCR7 but had higher levels of MHC II.

The mechanisms mediating the above described effects on behavior and the neuroimmune system are not known however past studies and present studies suggest that the

hippocampus is a primary target of disruption for low-levels of lead and that MHC II and CCR7 might mediate some of these effects. A small but growing body of literature is starting to reveal that MHC II and CCR7 play a role in behavior during health and disease and that trafficking of immune cells outside of brain and into deep cervical lymph nodes might be associated with behavioral deficits.

It has been known for decades that the CNS and immune system constantly communicate however it is only recently that the extent to which this communication occurs and its impact on behavior during health and disease is starting to be revealed. In fact for decades, it was thought that the brain and immune system did not have a specific route of communication. New evidence is suggesting that the communication between these systems is far more fluid than previously thought, that immune cells residing in meninges are critical for regulating cognition and behavior, and that cells might be constantly trafficking outside of brain and into deep cervical lymph nodes perhaps in order to promote at type of immune cell communication that modulates behavior. The extent to which behavioral deficits in early chronic low-level lead exposure occur as a result of disruption in these mechanisms is not yet known. The findings of the present study however suggested that mechanisms associated with trafficking and communication between immune cells via MHC II and CCR7 were altered. Whether these mechanisms directly affect behavior, and also perhaps body weight, remain a subject for further investigation.

4.5 Future Directions

Future studies of chronic low-level lead exposure in young animals should further examine behavioral deficits associated with cognitive functions such as “curiosity” and should use simpler olfactory memory tests to identify additional tests that are sensitive to the effects of

chronic low-level lead exposure. Studies examining effects on body weight perhaps via changes in CCR7 in lead-exposed mice will be important to conduct. Finally, future studies should examine whether lead-exposure induces the trafficking of microglia and/or macrophages outside of brain into deep cervical lymph nodes via CCR7 for antigen presentation of MHC II to T cells, or whether lead-exposure alters the expression or levels of these markers. Determination of these effects could provide the groundwork for the development of new and more effective treatments for cognitive deficits in children associated with chronic low-level lead exposure, and perhaps could prevent the development of secondary metabolic disorders.

REFERENCES

Agnish, N. D., & Keller, K. A. (1997). The rationale for culling rodent litters. *Fundamental and Applied Toxicology, 38,* 2-6.

Alves, R., Barbosa de Carvalho, J.B., & Campana Venditti M.A. (2012). High- and low-rearing rats differ in the brain excitability controlled by the allosteric benzodiazepine site in the $GABA_A$ receptor. *Journal of Behavioral and Brain Science, 2,* 315-325.

Aspelund, A., Antila, S., Proulx, S. T., Karlsen, T. V., Karaman, S., Detmar, M., … Alitalo, K. (2015). A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *The Journal of Experimental Medicine, 212,* 991–999.

Azzaoui, F. Z., Ahami, A. O., & Khadmaoui, A. (2009). Impact of lead sub-chronic toxicity on recognition memory and motor activity of Wistar rat. *Pakistan Journal of Biological Science, 12,* 173-177.

Bailey K. R., & Crawley, J. N. (2009). *Anxiety-related behaviors in mice. Methods of behavior analysis in neuroscience.* Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK5221/

Barbosa, F., Tanus-Santos, J. E., Gerlach, R. F., & Parsons, P. J. (2005). A critical review of biomarkers used for monitoring human exposure to lead: Advantages, limitations, and future needs. *Environmental Health Perspectives, 113,* 1669–1674.

Basgen, J. M., & Sobin, C. (2014). Early chronic low-level lead exposure produces glomerular hyperthrophy in young C57BL/6J mice. *Toxicology Letters, 225,* 48-56.

Bellinger, D.C., & Needleman, H.L. (2003). Intellectual impairment and blood lead levels. *New England Journal of Medicine, 348,* 1517-1526.

Beyer, M., Gimsa, U., Eyüpoglu, I. Y., Hailer, N. P., & Nitsch, R. (2000). Phagocytosis of neuronal or glial debris by microglial cells: Upregulation of MHC class II expression and multinuclear giant cell formation in vitro. *Glia, 31*, 262-266.

Braga, M. F. M., Pereira, E. F. R., Marchioro, M., & Albuquerque, E. X. (1999). Lead increases tetrodotoxin-insensitive spontaneous release of glutamate and GABA from hippocampal neurons. *Brain Research, 826*, 10-21.

Brennan, P. A., & Keverne, E. B. (1997). Neural mechanisms of mammalian olfactory learning. *Progress in Neurobiology, 51*, 457-481.

Broadbent, N. J., Squire, L. R., & Clark, R. E. (2004). Spatial memory, recognition memory, and the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 14515–14520.

Brodkin J. (1999). Assessing memory in mice using habituation of nosepoke responding. *Behavioural Pharmacology, 10*, 445-451.

Canfield, R.L., Henderson, C.R. Jr., Cory-Slechta, D.A., Cox, C., Jusko, T.A., & Lanphear, B.P. (2003). Intellectual impairment in children with blood lead concentrations below 10 micrograms per deciliter. *New England Journal of Medicine, 348*, 1517-1526.

Carlson, N. R. (2010). *Physiology of behavior*. Boston, MA: Allyn & Bacon.

Centers for Disease Control and Prevention (2012, October). What do parents need to know to protect their children? Retrieved from http://www.cdc.gov/nceh/lead/ACCLPP/blood_lead_levels.htm

Centers for Disease Control and Prevention (2013, December). Principles of pediatric environmental health why are children often especially susceptible to the adverse effects

of environmental toxicants? Retrieved from http://www.atsdr.cdc.gov/csem/csem.asp?csem=27&po=3

Clarkson, B. D., Walker, A., Harris, M. G., Rayasam, A., Hsu, M., Sandor, M., & Fabry, Z. (2017). CCR7 deficient inflammatory Dendritic Cells are retained in the Central Nervous System. *Scientific Reports, 7*, 42856.

Cox, F. F., Carney, D., Miller, A., & Lynch, M. A. (2012). CD200 fusion protein decreases microglial activation in the hippocampus of aged rats. *Brain, Behavior, and Immunity, 26*, 789-796.

Cronk, J., Derecki, N., Ji, E., Xu, Y., Lampano, A., Smirnov, I., . . . Kipnis, J. (2015). Methyl CpG binding protein 2 regulates microglia and macrophage gene expression in response to inflammatory stimuli. *Immunity, 42*, 679-691.

Crusio W. E. (1995). *Natural selection on hippocampal circuitry underlying exploratory behavior in mice: Quantitative-genetic analysis*. Behavioural Brain Research in Naturalistic and Seminaturalistic Settings. The Netherlands: Kluwer Academic Press.

Derecki, N., Cronk, J., & Kipnis, J. (2012). FACs of acutely isolated mouse microglia. *Protocol Exchange*. Retrieved from http://www.nature.com/protocolexchange/protocols/2309.

DiDonato, C., & Bogdanik, L (2011). Behavioral phenotyping for neonates: righting reflex. Treat NMD, neuromuscular network.

Dietert R. R., & Piepenbrink MS. (2006). Lead and immune function. *Critical Reviews in Toxicology, 36*, 359-385.

Dijkstra, I. M., de Haas, A. H., Brouwer, N, Boddeke, H. W., & Bieber, K. (2006). Challenge with innate and protein antigens induces CCR7 expression by microglia in vitro and in vivo. *Glia*, *54*, 861-872.

Du Y., Ge, M. M., Xue, W., Yang Q. Q., Wang, S., Xu, Y, & Wang, H. L. (2015). Chronic lead exposure and mixed factors of gender x age x brain regions interactions on dendrite growth, spine maturity and NDR kinase. *PLOS ONE*, *10*(9), 1-19.

Eichenbaum, H., Yonelinas, A. R., & Ranganath, C. (2007). The medial temporal lobe and recognition memory. *Annual Review of Neuroscience*, *30*, 123–152.

Emanuelli, T., Pagel, F. W., Porciúncula, L. O., & Souza, D. O. (2003). Effects of 5 aminolevulinic acid on the glutamatergic neurotransmission. *Neurochemistry International*, *42*, 115-121.

Engert, F., & Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long term synaptic plasticity. *Nature, 399*, 66-70.

Environmental Protection Agency (2006). Documentation for the final 2002 point source national emissions inventory. *Report of the EPA Documentation for the Final 2002 Point Source National Emissions Inventory.* Retrieved from ftp://ftp.epa.gov/EmisInventory/2002finalnei/documentation/point/2002nei_final_point source_documentation0206.pdf.

Environmental Protection Agency (2015, October). Learn about lead. Retrieved from https://www.epa.gov/lead/learn-about-lead#found

Etmad, S (2013). *Effects of lead on human microglia and neuronal precursor cells in vitro* (Doctoral dissertation). Retrieved from UWA Research Repository. https://repository.uwa.edu.au/R/-?func=dbin-jump full&object_id=40157&local_base=GEN01-INS01.

Faulk, C., Barks, A., Liu, K., Goodrich, J. M., & Dolinoy, D. C. (2013). Early-life lead exposure results in dose- and sex-specific effects on weight and epigenetic gene regulation in weanling mice. *Epigenomics*, *5*, 487-500.

Ferguson, S. A., & Bowman, R. E. (1990). Effects of postnatal lead exposure on open field behavior in monkeys. *Neurotoxicology and Teratology*, *12*, 91-97.

Flores-Montoya, M.G, & Sobin C. (2014). Early chronic lead exposure reduces exploratory activity in young C57BL/6J mice. *Journal of Applied Toxicology*, *35*, 759-765.

Flores-Montoya, M.G., Alvarez, J.M., & Sobin C. (2015). Olfactory recognition memory is disrupted in young mice with chronic low-level lead exposure. *Toxicology Letters, 236*, 69-74.

Franko, E., Palome, J., Brown, M., Kennedy, C., & Moore, L. (2000). Blood lead levels in young children-United States and selected states,1996-1999. *Morbidity and Mortality Weekly Report*, *49*,1133-1137.

Förster, R., Davalos-Misslitz, A., & Rot, A. (2008). CCR7 and its ligands: Balancing immunity and tolerance. *Nature Reviews Immunology, 8*, 362-371.

Fujimura, K. E., Slusher, N. A., Cabana, M. D., & Lynch, S. V. (2010). Role of the gut microbiota in defining human health. *Expert Review of Anti-Infective Therapy*, *8,* 435-454.

Gao. B.,Chi, L., Mahbub, R., Bian, X., Tu, P., Ru., H., & Lu, K. (2017). Multi-omics reveals that lead exposure disturbs gut microbiome development, key metabolites, and metabolic pathways. *Chemical Research in Toxicology*, *30*, 996–1005.

Ge M.M., Hu F., Lou Z-Y, Xue W., Yu H., Xu L., …Wang, H.L.(2015). Role of Wnt/ß-catenin signaling in the protective effects of epigallocatechin-3-gallate on lead-induced impairments of spine formation in the hippocampus of rats. *RSC Advances*, *5*, 31622 31628.

Gilbert M. E., Kelly, M. E., Samsan T. E., & Goodman, G. H. (2005). Chronic developmental lead exposure reduces neurogenesis in adult rat hippocampus but does not impair spatial learning. *Toxicological Science*, *86*, 365-374.

Gilbert, S.G., & Weiss, B., 2006. A rationale for lowering the blood lead action level from 10 to [mu]g/dL. *Neurotoxicology*, *27*, 693-701.

Griffin, R., Nally, R., Nolan, Y., McCartney, Y., Linden, J., & Lynch, M. A. (2006). The age related attenuation in long-term potentiation is associated with microglial activation. *Journal of Neurochemistry, 99*, 1263-1272.

Guinane, C. M., & Cotter, P. D. (2013). Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology*, *6*, 295–308.

Gurer, H., & Ercal, N. (2000). Can antioxidants be beneficial in the treatment of lead poisoning? *Free Radical Biology and Medicine*, *29*, 927-045.

Hadaczek, P., Yamashita, Y., Mirek, H., Tamas, L., Bohn, M. C., Noble, C., … Bankiewicz, K. (2006). The “perivascular pump” driven by arterial pulsation is a powerful mechanism for the distribution of therapeutic molecules within the brain. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *14*, 69–78.

Hager, R. Cheverud, J.M., Wolf, J. B. (2012). Genotype-dependent responses to levels of sibling competition over maternal resources in mice. *Heredity, 108*, 515-520.

Hanna-Attisha, M., LaChance, J., Richard Casey Sadler, & Allison Champney Schnepp. (2016) Elevated blood lead levels in children associated with the flint drinking water crisis: A spatial analysis of risk and public health response. *American Journal of Public Health, 106*, 283-290.

Harrison, E. L., Jaehne, E. J., Jawahar, M. C., Corrigan, F., & Baune, B. T. (2014). Maternal separation modifies behavioural and neuroendocrine responses to stress in CCR7 deficient mice. *Behavioural Brain Research, 263*, 169-175.

Harms, A. S., Cao, S., Rowse, A. L., Thome, A. D., Li, X., Mangieri, L. R., … Standaert, D. G. (2013). MHCII is required for α-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration. *The Journal of Neuroscience, 33*, 9592–9600.

Hasegawa, S., Sakuragi, S., Tominaga-Yoshino, K., & Ogura, A. (2015). Dendritic spine dynamics leading to spine elimination after repeated inductions of LTD. *Scientific Reports, 5*.

Hebb, D.O. (1949). *The organizatin of behavior; a nuerophsychological theory*. New York: Wiley.

Hitti, F. L., & Siegelbaum, S. A. (2014). The hippocampal CA2 region is essential for social memory. *Nature, 508*, 89-92.

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., & Audinat, E. (2012). Deficiency of the microglial receptor cx3cr1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. *Journal of Nueroscience, 32,* 15106-15111.

Hu, F., Xu, L., Liu, Z., Ge, M., Ruan, D., & Wang, H. (2014). Developmental lead exposure alters synaptogenesis through inhibiting canonical wnt pathway in vivo and in vitro. *Plos One, 9,* 1-8.

Hunsaker M.R., Rosenberg J. S., & Kesner R.P. (2008). The role of the dentate gyrus, CA3a,b, and CA3c for detecting spatial and environmental novelty. *Hippocampus, 18*:1064 -1073.

Iliff, J. J., Wang, M., Liao, Y., Plogg, B. A., Peng, W., Gundersen, G. A., … Nedergaard, M. (2012). A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. *Science Translational Medicine, 4,* 1-11. .

Ito, D., Imai, Y., Ohsawa, K., Nakajima, K., Fukuuchi, Y., & Kohsaka, S. (1998). Microglia specific localization of a novel calcium binding protein, Iba1. *Molecular Brain Research, 57,* 1-9.

Ito, D., Tanaka, D., Suzuki, S., Dembo, T., & Fukuuchi (2001). Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke, 32,* 1208-1215.

Jaehne, E. J., & Baune, B. T. (2014). Effects of chemokine receptor signaling on cognition-like, emotion-like and sociability behaviours of CCR6 and CCR7 knockout mice. *Brain Behavioral Research, 261,* 31-39.

Jusko, T.A., Henderson, C.R., Jr., Lanphear B.P., Cory-Slechta, D.A., Parsons, P.J., & Canfield, R.L., (2008). Blood lead concentrations < 10 mg/dL and child intelligence at 6 years of age. *Environ Health Perspectives*, *116*, 243-248.

Justin, T.R., Josh, M. M., Adam, D. B., Charles, E. H., Melinda, M. P., Bethany, A. G., … Gemma, C. (2011). CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *31*, 16241–16250.

Kasten-Jolly, J., Pabello N., Bolivar V.J., & Lawrence D.A., 2012. Developmental lead effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. *Neurotoxicology*, *33*, 1005-1020.

Kasten-Jolly, J., & Lawrence, D. A. (2014). Lead modulation of macrophages causes multiorgan detrimental health effects. *Journal of Biochemical and Molecular Toxicology, 28*, 355 372.

Kee, N., Teixeira, C. M., Wang, A. H., & Frankland, P. W. (2007). Preferential incorporation of adult generated granule cells into spatial memory networks in the dentate gyrus. *Nature neuroscience*, *10*, 355-362.

Kim, H., Lim, C.-S., & Kaang, B.-K. (2016). Neuronal mechanisms and circuits underlying repetitive behaviors in mouse models of autism spectrum disorder. *Behavioral and Brain Functions*, *12*, 3.

Klaassen, C.D. (2006). *Heavy metals and heavy-metal antagonists.* New York: McGraw Hill.

Kreutzberg G.W. (1995). Microglia, the first line of defence in brain pathologies, *Arzneimittelforschung*, *45*, 357-360.

Kumawat, K. L., Kaushik, D. K., Goswami, P., & Basu, A. (2014). Acute exposure to lead acetate activates microglia and induces subsequent bystander neuronal death via caspase 3 activation. *Neurotoxicology, 41*, 143-153.

Landrigan, P.J., Trasande, L., Thorpe L.E., Gwynn, C, Lioy P.G., Lipkind H.S., ... Susser E., (2006). The national children's study: A 21-year prospective study of 100 000 american children. *Pediatrics, 118*, 2173-2186.

Lanphear, B. P., Burgoon, D. A., Rust, S. W., Eberly, S., & Galke, W., (1998). Environmental exposures to lead and urban children's blood lead levels. *Environmental Research, 76*, 120-130.

Lanphear, B.P., Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, ... Roberts R. (2005). Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. *Environmental Health Perspectives, 113*, 894-899.

Lasley, S.M., & Gilbert, M.E. (1996). Presynaptic glutamatergic function in dentate gyrus *in vivo* is diminished by chronic exposure to inorganic lead. *Brain Research, 736*, 125 134.

Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience, 39*, 151-170.

Leasure, J. L., Giddabasappa, A., Chaney, S., Johnson, J.,Jerry E., Pothakos, K., Yuen, S. L., & Fox, D. A. (2008). Low-level human equivalent gestational lead exposure produces sex specific motor and coordination abnormalities and late-onset obesity in year-old mice. *Environmental Health Perspectives, 116*, 355-361.

Legare, M. E., Barhoumi, R., Hebert, E., Bratton, G. R., Burghardt, R. C., & Tiffany-Castiglioni, E. (1998). Analysis of Pb2+entry into cultured astroglia. *Toxicological Sciences, 4* 90-100.

Lever C., Burton S., O'Keefe, J. (2006). Rearing on hind legs, environmental novelty, and the hippocampal formation. *Reviews in the Neuroscience, 46,* 111-133.

Litman J. (2005). Curiosity and the pleasures of learning: Wanting and liking new information. *Cognition and Emotion, 19,* 793-814.

Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., . . . Kipnis, J. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature, 523,* 337-341.

Louveau, A., Harris, T. H., & Kipnis, J. (2015). Revisiting the mechanisms of CNS immune privilege. *Trends in Immunology, 36,* 569-577.

Lynch, M.A. (2004). Long-term potentiation and memory. *Psychological Review, 84,* 87-136.

Liu, M.-C., Liu, X.-Q., Wang, W., Shen, X.-F., Che, H.-L., Guo, Y.-Y., … Luo, W.-J. (2012). Involvement of microglia activation in the lead induced long-term potentiation impairment. *PLoS ONE,* 7.

Liu, H., Ni, Z., Chen, Y., Wang, D., Qi, Y., Zhang, Q., & Wang, S. (2012). Olfactory route for cerebrospinal fluid drainage into the cervical lymphatic system in a rabbit experimental model. *Neural Regeneration Research, 7,* 766–771.

Liu, J., Chen, B., Zhang, J., Kuang, F., & Chen, L. (2015). Lead exposure induced microgliosis and astrogliosis in hippocampus of young mice potentially by triggering TLR4–MyD88 NFκB signaling cascades. *Toxicology Letters, 239,* 97-107.

Mander, P.K., Jekabsone, A., & Brown, G.C. (2006). Microglia proliferation is regulated by peroxide from NADPH oxidase. *J Immunol*, *176*, 1046-1052.

Mathieu, E., Gupta, N., Macdonald, R. L., Ai, J., & Yücel, Y. H. (2013). In vivo imaging of lymphatic drainage of cerebrospinal fluid in mouse. *Fluids and Barriers of the CNS*, *10*, *35*, 1-5.

Magnus, T., Chan, A., Grauer, O., Toyka K.V., & Gold, R. (2001). Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. *The Journal of Immunology*, *167*, 5005-5010.

Merad, M., Sathe, P., Helft, J., Miller, J., & Mortha, A. (2013). The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual Review of Immunology*, *31*, 563-604.

Murgan M., Ling, E.A., & Kaur C. (2013). Glutamate receptors in microglia. *CNS NeurolDisord Drug Targets. 12*,773-784.

Mody, M., Cao, Y., Cui, Z., Tay, K., Shyong, A., Shimizu, E., . . . Tsien, J. Z. (2001). Genome Wide Gene Expression Profiles of the Developing Mouse. *Hippocampus*, *98*, 8862 8867.

National Research Council (U.S.). Institute for Laboratory Animal Research (U.S.), & National Academies Press (U.S.). (2011). Guide for the care and use of laboratory animals. Washington, D.C.: National Academies Press.

Naudé J, Tolu S, Dongelmans M, Torquet N, Valverde S, Rodriguez G, Pons S, Maskos U, Mourot A, Marti F[3], Faure P. (2016). Nicotinic receptors in the ventral tegmental area promote uncertainty-seeking. *Nature Neuroscience,* 19, 471-478.

Neal, A. P., Worley, P. F., & Guilarte, T. R. (2011). Lead exposure during synaptogenesis alters NMDA receptor targeting via NMDA receptor inhibition. *Neurotoxicology, 32,* 281 289.

Needleman, H. L., Schell, A., Bellinger, D., Leviton, A., & Allred, E. N., (1990). The long-term effects of exposure to low doses of lead in childhood. An 11-year follow-up report. *New England Journal of Medicine, 322,* 83-88.

Needleman, H. L., Riess, J. A., Tobin, M. J., Biesecker, G. E., & Greenhouse, J. B., (1996). Bone lead levels and delinquent behavior. *Jama, 275,* 363-369.

Nelson, R. J., & Chiavegatto, S. (2000). Aggression in knockout mice. *ILAR Journal, 41,* 153 162.

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., . . . Gross, C.T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science, 333,* 1456-1458.

Parkhurst, C. N., Yang, G., Ninan. I., Savas., J. N., Yates, J. R., Lafaille, J. J.,…Gan, W. B. (2013). Microglia promote learning-dependent synapse formation through BDNF. *Cell, 155,* 1596 1609.

Pignatelli & Belluzi, (2010). Neurogenesis in the adult olfactory bulb. *The neurobiology of olfaction.* Retireved from http://www.ncbi.nlm.nih.gov/books/NBK55966/

Prinz, M., & Priller, J. (2014). Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease. *Nature Reviews Neuroscience, 15,* 300-312.

Qian, Y., Harris, E. D., Zheng, Y., & Tiffany-Castiglioni, E. (2000). Lead targets GRP78, a molecular chaperone, in C6 rat glioma cells. *Toxicology and Applied Pharmacology, 163,* 260-266.

Ransohoff, R. M., & Cardona, A. E. (2010). The myeloid cells of the central nervous system parenchyma. *Nature, 468*, 253-262.

Reiter, L. W., Anderson, G. E., Laskey, J. W., & Cahill, D. F. (1975). Developmental and behavioral changes in the rat during chronic exposure to lead. *Environmental Health Perspectives, 12*, 119–123.

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., . . . Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement dependent manner. *Neuron, 74*, 691-705.

Schnaas, L., Rothenberg, S. J., Perroni, E., Martinez, S., Hernandez, C., & Hernandez, R. M., 2000. Temporal pattern in the effect of postnatal blood lead level on intellectual development of young children. *Neurotoxicology and Teratology*, *22*, 805-810.

Shreiner, A. B., Kao, J. Y., & Young, V. B. (2015). The gut microbiome in health and in disease. *Current Opinion in Gastroenterology*, *31*, 69–75.

Sierra, A., Encinas, J. M., Deudero, J. J. P., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L.S., . . . Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell, 7*, 483-495.

Simon, P., Dupuis, R., & Costentin, J. (1994). Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behavioural Brain Research, 61*, 59-64.

Smith, R. S., W. W. Kao, et al. (2002). *Ocular development. Systemic Evaluation of the Mouse Eye, Anatomy, Pathology, and Biomethods*. Boca Raton, FL: CRC Press.

Snyder, J. E., Filipov, N. M., Parsons, P. J., & Lawrence, D. A. (2000). The efficiency of maternal transfer of lead and its influence on plasma IgE and splenic cellularity of mice. *Toxicological Sciences, 57*, 87-94.

Sobin C., Parisi N., Schaub T., de la Riva E. (2011). A bland-altman comparison of the lead care® system and inductively coupled plasma mass spectrometry for detecting low-level lead in child whole blood samples. *Journal of Medical Toxicology, 7,* 24-32.

Sobin, C., Flores-Montoya, M. G., Parisi, N., Schaub, T., Cervantes, M., & Armijos, R. X. (2013). Microglial disruption in young mice with early chronic lead exposure. *Toxicology Letters, 220*, 44-52.

Sobin, C., Flores-Montoya, M. G., Gutierrez, M., Parisi, N., & Schaub, T., 2015. Δ Aminolevulinic acid dehydratase single nucleotide polymorphism 2 (ALAD2) and peptide transporter 2*2 haplotype (hPEPT2*2) differently influence neurobehavior in low-level lead exposed children. *Neurotoxicology and Teratology, 47*, 137-145.

Sobin C., Flores-Montoya, M. G., Alvarez J.M. (2017). Early chronic low-level pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in an object-in-place task in pre-adolescent C57BL/6J mice. *Neurotoxicology and Teratology, 61,* 104-114.

Takahashi, K., Rochford, C. D. P. & Neumman, H. (2005). Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *The Journal of Experimental Medicine, 201,* 647-657.

Takeda H, Tsuji M, & Matsumiya T. (1998). Changes in head-dipping behavior in the hole-board test reflect anxiogenic and/or anxiolytic state in mice. *Eur. J. Pharmacol., 350,* 21-29.

Takeda A, Nakamura M, Fujii H, Uematsu C, Minamino T, Adlard P. A., …Tamano, H. (2014). Amyloid β mediated Zn^{2+} influx into dentate granule cells transiently induces a short term cognitive deficit. *PLoS ONE, 9*.

Tanaka, S., Young, J.W., Halberstadt, A.L., Masten, V.L., Geyer, M.A. (2012). Four factors underlying mouse behavior in an open field. *Behav Brain Res. 233*, 55-61.

Torgan C. (2015). *Lymphatic vessels discovered in the central nervous system*. Retrieved from http://www.nih.gov/news events.

Turnbaugh, P. J., & Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *The Journal of Physiology, 587*, 4153–4158.

Villayandre, B. M., Paniagua, M. A., Fernández-López, A., & Calvo, P. (2005). Effect of δ aminolevulinic acid treatment on N-methyl-d-aspartate receptor at different ages in the rat brain. *Brain Research, 1061*, 80-87.

Voge C., & Marcotte E.M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics*, *13*, 227-232.

Volkow, N. D., Wang, G.-J., Tomasi, D., & Baler, R. D. (2013). The addictive dimensionality of obesity. *Biological Psychiatry*, *73*, 811–818.

Wang, Q., Gao, E., & Burkhalter, A. (2011). Gateways of ventral and dorsal streams in mouse visual cortex. *Journal of Neuroscience, 31*, 1905-1918.

Wasserman, G., Liu, X., Popovac, D., Factor-Litvak, P., Kline, J. K., Waternaux, C., . . . Graziano, J. H., (2000). The Yugoslavia prospective lead study: contributions of prenatal and postnatal lead exposure to early intelligence. *Neurotoxicology and Teratology*, *22*, 811-818.

Waterman, S. J., el-Fawal, H. A., & Snyder, C.A. (1994). Lead alters the immunogenicity of two neural proteins: a potential mechanism for the progression of lead-induced neurotoxicity. *Environmental Health Perspectives*, *102*, 1052-1056.

White, L. D., Cory-Slechta, D. A., Gilbert, M. E., Tiffany-Castiglioni, E., Zawia, N. H., Virgolini, M., . . . Basha, M. R. (2007). New and evolving concepts in the neurotoxicology of lead. *Toxicology and Applied Pharmacology*, *225*, 1-27.

Wlodarczyk, A., Cédile, O., Jensen, K. N., Jasson, A., Mony, J. T., Khorooshi, R., & Owens, T. (2015). Pathologic and protective roles for microglial subsets and bone marrow- and

blood-derived myeloid cells in central nervous system Inflammation. *Frontiers in Immunology, 6,* 463.

World Health Organization (WHO), 2010. Retrieved from http://www.who.int/ipcs/features/lead..pdf

Wu, J., William, X., Faulk, C., Boehnke, K., Zhang, H., Dolinoy, D. C., Xi, C. (2016). Perinatal lead exposure alters gut microbiota composition and results in sex specific body weight increases in adult mice. *Toxicological Science, 151,* 324-333.

Yang, M., & Crawley, J. N. (2009). Simple behavioral assessment of mouse olfaction. *Current protocols in neuroscience*. John Wiley & Sons, Inc.

APPENDIX A. SUPPLEMENTARY TABLES

Table 1. Body weight group differences in young C57BL/6J mice with and without early lead exposure examined with general linear model

	df	F	*p*		Parameter Estimate Solutions				
					Est	SE	df	t	p
Body weight									
PND	2, 60	347.3	***0.000***						
Group	2, 30	9.48	***0.001***						
PND x Group	4, 60	4.05	***0.006***						
PND14				*Intercept*	7.785	0.302			
				0 ppm	0	.	.	.	.
				30 ppm	-0.218	0.427	60	-0.511	0.613
				430 ppm	-0.397	0.427	60	-0.929	0.360
PND 21				*Intercept*	9.036	0.310			
				0 ppm	0	.	.	.	**.**
				30 ppm	1.195	0.439	60	2.723	***0.011***
				430 ppm	0.288	0.439	60	0.655	0.517
PND 28				*Intercept*	14.752	0.442			
				0 ppm	0	.	.	.	.
				30 ppm	1.625	0.624	60	2.603	***0.014***
				430 ppm	-1.063	0.624	60	-1.702	0.099

N = 33

Table 2. Righting reflex group differences in young C57BL/6J mice with and without early lead exposure examined with general linear model

	df	F	*p*		Parameter Estimate Solutions				
					Est	SE	df	t	p
Righting reflex									
PND	2, 60	14.31	***0.001***						
Group	2, 30	2.66	0.086						
PND x Group	4, 60	2.38	0.110						
PND7				*Intercept*	3.748	2.233			
				0 ppm	0	.	.	.	.
				30 ppm	-0.969	3.158	60	-0.307	0.761
				430 ppm	5.539	3.158	60	1.754	0.090
PND 14				*Intercept*	0.434	0.068			
				0 ppm	0	.	.	.	**.**
				30 ppm	0.207	0.096	60	2.154	***0.039***
				430 ppm	0.357	0.096	60	3.712	***0.001***
PND 21				*Intercept*	0.224	0.018			
				0 ppm	0	.	.	.	.
				30 ppm	-0.29	0.026	60	-1.14	0.263
				430 ppm	0.036	0.026	60	1.42	0.165

N = 33

Table 3. Open field behavior group differences in young C57BL6/J mice with and without early chronic lead exposure examined with generalized linear model

		Likelihood Ratio			Parameter Estimate Solutions				
	df	X^2	p		Est	SE	df	Wald X^2	p
Rear Number									
Periphery				*Intercept*	3.532	0.516			
Group	2	25.59	***0.00***	0 ppm	0	.	.	.	.
				30 ppm	-.393	0.0723	1	24.58	***0.000***
				430 ppm	-.133	0.0723	1	3.38	0.066
Center				Intercept	0.598	0.224			
Group	2	11.43	***0.00***	0 ppm	0	.	.	.	.
				30 ppm	-0.88	0.401	1	4.867	***0.027***
				430 ppm	-1.37	0.46	1	8.676	0.003
Distance travelled (cm)									
Total				*Intercept*	4092.131	157.69			
Group	2	1.19	0.55	0 ppm	0	.	.	.	.
				30 ppm	40.16	218.32	1	0.034	0.854
				430 ppm	217.72	214.26	1	1.033	0.310
Periphery				*Intercept*	3034.45	118.74			
Group	2	4.934	0.08	0 ppm	.	.	.	.	.
				30 ppm	153.64	164.38	1	0.87	0.350
				430 ppm	367.27	161.33	1	5.18	0.023
Time in periphery (s)				*Intercept*	0.772	0.018			
Group	2	5.608	0.061	0 ppm	0	.	.	.	.
				30 ppm	0.040	0.026	1	2.375	0.123
				430 ppm	0.063	0.025	1	5.988	0.014

N = 36

Table 4. Non-social odor exploration time (habituation) test of group differences in young C57BL6/J mice with and without early chronic lead exposure examined with general linear model

	df	F	*p*		Parameter Estimate Solutions				
					Est	SE	df	t	p
Water									
Trial	2, 60	13.03	***0.00***						
Group	2, 30	0.298	0.74						
Trial x Group	4, 60	0.148	0.96						
Trial 1				*Intercept*	16.66	6.37			
				0 ppm	0	.	.	.	.
				30 ppm	3.03	9.00	60	0.336	0.739
				430 ppm	-1.23	9.00	60	-.137	0.892
Trial 2				*Intercept*	8.44	2.08	60		
				0 ppm	0	.	.	.	.
				30 ppm	-0.64	2.94	60	-0.218	0.829
				430 ppm	-1.79	2.94	60	-6.1	0.55
Trial 3				*Intercept*	3.77	1.75	60		
				0 ppm	0	.	.	.	.
				30 ppm	3.54	2.48	60	1.43	0.163
				430 ppm	-.958	2.48	60	-0.387	0.702
Almond				*Intercept*	4.7	1.46	60		
Trial	2, 60	6.83	***0.02***			.	.	.	.
Group	2, 30	0.31	0.73						
Trial x Group	4, 60	1.35	0.26						
Trial1				0 ppm	0	.	.	.	.
				30 ppm	-1.48	2.07	60	-.72	0.478
				430 ppm	0.215	2.07	60	0.104	0.918
Trial 2				*Intercept*	1.94	0.66	60		
				0 ppm	0	.	.	.	.
				30 ppm	1.01	0.93	60	1.08	0.289
				430 ppm	-0.593	0.93	60	-0.632	0.532
Trial 3				*Intercept*	3.03	0.82	60		
				0 ppm	0	.	.	.	.
				30 ppm	-2.07	1.16	60	-1.77	0.086
				430 ppm	-1.19	1.16	60	-1.02	0.315
Orange				*Intercept*	1.24	0.91	60		
Trial	2, 60	1.41	0.25						
Group	2, 30	0.49	0.61						
Trial x Group	4, 60	0.28	0.88						
Trial 1				0 ppm	0	.	.	.	.
				30 ppm	0.79	1.28	60	0.619	0.541
				430 ppm	1.05	1.28	60	0.824	0.416
Trial 2				*Intercept*	.905	0.366	60	2.47	0.19
				0 ppm	0	.	.	.	.
				30 ppm	-0.44	0.518	60	-.853	0.40
				430 ppm	-0.299	0.518	60	-.577	0.569
Trial 3				*Intercept*	0.888	1.380	60	.644	0.525
				0 ppm	0	.	.	.	.
				30 ppm	1.339	1.95	60	.686	.498
				430 ppm	1.43	1.95	60	0.733	0.469

N = 33

Table 5. Social odor exploration time (habituation) test of group differences in young C57BL6/J mice with and without early chronic lead exposure examined with general linear model

		df	F	*p*		Solutions for Fixed Effects				
						Est	SE	df	t	p
Social Scent 1										
Trial		2, 60	2.62	0.08						
Group		2, 30	0.65	0.52						
Trial x Group		4, 60	0.62	0.65						
	Trial 1				*Intercept*	10.93	4.06			
					0 ppm	0	.	.	.	.
					30 ppm	-7.71	5.74	60	-1.34	0.19
					430 ppm	-3.05	5.74	60	-.532	.599
	Trial 2				*Intercept*	3.81	2.84	60		
					0 ppm	0	.	.	.	.
					30 ppm	0.76	4.01	60	0.19	0.85
					430 ppm	0.16	4.01	60	0.41	0.96
	Trial 3				*Intercept*	2.77	1.41	60		
					0 ppm	0	.	.	.	.
					30 ppm	-2.06	1.99	60	-1.03	0.31
					430 ppm	0.28	1.99	60	0.14	0.88
Social Scent 2					*Intercept*	2.91	2.22	60		
Trial		2, 60	0.109	0.89			.	.	.	.
Group		2, 30	0.317	0.73						
Trial x Group		4, 60	0.250	0.90						
	Trial 1				0 ppm	0	.	.	.	.
					30 ppm	2.65	3.13	60	0.84	0.40
					430 ppm	-.389	3.136	60	-.124	.902
	Trial 2				*Intercept*	2.82	1.73	60		
					0 ppm	0	.	.	.	.
					30 ppm	.378	2.45	60	0.154	0.87
					430 ppm	-.186	2.44	60	-0.76	0.94
	Trial 3				*Intercept*	2.17	2.32	60		
					0 ppm	0	.	.	.	.
					30 ppm	1.00	3.28	60	0.30	0.763
					430 ppm	1.80	3.28	60	0.55	0.587

N = 33

Table 6. Social odor exploration time (dishabituation) test of group differences in young C57BL6/J mice with and without early chronic lead exposure examined with general linear model analyses

	df	F	*p*		Solutions for Fixed Effects				
					Est	SE	df	t	p
Water T3 vs. Almond T1									
Trial	1, 30	0.076	0.785						
Group	2, 30	0.367	0.696						
Trial x Group	2, 30	2.317	0.116						
Water Trial 3				*Intercept*	3.76	1.75			
				0 ppm	0	.	.	.	.
				30 ppm	3.545	2.478	30	1.431	0.163
				430 ppm	-0.958	2.478	30	-.387	0.702
Almond Trial 1				*Intercept*	4.71	1.463			
				0 ppm	0	.	.	.	.
				30 ppm	-1.48	2.07	30	-.718	.478
				430 ppm	.215	2.07	30	.104	.918
Almond T3 vs. Orange T1									
Trial	1,30	0.015	0.905						
Group	2,30	0.315	0.732						
Trial x Group	2,30	1.586	0.221						
Almond Trial 3				*Intercept*	3.03	0.82			
				0 ppm	0	.	.	.	.
				30 ppm	-2.075	1.167	30	-1.77	0.086
				430 ppm	-1.192	1.167	30	-1.021	0.315
Orange Trial 1				*Intercept*	1.24	0.91		1.367	0.182
				0 ppm	0	.	.	.	.
				30 ppm	0.79	1.28	30	0.619	0.541
				430 ppm	1.05	1.28	30	0.824	0.416
Orange T3 vs. SS 1 T1									
Trial	1, 30	7.37	***0.011***						
Group	2,30	0.45	0.642						
Trial x Group	2, 30	1.64	0.210						
Orange Trial 3				*Intercept*	0.88	1.30			
				0 ppm	0	.	.	.	.
				30 ppm	1.339	1.95	30	0.686	0.498
				430 ppm	1.430	1.95	30	0.733	0.469
Social Scent 1 Trial 1				*Intercept*	10.932	4.065			
				0 ppm	0	.	.	.	.
				30 ppm	-7.715	5.748	30	-1.342	0.190
				430 ppm	-3.055	5.748	30	-0.532	0.599
SS 1 T3 vs. SS 2 T1									
Trial	1, 30	0.002	0.967						
Group	2, 30	1.465	0.247						
Trial x Group	2,30	1.611	0.216						
Social Scent 1 Trial 3				*Intercept*	1.500	0.501			
				0 ppm	0	.	.	.	.
				30 ppm	-1.364	0.708	30	-1.925	0.064
				430 ppm	-0.545	0.708	30	-0.770	0.447
Social Scent 2 Trial 1				*Intercept*	1.136	0.415			
				0 ppm	0	.	.	.	.
				30 ppm	-0.091	0.587	30	-0.155	0.878
				430 ppm	-0.733	0.587	30	-1.317	0.198

N = 33
T. refers to trial
SS. refers to social scent

Table 7. Hippocampal microglial cell median values for measured parameters in young C57BL6/J mice with and without chronic lead exposure*

	% Parent Population (Median)			Cell Count (Median)			Mean Fluorescence Intensity (MFI) (GM/CV)**		
	0 ppm	**30 ppm**	**430 ppm**	**0 ppm**	**30 ppm**	**430 ppm**	**0 ppm**	**30 ppm**	**430 ppm**
Overall									
Microglia	.4425	.3945	.6035	4656	1846	12883	…	…	…
MHC II	…	…	…	…	…	…	6341 ±117	4878 ±114	4987 ±69
CCR7	…	…	…	…	…	….	5604 ± 38	4656 ± 36	3938 ± 36
Quadrant									
MHC II	.0068	.0053	.0050	26	15	83	27946 ± 168	21386 ± 0136	16739±144
CCR7	.0160	.0067	.0035	25	9	45	14454 ± 544	11326 ± 8	10927±30
MHCII/CCR7	.0031	.0016	.0006	7	1	8	…	…	…
MHC II	…	…	…	…	…	…	18395±34	13532 ±0	18545±32
CCR7	…	…	…	…	…	…	15108±7	11080±0	11201.5± 0

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Geometric mean/Coefficient of variation*

Table 8. Brain microglial cell median values for measured parameters in young C57BL6/J mice with and without chronic lead exposure*

	%			Count			Mean Fluorescence Intensity (MFI) (GM/CV)**		
	0 ppm	**30 ppm**	**430 ppm**	**0 ppm**	**30 ppm**	**430 ppm**	**0 ppm**	**30 ppm**	**430 ppm**
Overall									
Microglia	0.8515	0.8285	0.7800	46068	44507	44507	…	…	…
MHC II	…	…	…	…	…	…	5307 ± 78	4722 ±108	5371± 66
CCR7	…	…	…	…	…	…	4163 ± 41	4278 ±49	4329 ± 41
Quadrant									
MHC II	0.0039	0.0048	0.0024	164	199	159	18694 ± 113	18987±207	23095 ±153
CCR7	0.0018	0.0022	0.0023	72	97	141	12320 ± 109	13286±145	12006 ±127
MHCII/CCR7	0.0006	0.0007	0.0005	21	28	29	…	…	…
MHC II	…	…	…	…	…	…	29374 ± 125	28039±125	23420 ± 71
CCR7	…	…	…	…	…	…	12351 ± 18	13100±24	12604 ±19

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Geometric mean/Coefficient of variation*

Table 9. Hippocampal macrophage median values for measured parameters in young C57BL6/J mice with and without chronic lead exposure*

	%			Count			Mean Fluorescence Intensity (MFI) (GM/CV)**		
	0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm
Overall									
Macrophages	.0408	.0506	.0298	171	210	520	…	…	…
MHC II	…	…	…	…	…	…	10786 ±175	10920±247	15964±267
CCR7	…	…	…	…	…	…	9695 ± 247	7342±67	6969±165
Quadrant									
MHC II	.3025	.4535	.5010	60	86	262	19555±181	17908±184	18879±288
CCR7	.0524	.0746	.0032	22	21	2	20778±128	19903±40	45687±132
MHC II/CCR7	.2655	.1510	.2380	42	35	136	…	…	…
MHC II	…	…	…	…	…	…	21371±79	21720± 49	29264±100
CCR7	…	…	…	…	…	…	14753±89	13880±51	13360±28

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Geometric mean/Coefficient of variation*

Table 10. Brain macrophage median values for measured parameters in young C57BL6/J mice with and without chronic lead exposure*

	%			Count			Mean Fluorescence Intensity (MFI) (GM/CV)**		
	0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm
Overall									
Macrophages	.0390	.0334	.0389	1878	1569	3118	…	…	…
MHC II	…	…	…	…	…	…	7940 ± 275	7763 ± 357	8946 ± 311
CCR7	…	…	…	…	…	…	5346 ± 150	5315 ± 284	5722 ± 241
Quadrant									
MHC II	.2810	.3040	2865	507	344	745	19088 ± 208	18678 ± 274	20745 ± 235
CCR7	.0282	.0265	.0049	61	34	15	17357 ± 167	19970 ± 222	26494 ± 189
MHC II/CCR7	.0807	.0578	.1465	131	81	337	…	…	…
MHC II	…	…	…	…	…	…	24117 ± 228	18752 ± 104	25588 ± 159
CCR7	…	…	…	…	…	…	3388 ± 53	13247 ± 31	12446 ± 47

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Geometric mean/Coefficient of variation*

Table 11. Hippocampal and brain immune cell median values for measured parameters in young C57BL6/J mice with and without chronic lead exposure*

	%			Count		
	0 ppm	**30 ppm**	**430 ppm**	**0 ppm**	**30 ppm**	**430 ppm**
Hippocampal immune cells	.4640	.3330	.5665	10882	4742	21934
Brain immune cells	.7530	.7685	.8360	53304	53136	80001

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Geometric mean/Coefficient of variation*

Table 12. Chi-square results for differences in microglial cell percentage of parent population measured in hippocampus and brain in young C57BL6/J mice with and without early chronic lead exposure*

			Chi-square	df	p-value
Hippocampus	Microglia		1437.326	2	***.000***
		MHC II	6.494	2	***.039***
		CCR7	46.801	2	***.000***
		MHCII/CCR7	2.036	2	.361
Brain	Microglia		879.414	2	***.000***
		MHC II	859.479	2	***.000***
		CCR7	40.006	2	***000***
		MHCII/CCR7	19.837	2	***.000***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

Table 13. Chi-square results for differences in macrophage percentage of parent population measured in hippocampus and brain in young C57BL6J mice with and without early chronic lead exposure*

		Chi-square	df	p-value
Hippocampus	Macrophages	215.970	2	***.000***
	MHC II	14.066	2	***.001***
	CCR7	74.665	2	***.000***
	MHCII/CCR7	56.273	2	***.000***
Brain	Macrophages	434.930	2	***.000***
	MHC II	14.066	2	***.001***
	CCR7	42.695	2	***.000***
	MHCII/CCR7	169.415	2	***.000***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

Table 14. Chi-square results for differences in immune cell percentage measured in hippocampus and brain in young C57BL6J mice with and without early chronic lead exposure*

		Chi-square	df	p-value
Hippocampus	Immune cells	2585.208	2	***.000***
Brain	Immune cells	2876.674	2	***.000***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

Table 15. Hippocampal microglial cell results examining differences between groups with independent samples median tests in young C57BL6/J mice with and without chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Microglia	%	2.00	2	0.368
	Cell Count	8.00	2	***0.018***
MHC II	MFI (GM)**	2.00	2	0.368
CCR7	MFI (GM)	6.00	2	***0.050***
Quadrant Analyses				
"Single positive" MHC II	%	0.005	2	*0.368*
	Cell Count	8.00	2	***0.018***
	MFI (GM)	2.00	2	0.368
"Single positive" CCR7	%	0.69	2	0.71
	Cell Count	6.00	2	***0.050***
	MFI (GM)	0.00	2	1.00
"Double positive" MHCII/CCR7* **				
MHC II	MFI (GM)	6.00	2	***0.050***
CCR7	MFI (GM)	2.00	2	0.368

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Mean fluorescence intensity/geometric mean.*

****Chi-square did not show significant differences between group (see Table 12) thus double positives percentage and count were not examined with independent samples median tests.*

Table 16. Brain microglial cell results examining differences between groups with independent samples median tests in young C57BL6/J mice with and without chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Microglia	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
MHC II	MFI (GM)**	2.00	2	0.368
CCR7	MFI (GM)	2.00	2	0.368
Quadrant Analyses				
"Single positive"				
MHC II	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
	MFI (GM)	2.00	2	0.368
"Single positive"				
CCR7	%	6.00	2	***0.050***
	Cell Count	2.00	2	0.368
	MFI (GM)	2.00	2	0.369
"Double positive"				
MHCII/CCR7	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
MHC II	MFI (GM)	2.00	2	0.368
CCR7	MFI (GM)	0.00	2	1.00

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

*** Mean fluorescence intensity/geometric mean*

Table 17. Hippocampal macrophages results examining differences between groups with independent samples median tests in young C57BL6/J mice with and without chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Macrophages	%	6.00	2	***0.050***
	Cell Count	6.00	2	***0.050***
MHC II	MFI (GM)	2.00	2	0.368
CCR7	MFI (GM)	2.00	2	0.368
Quadrant Analyses				
"Single positive"				
MHC II	%	2.00	2	0.368
	Cell Count	6.00	2	***0.050***
	MFI (GM)	2.00	2	0.368
"Single positive"				
CCR7	%	8.00	2	***0.018***
	Cell Count	2.00	2	0.368
	MFI (GM)	1.253	2	0.535
"Double positive"	%	0.00	2	1.00
MHCII/CCR7				
	Cell Count	2.00	2	0.368
MHC II	MFI (GM)	6.00	2	***0.050***
CCR7	MFI (GM)	0.00	2	1.00

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

*** Mean fluorescence intensity/geometric mean*

Table 18. Brain macrophages results examining differences between groups with independent samples median tests in young C57BL6/J mice with and without chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Macrophages	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
MHC II	MFI (GM)	2.00	2	0.368
CCR7	MFI (GM)	0.00	2	1.00
Quadrant Analyses				
"Single positive" MHC II	%	0.00	2	1.00
	Cell Count	2.00	2	0.368
	MFI (GM)	2.00	2	0.368
"Single positive" CCR7	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
	MFI (GM)	2.00	2	0.368
"Double positive" (MHCII/CCR7)	%	2.00	2	0.368
	Cell Count	2.00	2	0.368
MHC II	MFI (GM)	8.00	2	***0.018***
CCR7	MFI (GM)	2.00	2	0.368

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

*** Mean fluorescence intensity/geometric mean*

Table 19. Hippocampal and brain immune cell results examining differences between groups with independent samples median tests in young C57BL6/J mice with and without chronic lead exposure*

		Test-statistic	df	p-value
Hippocampal Immune cells	%	8.00	2	***0.018***
	Cell Count	8.00	2	***0.018***
Brain Immune cells	%	2.00	2	0.368
	Cell Count	6.00	2	***0.050***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

*** Mean fluorescence intensity/geometric mean*

Table 20. Post-hoc analyses for significant main effect group comparisons in hippocampal microglial cells of young C57BL6/J mice with and without chronic lead exposure*

		Group Comparison Statistics					
		0 ppm vs. 30 ppm		0 ppm vs. 430 ppm		30 ppm vs. 430 ppm	
		test	*p*	*test*	*p*	*test*	*p*
Overall							
Microglia	**Cell Count**	0.00	1.00	2.00	0.427	8.00	***0.014***
CCR7	**MFI (GM)**	8.00	***0.014***	2.00	0.472	2.00	0.472
Quadrant							
MHC II	**Cell Count**	0.00	1.00	2.00	0.472	8.00	***0.014***
CCR7	**Cell Count**	2.00	0.472	2.00	0.472	2.00	0.472
MHC II[1]	**MFI (GM)**	8.00	***0.014***	0.00	1.00	2.00	0.472

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

[1] Refers to MHCII measure of double positive microglial cell measured in hippocampus.

Table 21. Post-hoc analyses for significant main effect group comparisons in brain microglial cells of young C57BL6/J mice with and without chronic lead exposure*

Variables		Group Comparisons Statistics					
		0 ppm vs. 30 ppm		0 ppm vs. 430 ppm		30 ppm vs. 430 ppm	
		test	***p***	***test***	***p***	***test***	***p***
Quadrant							
CCR7	%	2.00	0.472	2.00	0.472	0.533	1.00

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

[1] Refers to MHCII measure of double positive microglial cell measured in brain.

Table 22. Post-hoc analyses for significant main effect group comparisons in hippocampal macrophages of young C57BL6/J mice with and without chronic lead exposure*

		Group Comparison Statistics					
		0 ppm vs. 30 ppm		0 ppm vs. 430 ppm		30 ppm vs. 430 ppm	
		Test	*p*	*Test*	*p*	*Test*	*p*
Overall							
Macrophages	**%**	0.00	1.00	2.00	0.472	2.00	0.472
Macrophages	**Cell Count**	0.00	1.00	2.00	0.472	8.00	***0.014***
	*Simulated***	***6.00***	***0.043***	0.00	1.000	24.00	***0.000***
Quadrant							
MHC II	**Cell Count**	0.00	1.00	2.00	0.472	8.00	***0.014***
	Simulated	0.66	1.00	2.67	0.310	24.00	***0.000***
CCR7	**%**	0.00	1.00	8.00	***0.014***	2.00	0.472
	Simulated	0.67	1.00	24.00	***0.000***	24.00	***0.003***
MHCI I[1]	**MFI (GM)**	0.00	1.00	8.00	***0.014***	2.00	0.472
	Simulated	0.67	1.00	16.67	***0.000***	24.00	***0.000***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

***Simulated refers to database representing N = 36.*

[1] Refers to MHCII measure of double positive microglial cell measured in brain.

Table 23. Post-hoc analyses for significant main effect group comparisons in brain macrophages of young C57BL6/J mice with and without chronic lead exposure*

Variables		Group Comparison Statistics					
		0 ppm vs. 30 ppm		0 ppm vs. 430 ppm		30 ppm vs. 430 ppm	
		Test	***p***	***Test***	***p***	***Test***	***p***
MHC II[1]	**MFI (GM)**	4.00	0.350	-3.25	0.61	-7.25	***0.013***

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests.*

[1] Refers to MHCII measure of double positive microglial cell measured in brain.

Table 24. Post-hoc analyses for significant main effect group comparisons hippocampal and brain immune cells of young C57BL6/J mice with and without chronic lead exposure*

		Group Comparison Statistics					
		0 ppm vs. 30 ppm		0 ppm vs. 430 ppm		30 ppm vs. 430 ppm	
		Test	***p***	***Test***	***p***	***Test***	***p***
Hippocampal immune cells	**%**	0.00	1.00	0.00	1.00	8.00	***0.014***
	Count	2.00	0.472	2.00	0.472	8.00	***0.014***
Brain immune cells	**Count**	0.00	1.00	8.00	***0.014***	2.00	0.472

**N = 12 samples, from 36 animals combined into groups of 3 to ensure sufficient cells for hippocampus tests*.

[1] Refers to MHCII measure of double positive microglial cell measured in brain.

Table 25. Tests of homogeneity of variance for behavioral outcomes measured at one time point in young C57BL6/J mice with and without early chronic lead exposure*

	Test statistic	df	P
Inverted screen test	4.677	2, 30	***0.017***
Open Field			
Rearing			
Periphery	1.801	2, 33	0.181
Center	2.691	2, 33	0.083
Distance travelled			
Total	2.521	2, 33	0.096
Periphery	1.768	2, 33	0.076
Time in Periphery	0.165	2, 33	0.848

**Levene's statistic was used to examine homogeneity of variance in outcome variables measured at one time point (PND 28).*

Table 26. Tests of sphericity for behavioral outcomes measured at different time points in young C57BL6/J mice with and without early chronic lead exposure*

	Test statistic	df	p
BLLs			
Developmental Milestones			
Body weight	**0.957**	2	0.532
Righting reflex	**0.003**	2	***0.000***
Odor habituation/dishabituation			
Habituation			
Water	0.273	2	***0.000***
Almond	0.765	2	***0.021***
Orange	0.652	2	***0.002***
Social Scent 1	0.496	2	***0.000***
Social Scent 2	0.990	2	0.859
Dishabituation			
WT3 TO AT1	1.00	0	.
AT3 OT1	1.00	0	.
OT3 SST1	1.00	0	.
SS1 T3 SS2 T1	1.00	0	.

**Mauchly's test of sphericity statistic was used to examine homogeneity of variance in outcome variables measured at different time points.*

T. refers to trial.

SS. refers to social scent.

Table 27. Tests of normality distribution for behavioral outcomes on developmental milestones and open field in young C57BL6/J mice with and without early chronic lead exposure*

Developmental Milestones		**Test statistic**	**df**	**P**
Eye-opening				
Body weight				
PND 14	0 ppm	0.183	11	0.200
	30 ppm	0.363	11	***0.000***
	430 ppm	0.153	11	0.200
PND 21	0 ppm	0.238	11	0.083
	30 ppm	0.184	11	0.200
	430 ppm	0.231	11	0.104
PND 28	0 ppm	0.197	11	0.200
	30 ppm	0.167	11	0.200
	430 ppm	0.201	11	0.189
Righting reflex				
PND 7	0 ppm	0.255	11	***0.044***
	30 ppm	0.255	11	***0.044***
	430 ppm	0.280	11	***0.041***
PND 14	0 ppm	0.208	11	0.198
	30 ppm	0.158	11	0.200
	430 ppm	0.131	11	0.200
PND 21	0 ppm	0.302	11	***0.006***
	30 ppm	0.263	11	***0.032***
	430 ppm	0.318	11	***0.003***
Inverted screen test	0 ppm	0.197	11	2.000
	30 ppm	0.325	11	***0.002***
	430 ppm	0.158	11	2.000
Open Field				
Rearing				
Periphery	0 ppm	0.200	11	0.200
	30 ppm	0.226	11	0.092
	430 ppm	0.140	11	0.200
Center	0 ppm	0.339	11	***0.001***
	30 ppm	0.396	11	***0.000***
	430 ppm	0.428	11	***0.000***
Distance travelled				
Total	0 ppm	0.943	11	0.551
	30 ppm	0.906	11	0.192
	430 ppm	0.911	11	0.188
Periphery	0 ppm	0.154	11	0.492
	30 ppm	0.200	11	0.391
	430 ppm	0.204	11	0.249
Time in Periphery	0 ppm	0.153	11	0.200
	30 ppm	0.159	11	0.200
	430 ppm	0.102	11	0.200

**Kolmogorov-smirnov test was used to examine distribution properties.*

Table 28. Tests of normality distribution for behavioral outcomes on odor habituation/dishabituation task (non-social odors) in young C57BL6/J mice with and without early chronic lead exposure.

		Group	Test statistic	df	p
Water					
	Trial 1	0 ppm	0.150	11	0.200
		30 ppm	0.310	11	***0.004***
		430 ppm	0.360	11	***0.000***
	Trial 2	0 ppm	0.137	11	2.000
		30 ppm	0.201	11	2.000
		430 ppm	0.187	11	***0.012***
	Trial 3	0 ppm	0.284	11	***0.013***
		30 ppm	0.324	11	***0.002***
		430 ppm	0.208	11	0.200
Almond					
	Trial 1	0 ppm	0.189	11	2.00
		30 ppm	0.153	11	2.00
		430 ppm	0.314	11	***0.003***
	Trial 1	0 ppm	0.280	11	***0.016***
		30 ppm	0.166	11	0.200
		430 ppm	0.182	11	0.200
	Trial 3	0 ppm	0.326	11	***0.002***
		30 ppm	0.255	11	***0.043***
		430 ppm	0.253	11	***0.048***
Orange					
	Trial 1	0 ppm	0.349	11	***0.001***
		30 ppm	0.238	11	0.081
		430 ppm	0.280	11	***0.016***
	Trial 2	0 ppm	0.405	11	***0.000***
		30 ppm	0.312	11	***0.004***
		430 ppm	0.332	11	***0.001***
	Trial 3	0 ppm	0.257	11	***0.042***
		30 ppm	0.478	11	***0.000***
		430 ppm	0.411	11	***0.000***

**Kolmogorov-smirnov test was used to examine distribution properties.*

Table 29. Tests of normality distribution for behavioral outcomes on odor habituation/dishabituation task (social odors) in young C57BL6/J mice with and without early chronic lead exposure.

	Group	Test statistic	df	p
Social Scent 1				
	0 ppm	0.325	11	***0.002***
	30 ppm	0.494	11	***0.000***
	430 ppm	0.355	11	***0.000***
	0 ppm	0.403	11	***0.000***
	30 ppm	0.442	11	***0.000***
	430 ppm	0.294	11	***0.009***
	0 ppm	0.393	11	***0.000***
	30 ppm	0.450	11	***0.000***
	430 ppm	0.406	11	***0.000***
Social scent 2				
	0 ppm	0.528	11	***0.000***
	30 ppm	0.379	11	***0.000***
	430 ppm	0.305	11	***0.000***
	0 ppm	0.416	11	***0.000***
	30 ppm	0.352	11	***0.000***
	430 ppm	0.350	11	***0.000***
	0 ppm	0.457	11	***0.000***
	30 ppm	0.444	11	***0.000***
	430 ppm	0.332	11	***0.001***

**Kolmogorov-smirnov test was used to examine distribution properties.*

Table 30. Tests of homogeneity of variance for hippocampal microglia outcomes in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Microglia	%	5.57	2, 9	***0.027***
	Cell Count	2.94	2. 9	0.104
MHC II	MFI (GM)	0.77	2, 9	0.490
CCR7	MFI (GM)	1.53	2, 9	0.260
Quadrant Analyses				
"Single positive"				
MHC II	%	2.61	2, 9	0.127
	Cell Count	2.43	2, 9	***0.146***
	MFI (GM)	1.91	2, 9	0.204
"Single positive"		5.00	2, 9	***0.035***
CCR7	%			
	Cell Count	5.05	2, 9	***0.034***
	MFI (GM)	17.17	2, 9	***0.001***
"Double positive"				
MHCII/CCR7	%	3.30	2, 9	0.084
	Cell Count	1.41	2, 9	0.294
MHC II	MFI (GM)	0.83	2, 9	0.465
CCR7	MFI (GM)	1.76	2, 9	0.225

** Levene's test statistic was used to examine possible unequal variances.*

Table 31. Tests of homogeneity of variance for brain microglia outcomes in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Brain Microglia	%	3.267	2, 9	0.086
	Cell Count	2.902	2, 9	0.106
MHC II	MFI (GM)	1.356	2, 9	0.306
CCR7	MFI (GM)	0.854	2, 9	0.458
Quadrant Analyses				
"Single positive"				
MHC II	%	7.67	2, 9	***0.011***
	Cell Count	8.10	2, 9	***0.010***
	MFI (GM)	3.05	2, 9	0.097
"Single positive"				
CCR7	%	1.16	2, 9	0.354
	Cell Count	2.237	2, 9	0.163
	MFI (GM)	4.41	2, 9	***0.046***
"Double positive"				
MHCII/CCR7	%	3.31	2, 9	0.084
	Cell Count	3.34	2, 9	0.082
MHC II	MFI (GM)	0.626	2, 9	0.556
CCR7	MFI (GM)	2.74	2, 9	0.117

** Levene's test statistic was used to examine possible unequal variances.*

Table 32. Tests of homogeneity of variance for hippocampal macrophages outcomes in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Hippocampal				
macrophages	%	2.07	2, 9	0.182
	Cell Count	6.01	2. 9	***0.022***
MHC II	MFI (GM)	0.29	2, 9	0.374
CCR7	MFI (GM)	0.093	2, 9	0.912
Quadrant Analyses				
"Single positive"				
MHC II	%	0.835	2, 9	0.465
	Cell Count	2.940	2, 9	0.104
	MFI (GM)	0.123	2, 9	0.886
"Single positive"			2, 9	
CCR7	%	4.20		0.051
	Cell Count	8.49	2, 9	***0.009***
	MFI (GM)	4.526	2, 9	0.055
"Double positive"				
MHCII/CCR7	%	2.32	2, 9	0.154
	Cell Count	4.23	2, 9	0.051
MHC II	MFI (GM)	1.169	2, 9	0.354
CCR7	MFI (GM)	1.869	2, 9	0.210

** Levene's test statistic was used to examine possible unequal variances.*

Table 33. Tests of homogeneity of variance for brain macrophages outcomes in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Overall Analyses				
Brain				
macrophages	%	0.355	2, 9	0.711
	Cell Count	0.258	2. 9	0.778
MHC II	MFI (GM)	0.784	2, 9	0.486
CCR7	MFI (GM)	0.421	2, 9	0.669
Quadrant Analyses				
"Single positive"				
MHC II	%	1.275	2, 9	0.326
	Cell Count	0.170	2, 9	0.846
	MFI (GM)	6.20	2, 9	***0.020***
"Single positive"			2, 9	
CCR7	%	6.99		***0.015***
	Cell Count	0.67	2, 9	0.534
	MFI (GM)	2.25	2, 9	0.161
"Double positive"				
MHCII/CCR7	%	1.37	2, 9	0.300
	Cell Count	4.349	2, 9	***0.048***
MHC II	MFI (GM)	0.659	2, 9	0.541
CCR7	MFI (GM)	4.476	2, 9	***0.045***

** Levene's test statistic was used to examine possible unequal variances.*

Table 34. Tests of homogeneity of variance for hippocampal and immune cell outcomes in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Hippocampal				
Immune cells	%	5.601	2, 9	***0.026***
	Cell Count	7.855	2. 9	***0.011***
Brain				
Immune cells	%	2.786	2, 9	0.114
	Cell Count	0.563	2, 9	0.589

* *Levene's test statistic was used to examine possible unequal variances.*

Table 35. Tests of normality distribution for hippocampal microglia outcomes (overall analyses) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	df	p-value
		0 ppm	0.983	4	0.917
Hippocampal	%	30 ppm	0.805	4	0.110
Microglia		430 ppm	0.977	4	0.884
	Cell Count	0 ppm	0.867	4	0.287
		30 ppm	0.896	4	0.412
		430 ppm	0.976	4	0.876
MHC II	MFI (GM)	0 ppm	0.387	4	0.685
		30 ppm	0.404	4	0.653
		430 ppm	0.436	4	0.056
CCR7	MFI (GM)	0 ppm	0.782	4	0.074
		30 ppm	0.918	4	0.527
		430 ppm	0.981	4	0.906

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 36. Tests of normality distribution for hippocampal microglia outcomes (single positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Hippocampal Microglia					
"Single positive"		0 ppm	0.288	4	***0.039***
MHC II	%	30 ppm	0.346	4	***0.012***
		430 ppm	0.282	4	***0.002***
	Cell Count	0 ppm	0.891	4	0.347
		30 ppm	0.742	4	0.339
		430 ppm	0.926	4	0.547
	MFI (GM)	0 ppm	0.964	4	0.804
		30 ppm	0.901	4	0.435
		430 ppm	0.969	4	0.837
"Single positive"		0 ppm	0.864	4	0.276
CCR7	%	30 ppm	0.787	4	0.080
		430 ppm	0.939	4	0.650
	Cell Count	0 ppm	0.963	4	0.797
		30 ppm	0.963	4	0.972
		430 ppm	0.963	4	0.205
	MFI (GM)	0 ppm	0.782	4	0.074
		30 ppm	0.918	4	0.527
		430 ppm	0.981	4	0.906

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 37. Tests of normality distribution for hippocampal microglia outcomes (double positive) in young C57BL6/J mice with and without early chronic lead exposure*

			T-statistic	*df*	*p-value*
Hippocampal Microglia					
"Double positive"		0 ppm	0.874	4	0.312
MHCII/CCR7	%	30 ppm	0.808	4	0.117
		430 ppm	0.882	4	0.349
	Cell Count	0 ppm	0.916	4	0.512
		30 ppm	0.737	4	***0.029***
		430 ppm	0.920	4	0.539
MHC II	MFI (GM)	0 ppm	0.891	4	0.387
		30 ppm	0.742	4	***0.032***
		430 ppm	0.926	4	0.574
CCR7	MFI (GM)	0 ppm	0.944	4	0.677
		30 ppm	0.645	4	***0.002***
		430 ppm	0.987	4	***0.039***

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 38. Tests of normality distribution for brain microglia outcomes (overall analyses) in young C57BL6/J mice with and without early chronic lead exposure*

		Test-statistic	df	p-value
Brain Microglia	%	0 ppm	4	***0.026***
		30 ppm	4	0.070
		430 ppm	4	0.304
	Cell Count	0 ppm	4	0.446
		30 ppm	4	0.888
		430 ppm	4	0.129
MHC II	MFI (GM)	0 ppm	4	0.669
		30 ppm	4	0.400
		430 ppm	4	0.822
CCR7	MFI (GM)	0 ppm	4	0.151
		30 ppm	4	0.424
		430 ppm	4	0.056

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 39. Tests of normality distribution for brain microglia outcomes (single positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Brain Microglia					
"Single positive"			0 ppm	4	0.687
MHC II	%		30 ppm	4	0.055
			430 ppm	4	***0.002***
		Cell Count	0 ppm	4	0.693
			30 ppm	4	0.275
			430 ppm	4	***0.002***
		MFI (GM)	0 ppm	4	0.080
			30 ppm	4	0.185
			430 ppm	4	***0.058***
"Single positive"			0 ppm	4	0.062
CCR7	%		30 ppm	4	0.681
			430 ppm	4	0.759
		Cell Count	0 ppm	4	0.598
			30 ppm	4	0.681
			430 ppm	4	0.759
		MFI (GM)	0 ppm	4	***0.016***
			30 ppm	4	0.164
			430 ppm	4	0.355

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 40. Tests of normality distribution for brain microglia outcomes (single positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Brain Microglia					
"Double positive"			0 ppm	4	0.717
MHCII/CCR7	%		30 ppm	4	0.921
			430 ppm	4	***0.012***
	Cell Count		0 ppm	4	0.497
			30 ppm	4	0.670
			430 ppm	4	***0.005***
MHC II	MFI (GM)		0 ppm	4	0.852
			30 ppm	4	0.339
			430 ppm	4	0.867
CCR7	MFI (GM)		0 ppm	4	***0.029***
			30 ppm	4	0.564
			430 ppm	4	0.103

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 41. Tests of normality distribution for hippocampal macrophages outcomes (overall analyses) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Hippocampal		0 ppm	0.995	4	0.983
Macrophages	%	30 ppm	0.801	4	0.116
		430 ppm	0.902	4	0.391
	Cell Count	0 ppm	0.686	4	***0.008***
		30 ppm	0.938	4	0.518
		430 ppm	0.846	4	0.229
MHC II	MFI (GM)	0 ppm	0.681	4	***0.007***
		30 ppm	0.803	4	0.121
		430 ppm	0.771	4	***0.048***
CCR7	MFI (GM)	0 ppm	0.941	4	0.663
		30 ppm	0.928	4	0.481
		430 ppm	0.914	4	0.433

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 42. Tests of normality distribution for hippocampal macrophages outcomes (single positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Hippocampal Macrophages					
"Single positive"		0 ppm	0.990	4	0.958
MHC II	%	30 ppm	0.963	4	0.633
		430 ppm	0.998	4	0.906
	Cell Count	0 ppm	0.736	4	***0.029***
		30 ppm	0.900	4	0.384
		430 ppm	0.910	4	0.417
	MFI (GM)	0 ppm	0.975	4	0.870
		30 ppm	0.938	4	0.519
		430 ppm	0.984	4	0.757
"Single positive"		0 ppm	0.796	4	0.095
CCR7	%	30 ppm	0.996	4	0.876
		430 ppm	0.929	4	0.485
					0.485
	Cell Count	0 ppm	0.937	4	0.634
		30 ppm	0.987	4	0.780
		430 ppm	0.750	4	***0.000***
	MFI (GM)	0 ppm	0.876	4	0.323
		30 ppm	1.000	4	0.242
		430 ppm	0.970	4	0.074

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 43. Tests of normality distribution for hippocampal macrophages outcomes (double positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Hippocampal Macrophages					
"Double positive"		0 ppm	0.820	4	0.142
MHCII/CCR7	%	30 ppm	0.999	4	0.942
		430 ppm	0.891	4	0.537
	Cell Count	0 ppm	0.650	4	***0.003***
		30 ppm	0.792	4	0.094
		430 ppm	0.953	4	0.581
MHC II	MFI (GM)	0 ppm	0.926	4	0.574
		30 ppm	0.986	4	0.777
		430 ppm	0.899	4	0.382
CCR7	MFI (GM)	0 ppm	0.952	4	0.729
		30 ppm	0.989	4	0.797
		430 ppm	0.983	4	0.753

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 44. Tests of normality distribution for brain macrophages outcomes (overall analyses) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	*df*	*p-value*
Brain Macrophages	%	0 ppm	0.917	4	0.519
		30 ppm	0.985	4	0.930
		430 ppm	0.970	4	0.839
	Cell Count	0 ppm	0.965	4	0.810
		30 ppm	0.937	4	0.634
		430 ppm	0.930	4	0.596
MHC II	MFI (GM)	0 ppm	0.954	4	0.740
		30 ppm	0.955	4	0.750
		430 ppm	0.959	4	0.775
CCR7	MFI (GM)	0 ppm	0.977	4	0.887
		30 ppm	0.831	4	0.171
		430 ppm	0.830	4	0.161

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 45. Tests of normality distribution for brain macrophages outcomes (single positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test-statistic	df	*p-value*
Brain Macrophages					
"Single positive"		0 ppm	0.895	4	0.407
MHC II	%	30 ppm	0.915	4	0.510
		430 ppm	0.788	4	0.083
	Cell Count	0 ppm	0.959	4	0.774
		30 ppm	0.822	4	0.148
		430 ppm	0.959	4	0.776
	MFI (GM)	0 ppm	0.858	4	0.253
		30 ppm	0.681	4	***0.007***
		430 ppm	0.903	4	0.448
"Single positive"		0 ppm	0.996	4	0.985
CCR7	%	30 ppm	0.734	4	***0.027***
		430 ppm	0.855	4	0.242
	Cell Count	0 ppm	0.948	4	0.703
		30 ppm	0.936	4	0.630
		430 ppm	0.914	4	0.502
	MFI (GM)	0 ppm	0.841	4	0.198
		30 ppm	0.777	4	0.066
		430 ppm	0.823	4	0.150

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 46. Tests of normality distribution for brain macrophages outcomes (double positive) in young C57BL6/J mice with and without early chronic lead exposure.*

			Test-statistic	*df*	*p-value*
Brain Macrophages					
"Double positive"		0 ppm	0.833	4	0.176
MHCII/CCR7	%	30 ppm	0.830	4	0.169
		430 ppm	0.861	4	0.265
	Cell Count	0 ppm	0.970	4	0.840
		30 ppm	0.921	4	0.540
		430 ppm	0.973	4	0.863
MHC II	MFI (GM)	0 ppm	0.891	4	0.389
		30 ppm	0.923	4	0.556
		430 ppm	0.910	4	0.481
CCR7	MFI (GM)	0 ppm	0.943	4	0.674
		30 ppm	0.887	4	0.369
		430 ppm	0.998	4	0.992

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties.*

Table 47. Tests of normality distribution for brain macrophages outcomes (double positive) in young C57BL6/J mice with and without early chronic lead exposure*

			Test statistc	df	p-value
Hippocampal Immune Cells					
		0 ppm	0.983	4	0.918
	%	30 ppm	0.899	4	0.424
		430 ppm	0.966	4	0.814
	Cell	0 ppm	0.959	4	0.774
	Count	30 ppm	0.973	4	0.861
		430 ppm	0.972	4	0.852
Brain Immune Cells					
	%	0 ppm	0.837	4	0.188
		30 ppm	0.966	4	0.817
		430 ppm	0.707	4	***0.014***
	Count	0 ppm	0.935	4	0.623
		30 ppm	0.993	4	0.971
		430 ppm	0.791	4	0.088

** Shapiro-Wilk test statistic (for small sample sizes) was used to examine distribution properties*

APPENDIX B. SUPPLEMENTARY FIGURES

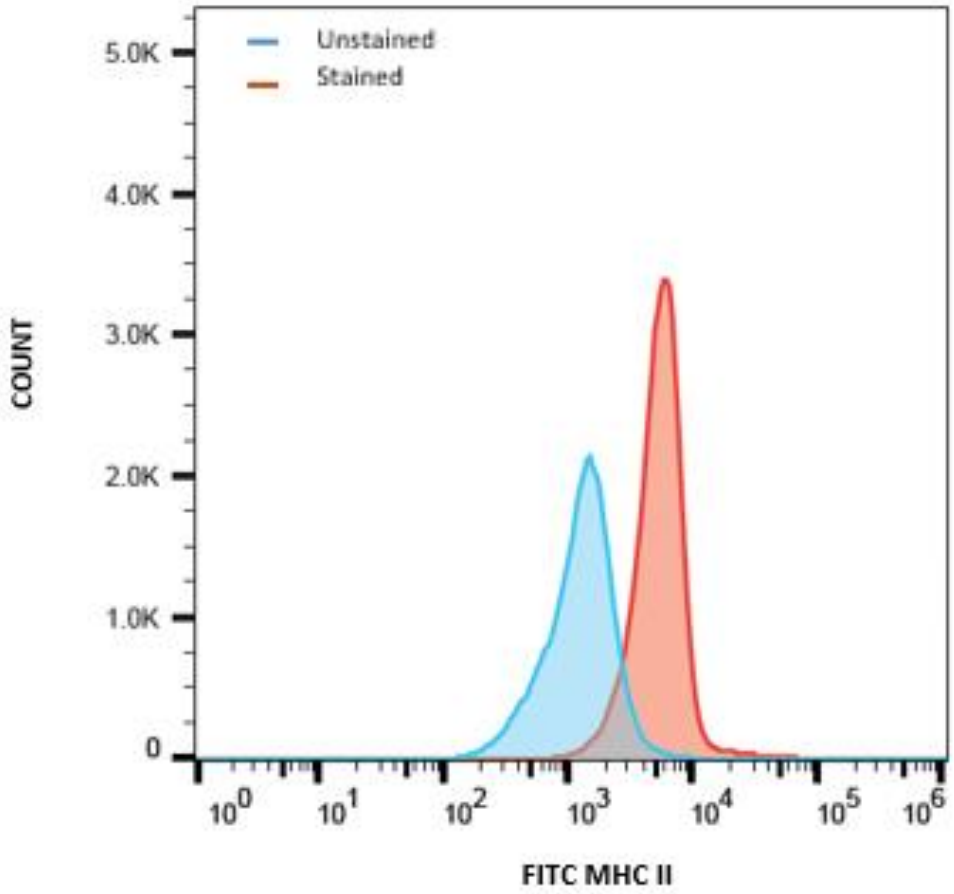


Figure 1. Fluorescence intensity of stained and unstained immune cells with FITC MHC II. As shown in the figure there was a shift towards the right for cells stained with FITC MHC II as compared to unstained cells. This shift indicates that the antibody FITC MHC II did bind to immune cells and that the fluorophore FITC emitted higher fluorescence intensity than the potential autofluoresence emitted by unstained cells.

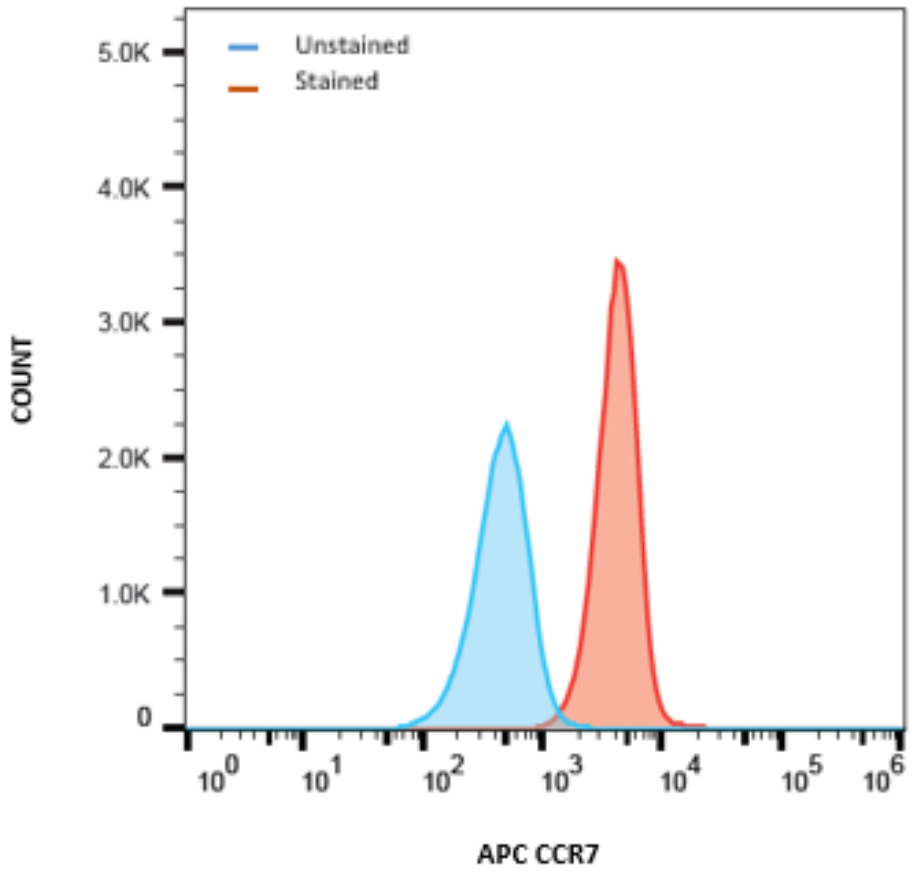


Figure 2. Fluorescence intensity of stained and unstained immune cells with APC CCR7. As shown in the figure there was a shift towards the right for cells stained with APC CCR7 as compared to unstained cells. This shift towards the right indicates that the antibody APC CCR7 did bind to immune cells and that the antibody emitted higher fluorescence intensity than the potential autofluoresence emitted by unstained cells.

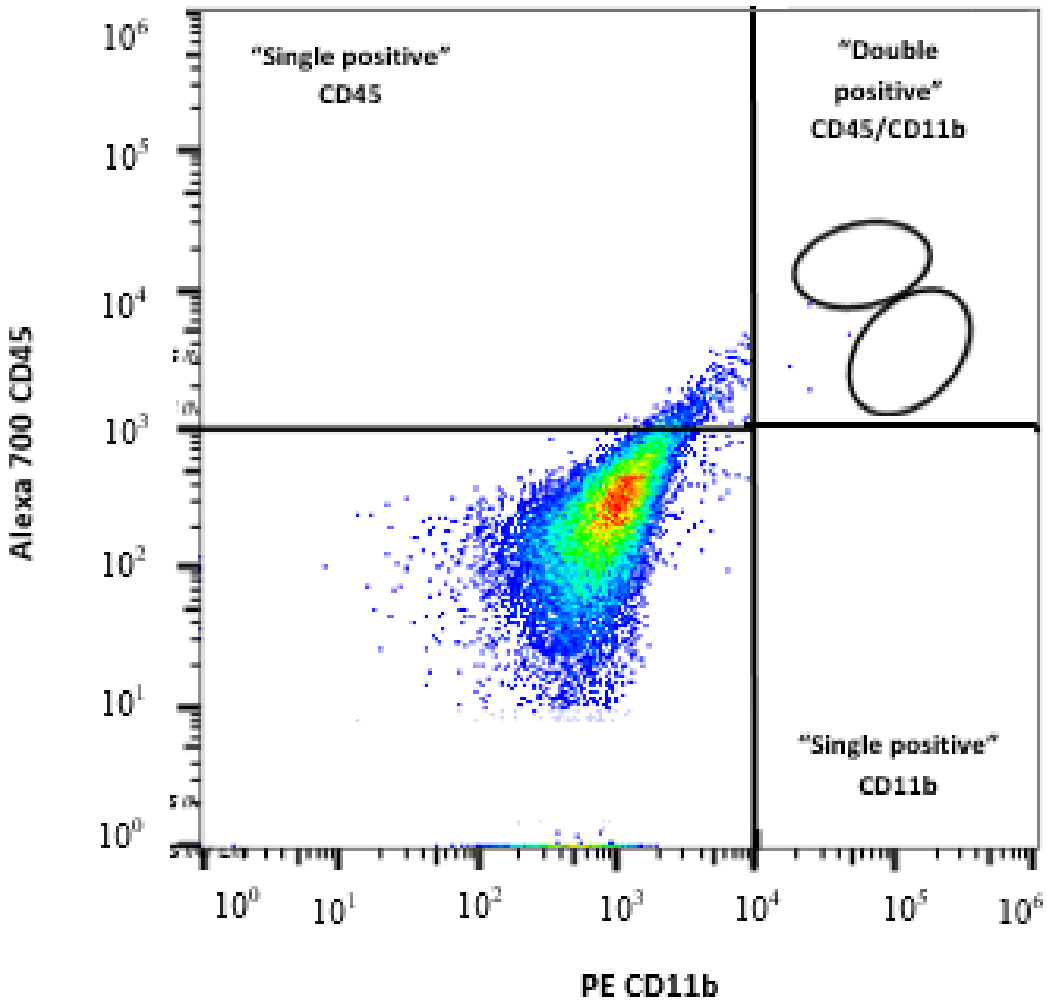


Figure 3. Unstained sample of cells plotted against PE CD11b and A700 CD45. As shown in the figure PE CD11b was plotted on the *x-axis* whereas Alexa 700 CD45 was plotted on the *y-axis*. Unstained cells had negative values for PE CD11b and A700 CD45. The cells of interest were gated outside of the range of fluorescence emitted by the unstained cells. The circular gates show areas where microglia and macrophages were gated for fully stained cells.

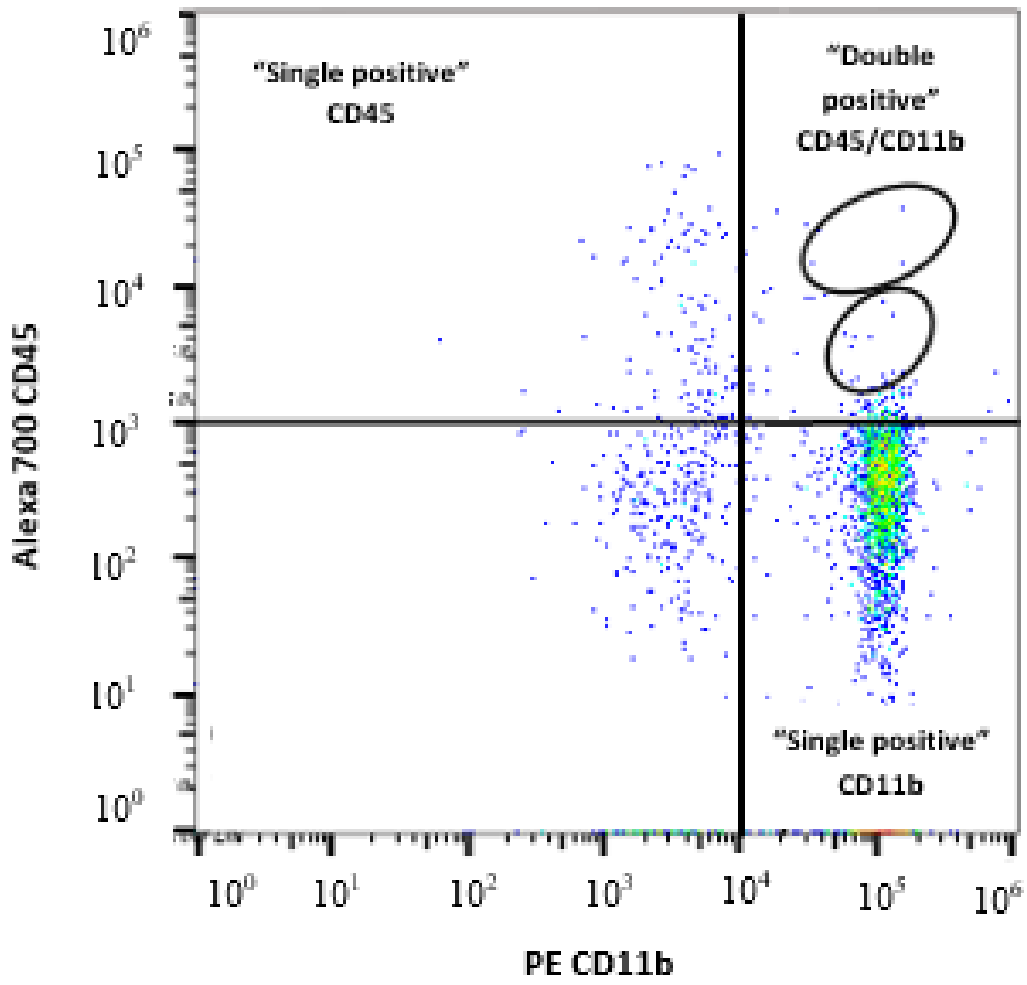


Figure 4. Fluorescence minus one for Alexa 700 CD45. Cells were stained with all the markers of interest with the exception of Alexa 700 CD45 to delimit the gates for double positive cells. That is, for the selection of microglia and macrophages that are positive for both CD45 and CD11b. As shown in the figure there was very little unspecific signal for CD45. The circular gates show areas where microglia and macrophages were gated for fully stained cells.

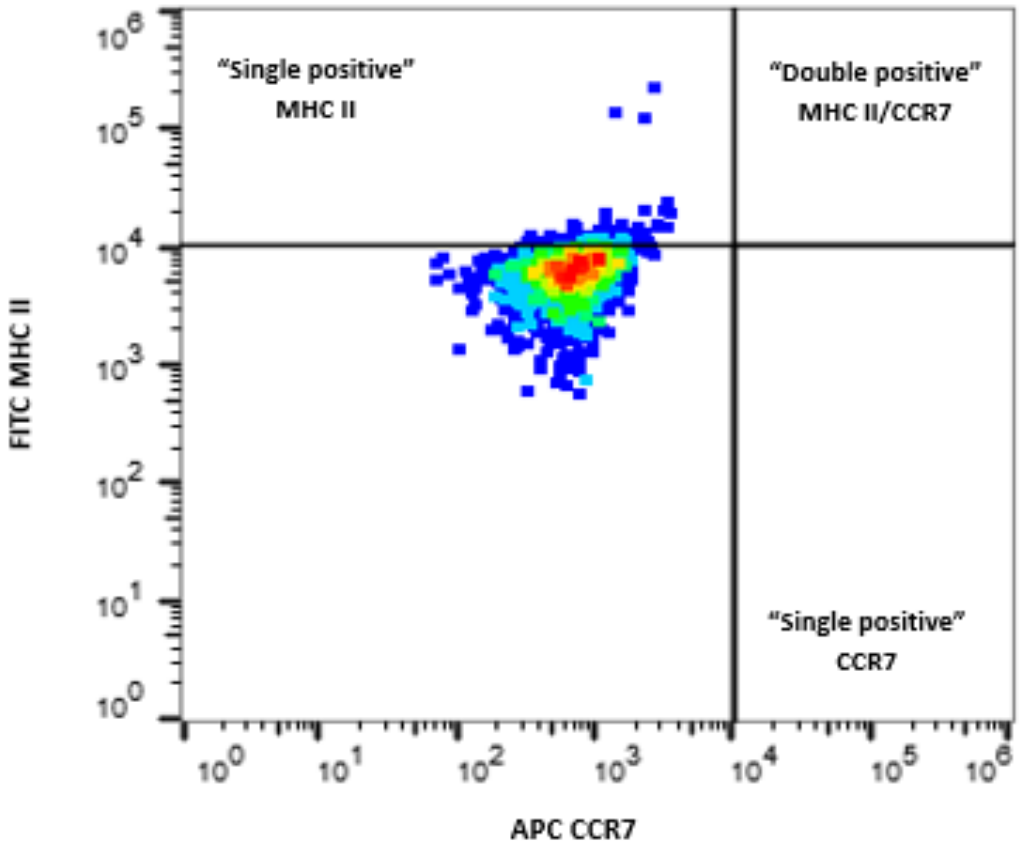


Figure 4. Fluorescence minus one for APC CCR7 in microglial cells. Cells were stained with all the markers of interest with the exception of APC CCR7 to delimit the gates for CCR7 and double positive cells (MHC II/CCR7). As shown in the figure there was no unspecific signal for CCR7.

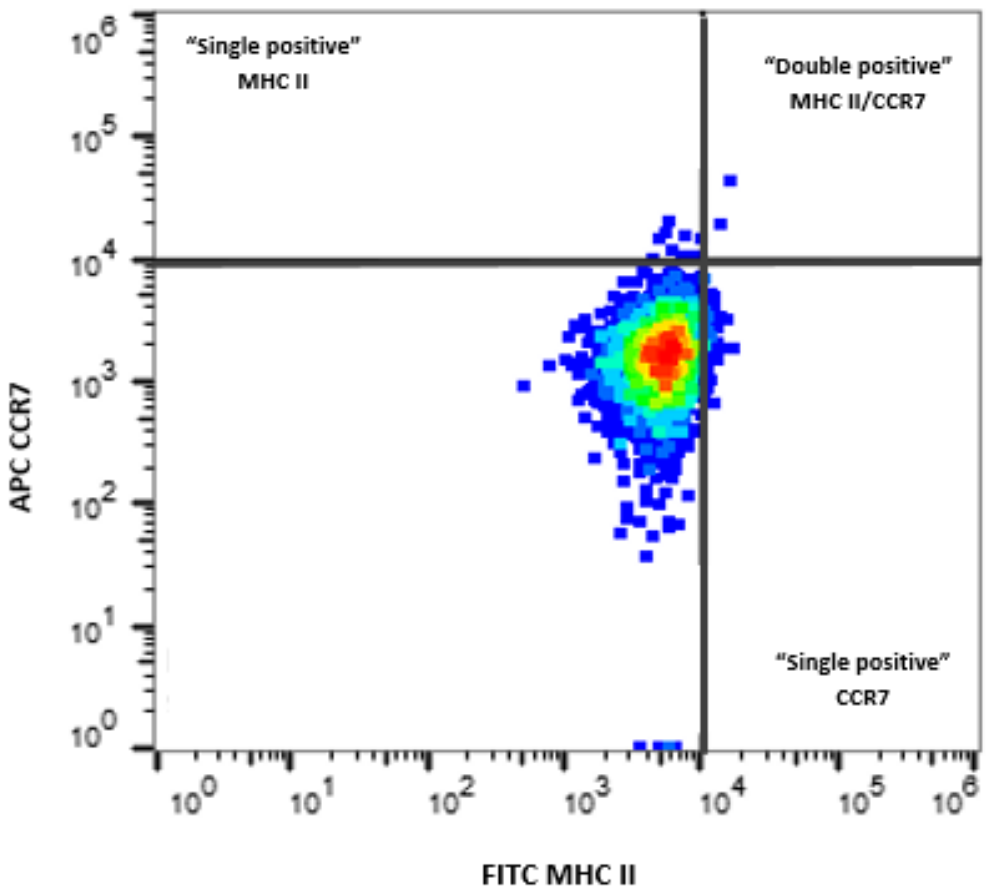


Figure 5. Fluorescence minus one for FITC MHC II in microglial cells. Cells were stained with all the markers of interest with the exception of FITC MHC II to delimit the gates for MHC II and double positive cells (MHC II/CCR7). As shown in the figure there was little unspecific staining for MHC II.

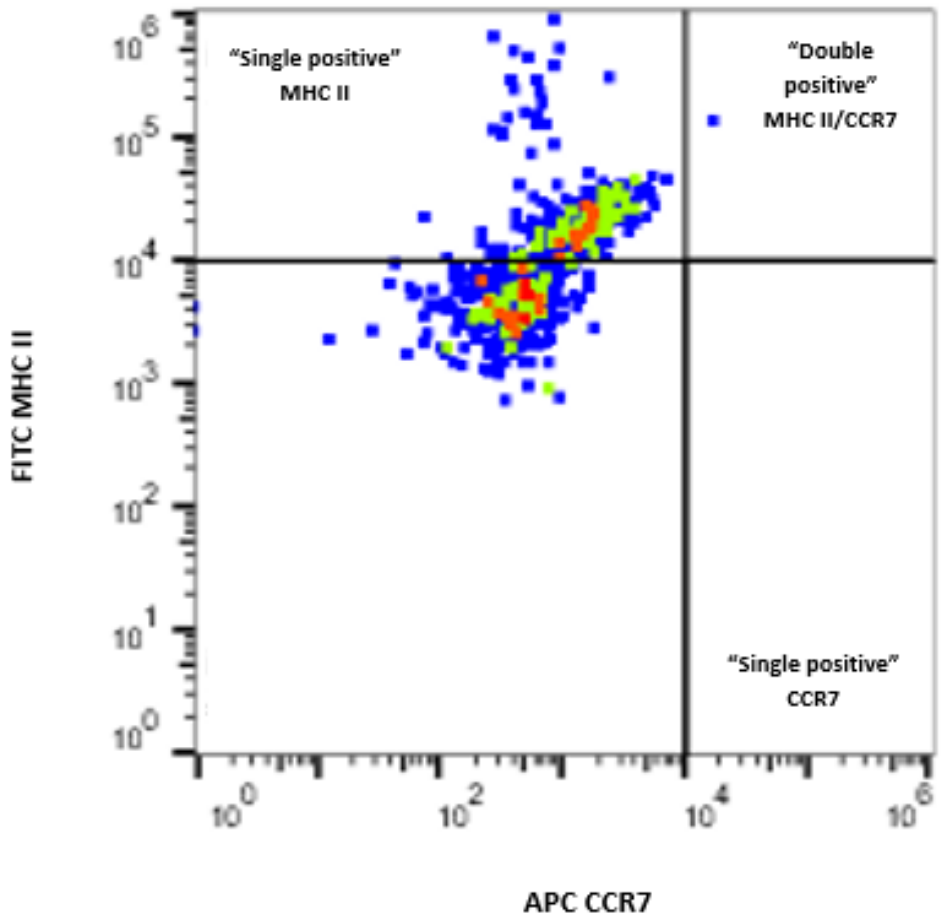


Figure 4. Fluorescence minus one for APC CCR7 in macrophages. Cells were stained with all the markers of interest with the exception of APC CCR7 to delimit the gates for CCR7 and double positive cells (MHC II/CCR7). As shown in the figure there was no unspecific staining for CCR7.

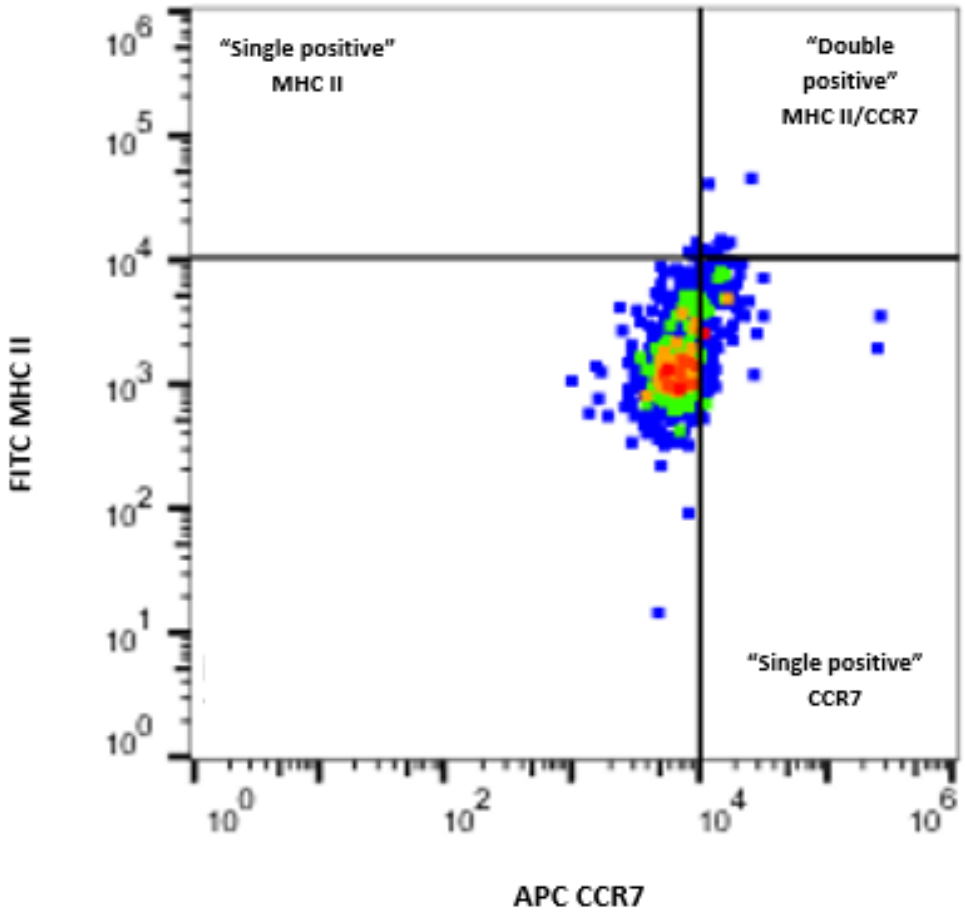


Figure 6. Fluorescence minus one for FITC MHC II in macrophages cells. Cells were stained with all the markers of interest with the exception of FITC MHC II to delimit the gates for MHC II and double positive cells (MHC II/CCR7). As shown in the figure there was little unspecific staining for MHC II.

APPENDIX C. RELEVANT AUTHOR PUBLICATIONS

Research Article

Journal of Applied Toxicology

Received: 11 April 2014, Revised: 10 July 2014, Accepted: 16 July 2014 Published online in Wiley Online Library: 12 September 2014

(wileyonlinelibrary.com) DOI 10.1002/jat.3064

Early chronic lead exposure reduces exploratory activity in young C57BL/6J mice

Mayra Gisel Flores-Montoya[a,b] and Christina Sobin[a,c,d]*

ABSTRACT: Research has suggested that chronic low-level lead exposure diminishes neurocognitive function in children. Tests that are sensitive to behavioral effects at lowest levels of lead exposure are needed for the development of animal models. In this study we investigated the effects of chronic low-level lead exposure on exploratory activity (unbaited nose poke task), exploratory ambulation (open field task) and motor coordination (Rotarod task) in pre-adolescent mice. C57BL/6J pups were exposed to 0 ppm (controls), 30 ppm (low-dose) or 230 ppm (high-dose) lead acetate via dams' drinking water administered from birth to postnatal day 28, to achieve a range of blood lead levels (BLLs) from not detectable to 14.84 μg dl^{-1}). At postnatal day 28, mice completed behavioral testing and were killed (n = 61). BLLs were determined by inductively coupled plasma mass spectrometry. The effects of lead exposure on behavior were tested using generalized linear mixed model analyses with BLL, sex and the interaction as fixed effects, and litter as the random effect. BLL predicted decreased exploratory activity and no threshold of effect was apparent. As BLL increased, nose pokes decreased. The C57BL/6J mouse is a useful model for examining effects of early chronic low-level lead exposure on behavior. In the C57BL/6J mouse, the unbaited nose poke task is sensitive to the effects of early chronic low-level lead exposure. This is the first animal study to show behavioral effects in pre-adolescent lead-exposed mice with BLL below 5 μg dl^{-1}. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: developmental lead exposure; mouse model; exploratory activity; neurobehavioral toxicity; cognition

Introduction

Substantial progress has been made in reducing the numbers of children exposed to higher levels of environmental lead; however, early chronic low-level lead exposure remains an unresolved child public health problem and child health disparity. Over the past 30 years more than 50 longitudinal and cross-sectional studies have shown that blood lead levels (BLLs) as low as 2 μg dl^{-1} are associated with lower measured intelligence, reduced neurocognitive function and/or impaired motor functions (Bellinger and Needleman, 2003; Canfield *et al.*, 2003; CDC, 2005; Gilbert and Weiss, 2006; Jusko *et al.*, 2008; Landrigan *et al.*, 2006; Lanphear *et al.*, 2005). In response to these findings, in January 2012, the Centers for Disease Control and Prevention (CDC) recommended 5 μg dl^{-1} as a reference value for identifying children with elevated blood lead (CDC, 2012). How and why early chronic low-level lead exposure alters behavior and brain is not yet understood however.

Very few animal studies have examined the effects on behavior at the lowest levels of lead exposure. In a recent comprehensive review of the literature, five animal studies were identified and included two mouse studies, two rat studies and one monkey study. In mouse studies, as compared with controls, developmental exposure to 27 ppm lead acetate delivered in dams' drinking water, yielding mean BLL ≤ 10 μg dl^{-1} (specific BLL values not reported), was associated with decreased exploratory ambulation (open field task) and decreased motor coordination (Rotarod task) in adult male but not female C57BL/6J mice (Leasure *et al.*, 2008). In a second mouse study, as compared with controls, adult BALB/c mice with developmental exposure to 20 ppm lead acetate delivered in dams' drinking water (specific BLL values were not reported) had decreased exploratory ambulation (open field task) and decreased memory (water maze task) (Kasten-Jolly, Pabello, Bolivar, and Lawrence, 2012).

In a study of adult Wistar rats with and without exposure to 20 ppm lead acetate delivered in dams' drinking water during development, low-level lead-exposed animals had decreased recognition memory (novel object recognition task), and increased exploratory ambulation (open field task) (Azzaoui, Ahami, and Khadmaoui, 2009). In another study of adult Sprague–Dawley rats exposed to 5 and 50 ppm lead acetate delivered in dams' drinking water, the lowest exposure group had decreased exploratory ambulation on an open field task (Reiter, Anderson, Laskey, and Cahill, 1975) (specific BLL values not reported). In a study of adult Rhesus monkeys with developmental exposure to lead acetate (0.7 mg kg^{-1}) and with mean BLL < 5 μg dl^{-1}, exploration of a novel space was increased following habituation (Ferguson and Bowman, 1990).

In these five studies, the effects of chronic low-level lead exposure on behavior in adult animals were assessed. Consistent effects on memory and inconsistent effects on exploratory

*Correspondence to: Christina Sobin, Ph.D., University of Texas, El Paso, 500 West University, El Paso, TX 79902, USA.
Email: casobin@utep.edu

[a]Border Biomedical Research Center, Toxicology Core, University of Texas, El Paso, TX, USA

[b]Department of Psychology, University of Texas, El Paso, TX, USA

[c]Department of Public Health Sciences, College of Health Sciences, University of Texas, El Paso, TX, USA

[d]Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY, USA

ambulation were observed. To increase relevance to the findings observed in the child clinical literature, and to promote the development of animal models, studies are needed examining effects of early chronic low-level lead exposure in young animals. The mouse as a model organism has several advantages. Mouse physiology, anatomy and genetics closely approximate human systems; the relatively short gestation and accelerated lifespan of mice reduce expense and improve feasibility. Importantly, studies of proteins that influence lead absorption, including δ-aminolevulinic acid dehydratase (ALAD) and metallothioneins, have been modeled in mice (Takahashi, 2012).

The goal of this study was to contribute to the small but growing animal literature of studies examining effects on behavior of early chronic low-level lead exposure yielding BLLs similar to those observed in the child clinical literature. We attempted to predict behavioral performance from BLL at death in pre-adolescent C57BL/6J mice, with and without early chronic low-level lead exposure. Behavioral tasks used in this study included exploratory ambulation (open field task), exploratory activity (unbaited nose poke task) and motor coordination (Rotarod task). We hypothesized linear inverse relationships between BLL and exploratory ambulation (number of quadrants crossed in open field), exploratory activity (number of nose pokes) and motor coordination (number of seconds on Rotarod).

Materials and Methods

Animals

This study was done in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 2011) and with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at El Paso (UTEP). C57BL/6J mice were purchased at the Jackson Laboratory, and then bred and housed in the animal vivarium of the Biosciences Research Building at UTEP. Animals were housed in a room that was kept at a temperature that ranged from 20 to 26 °C and at a relative humidity that ranged from 30 to 70% with a 12 h light–dark cycle. Animals were housed in individual ventilated cages (22.22 cm × 36.83 cm × 13.97 cm) with an air handling that provided the air supply to the cages and exhausted the ammonia gases and CO_2 buildup within the cage. All animals were fed ad libitum with Harlan irradiated global 18% protein rodent diet.

Dams were mated at postnatal day (PND) 30. Each dam was housed with a male mouse in individual cages until a vaginal plug was detected, at which time the male mouse was removed from the cage. At birth, eight unculled litters (36 males and 25 females) were assigned to one of three treatment groups, including 0 ppm lead acetate (controls), 30 ppm lead acetate (low dose) and 230 ppm lead acetate (high dose).

Lead Exposure

Water bottles of experimental groups were filled with lead acetate treated distilled water (30 ppm or 230 ppm) at PND 0. Water bottles of control pups were treated with 30 ppm sodium acetate. Pups were weaned at PND 21, and males and females were separated and group housed. Exposure to lead continued until PND 28 when mice were placed in individual cages, tested behaviorally and killed.

Behavioral Tests

Mice completed three behavioral tests, including the open field task (5 min), the unbaited nose poke task (3 min) and the Rotarod task (four trials of maximum 300 s each). All animals completed the three tests in the same order (open field, nose poke and Rotarod) with a 5 min break between tasks. Animals completed behavioral testing in a room with standard-level fluorescent overhead lighting. All testing occurred between 10.00 and 13.00 h.

Nose Poke Task

The test was conducted in a 16 × 16 × 16 inch square Plexiglas arena with a fitted raised platform with 16 evenly spaced unbaited holes (1 inch in diameter). At the start of testing, each mouse was released into the lower right corner of the arena and left to explore the platform freely for 3 min. A video camera mounted at the level of the platform recorded each testing session. Following the completion of all behavioral testing, raters trained to reliability and blind to experimental condition rated the number of nose pokes (head dips) per minute. Video recordings for each mouse were projected on to a 45 × 60 in high-resolution and high-reflectivity projection screen by a Toshiba DLP high-resolution projector.

Open Field Task

The test was conducted in a 16 × 16 × 16 inch square Plexiglas arena with four quadrant markings. At the start of testing, each mouse was released into the lower right corner of the field and allowed to explore it freely for 5 min.

A video camera mounted at the level of the open field recorded each testing. Following the completion of all behavioral testing, raters trained to reliability and blind to experimental condition rated the number of crosses from one quadrant into another with all four paws. Video recordings for each mouse were projected on to a 45 × 60 inch high-resolution and high-reflectivity projection screen by a Toshiba DLP high-resolution projector.

Rotarod Task

The Rotarod device used in this study was stand-alone unit designed for mouse testing (Med Associates, Inc., St. Albans, VT, USA), including five testing stations each with its own beam-activated timer. The rotating barrel was 3.2 cm in diameter and the lane widths were 5.7 cm; the fall height was 16.5 cm. Mice were tested on an accelerating rod that increased from 3.5 to 35 rpm over the 300 s trial. Three researchers observed the mice throughout the procedure and noted session details. At the start of each trial, researchers placed one mouse per station facing towards the wall and the motor was started. Mice were given a 10 s stabilization period on the rod before trial timing began. If a mouse fell during the 10 s stabilization period, the mouse was replaced on the rotating rod. After the 10 s stabilization, a mouse drop from the rod triggered the electric beam and stopped the timer at the bottom of each station. The trial time for each mouse was recorded. Each mouse completed four trials for a maximum of 300 s per trial, with a 3 min inter-trial interval.

Journal of AppliedToxicology

Blood Collection

After behavioral testing was completed, animals were anesthetized with Avertin ranging from 5 to 10 ml depending on body weight. When mice were unresponsive to corneal touch and paw pinch tests, mice were sexed and weighed. The chest was opened and heart blood was extracted via syringe puncture at the heart apex, yielding approximately 50 µl of whole blood per animal. Blood samples were refrigerated until processing for inductively coupled plasma mass spectrometry (ICP-MS) analysis, which occurred with 72 h of sample collection.

Inductively Coupled Plasma Mass Spectrometry Analysis of Blood Lead

A complete description of ICP-MS apparatus and procedures were previously provided (Sobin, Parisi, Schaub, and de la Riva E, 2011a). Briefly, an Agilent 7500ce ICP-MS with an octopole reaction system and a CETAC ASX-520 autosampler was used (Agilent Technologies, Inc., Santa Clara, CA). A Micro Mist U-series nebulizer and a double-pass quartz spray chamber were used to introduce the samples into plasma. The instrument parameters were a carrier gas of 0.78 l min^{-1}, makeup gas of 0.15 l min^{-1}, RF power of 1420 W and a spray chamber that was set at a temperature of 2 °C. For sample processing, a propylene tube was filled with 5.58 ml of water, 300 µl of blood, 60 µl of aqueous internal standard solution and 60 µl of aqueous 10 ppm gold in 3% hydrochloric acid solution. The samples were vortexed and centrifuged for 1 min at 2000 g and the supernatant was analyzed by ICP-MS. BLLs were determined in µg dl^{-1}.

Data Analyses

Data were analyzed using *SAS Version 9.3* and *R* (R Development Core Team, 2009) with package lme4 (Bates, Maechler, and Bolker, 2011). Distributions of variables were examined and residual plots were visually inspected. No obvious deviations from homoscedasticity or normality were observed. BLLs and body weights were compared for males and females by exposure group. Generalized linear mixed model regression analyses were used with BLL, sex and the interaction as fixed effects and litter as the random effect (to account for possible non-independence of behavior among litter mates). Random intercepts and random slopes were included. For each of the three behavioral tests, one dependent variable (task parameter score) was initially tested. When a significant effect was observed, additional models were conducted to test for effects during each minute of task performance. The purpose of this approach was to determine whether behavioral differences occurred consistently throughout the task, or were evident only in selected task segments. Discrete data (number of nose pokes and number of quadrants crossed) were modeled with a Poisson distribution; Rotarod time was modeled with a Gaussian distribution. Residual pseudo-likelihood estimation and dual quasi-Newton optimization were used. Fit was evaluated by examining the ratio of the generalized chi-squared test to its degrees of freedom; a value close to 1.0 indicated that variability in the data had been adequately modeled with no residual overdispersion (Schabenberger, 2005). Covariance parameter estimates were evaluated. Finally, the significance of added variance explained in models with vs. without BLL was determined in two ways, by evaluation of the Akaike Information Criterion (AIC) value decrease (>2 indicating significant model improvement) (Burnham and Anderson, 2002), and the reduction in model deviance tested with the likelihood ratio test ($P < 0.05$) (Bolker *et al.*, 2009; Pinheiro and Bates, 2000).

Results

Animals

Eight unculled litters (36 males and 25 females) were randomly assigned to one of three treatment conditions resulting in 19 control animals (eight males, 11 females), 26 low-dose animals (16 males, 10 females) and 16 high-dose animals (12 males, four females). Three treatment conditions were used to produce a distribution of BLLs for regression analyses. Lead at the levels administered in this study had no observable effects on mortality or morbidity of the exposed mice, and had no observable adverse physical effects, such as abnormal motor function or ataxia. Table 1 shows BLLs after 28 days of lead exposure treatment and body weights at death in males and females. As shown, BLLs and body weights differed between exposure groups, and body weight was dependent on BLL (males, $r = -0.77$; females $r = -0.78$). The ranges of BLL for males and females did not differ significantly (means ± SD for males = 4.9 ± 3.7 and females = 3.3 ± 4.3, $t_{59} = 1.59$, P = NS). All animals completed the open field and nose poke tasks. One control animal did not stabilize on the rotating rod (repeatedly fell off during the 10 s stabilization period) and did not complete the task. Overall, the

Table 1. Blood lead levels and body weight of males and females by exposure group after 28 days of lead exposure

Lead exposure group	Blood lead levels (µg dl^{-1})		Body weight (g)	
	Males	Females	Males	Females
230 ppm	9.39 ± 1.90	12.14 ± 2.90 [a]** [b]**	8.65 ± 1.94	8.83 ± 1.11 [d]**
30 ppm	3.93 ± 0.91	3.19 ± 0.75 [c]**	14.97 ± 1.59	12.50 ± 1.82
0 ppm	0.20 ± 0.12	0.19 ± 0.09	15.75 ± 1.57	13.93 ± 1.09

Each value represents the least square mean ± SD of the blood lead levels (µg dl^{-1}) and body weight (g) for males and females by lead exposure group.
[a]Compared with 30 ppm group and 0 ppm group.
[b]Compared with 230 ppm lead-exposed males.
[c]Compared with 0 ppm group.
[d]Compared with 30 ppm group and 0 ppm group.
** $P < 0.001$.

Table 2. Performance for males and females on the three behavioral tests with and without detectable blood lead measured after 28 days of exposure to lead acetate or sodium

	Mean ± SD		
Behavioral test	Total	Males	Females
Nose poke (number of head-dips)	12.01 ± 4.38	11.67 ± 3.99	12.51 ± 4.93
Open field (quadrants crossed)	13.89 ± 4.05	13.70 ± 3.97	14.18 ± 4.24
Rotarod (seconds spent on the rod)	90.42 ± 55.68	78.37 ± 40.77	107.28 ± 68.98

Each value represents the mean ± SD for the performance of males and females on each of the behavioral tests.

results showed that BLL affected nose poke behavior; no effects of BLL on open field behavior or Rotarod behavior were observed. Table 2 shows the means ± SD from each of the behavioral tests for males and females, illustrated in Figs. 1–3.

Nose Poke Task

Animals were placed in the lower right corner of the arena. All animals moved either horizontally or vertically from the initial placement corner moving at a relatively steady pace, moving from hole to hole. There were no notable differences in how animals completed the task and no animals fell through the unbaited holes to the arena floor below. The model solution for nose poke average required five iterations with no restarts, and convergence criterion met. The fit statistics suggested that the variability was adequately modeled (chi-squared/df. = 1.4). The litter random effect covariance parameter estimate was small (est = 0.06, SE = 0.04). Controlling for litter, the main effect for BLL was significant (type III SS $F = 4.60$, $P = 0.037$); no effect was observed for sex ($F = 0.49$), or the interaction of BLL by sex ($F = 0.73$). Comparison of models without (AIC = 334.4) and with (AIC = 331.6) BLL showed that BLL affected the nose pokes (chi-squared/1 = 4.79, $P = 0.03$), lowering the average by approximately 0.55 ± 0.26. Figure 4 shows that as BLL increased, the number of nose pokes decreased for males and females. Given the significant findings for the model testing average nose pokes, each minute of nose poke performance was also modeled to determine whether effects were present consistently throughout the task. For the model examining effects of BLL on nose pokes during minute 1 (five iterations, no restarts, convergence criterion met), fit statistics suggested that the variability was adequately modeled (chi-squared/df. = 0.70) and the litter random effect covariance parameter estimate was small (est = 0.03, SE = 0.03). Controlling for litter, a main effect for BLL was found (type III SS $F = 10.6$, $P < 0.01$), with no effect for sex ($F = 0.33$) and no effect for the interaction ($F = 0.31$). Comparison of models without (AIC = 348.4) and with (AIC = 341.4) BLL showed that BLL affected nose pokes during minute 1 (chi-squared/1 = 9.01, $P = 0.003$), lowering the average by approximately 0.71 ± 0.17. During minute 1 of the nose poke task,

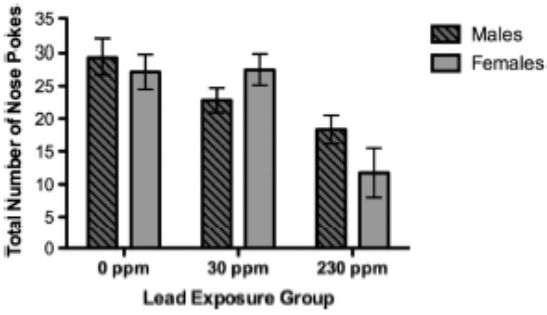


Figure 1. Total number of nose pokes of males and females by lead exposure group during 3 min. Data are expressed as least square means ± SEM 230 ppm (12 males, four females), 30 ppm (16 males, 10 females) and 0 ppm (eight males, 11 females).

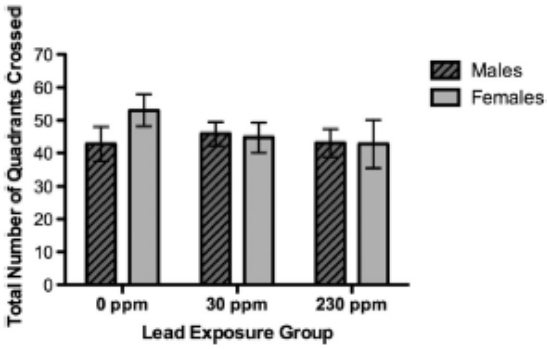


Figure 2. Total number of quadrants crossed of males and females by lead exposure group during 5 min. Data are expressed as least square means ± SEM 230 ppm (12 males, four females), 30 ppm (16 males, 10 females) and 0 ppm (eight males, 11 females).

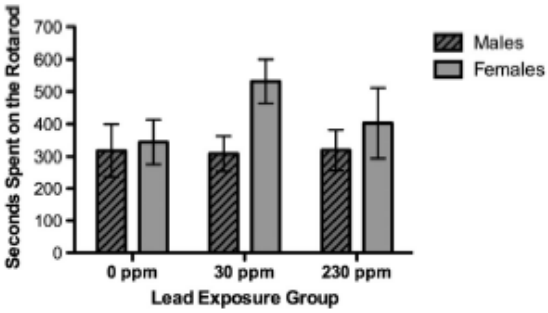


Figure 3. Seconds spent on the Rotarod of males and females by lead exposure group. Data are expressed as least square means ± SEM 230 ppm (12 males, four females), 30 ppm (16 males, 10 females) and 0 ppm (eight males, 11 females).

Journal of AppliedToxicology

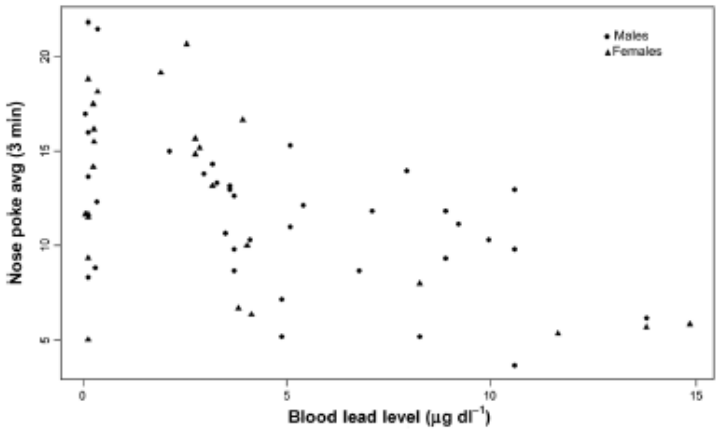


Figure 4. Association between blood lead levels and number of nose pokes during 3 min (total exploration). As blood lead levels increased, the number of nose pokes decreased for males and females (chi-squared/1 = 4.79, $P = 0.03$). Males ($n = 36$) and females ($n = 25$).

as BLL increased, the number of nose pokes decreased for males and for females. For the nose poke task minute 2 model (six iterations no restarts, convergence criterion met), fit statistics suggested the data variability was adequately modeled (chi-squared/df. = 1.10) and the litter random effect covariance parameter estimate was small (est = 0.04, SE = 0.04). In contrast to minute 1, no effects for BLL (type III SS $F = 0.58$), sex (type III SS $F = 0.02$) or the interaction (type III SS $F = 0.01$) were observed for minute 2 nose pokes. Comparison of models without (AIC = 372.8) and with (AIC = 373.9) BLL confirmed that BLL did not affect nose pokes during minute 2 (chi-squared/1 = 0.90, P = NS). Thus, there was no measureable association between BLL and nose poke behavior during minute 2. For the nose poke task minute 3 model (six iterations, no restarts, convergence criterion met) fit statistics again suggested that the data variability was adequately modeled (chi-squared/df. = 0.89) and the litter random effect covariance parameter estimate was small (est = 0.06, SE = 0.05). Similar to minute 1, in minute 3, a main effect for BLL was observed (type III SS $F = 12.7$, $P < 0.01$), with no effect for sex ($F = 0.09$) or the interaction ($F = 1.42$). Comparison of models without (AIC = 364.5) and with (AIC = 360.9) BLL showed that BLL affected nose pokes during minute 3 of the task (chi-squared/1 = 5.63, $P = 0.02$), lowering the average by approximately 0.78 ± 0.19. As for behavior in minute 1 of the task, as BLL increased, the number of nose pokes during minute 3 decreased for males and for females.

Open Field Task

Animals were placed in the lower right corner of the arena and allowed to explore freely. The immediacy with which animals explored the open field varied considerably across animals; however, all animals moved in the space to some extent during each minute of the task. For the model testing average number of quadrants crossed (seven iterations, no restarts, convergence criterion met) fit statistics suggested that the data variability was adequately modeled (chi-squared/df. = 3.13) and the litter random effect covariance parameter estimate was small (est = 0.04, SE = 0.03). No effects were observed for BLL (type III SS $F = 0.37$), sex ($F = 1.71$) or the interaction ($F = 1.73$). Comparison of models with and without BLL confirmed that BLL did not affect open field behavior (chi-squared/1 = 0.38, P = NS).

Rotarod Task

Animals were placed on the rod in one of five bin sections by three researchers and positioned to be facing away from the experimenters. Placing the mice took less than 20 s; when all mice were in place, the rod was started. Mice were allowed a 10 s stabilization period and after this, task timing began. Twelve animals (20%) fell and had to be replaced on the rod during the stabilization period; one animal fell twice and then completed the task. One control animal fell repeatedly and did not complete the task. Timing began after the 10 s stabilization. The ranges of time on the accelerating rod were large for all groups across all trials. Among the control animals, time on the rod ranged from 2 to 300 s; among low-dose animals from 3 to 300 s; and among high-dose animals from 10 to 300 s. For trial 1, no animal stayed on the rod for the full trial period (300 s). For trials 2 and 3, five animals stayed on the rod for each full trial period; for trial 4, seven animals stayed on the rod for the full trial period. No animal stayed on the rod for the full period more than once. For the model of average time on rod (in seconds) for four trials (four iterations, no restarts, convergence criterion met) fit statistics suggested that the data variability was not adequately modeled (chi-squared/df. = 42764). The litter random effect covariance parameter estimate was large and the standard error exceeded the parameter (est = 8145, SE = 8437). The model was not evaluated further (and did not suggest significant main effects).

Discussion

The findings from this study contribute to the small but growing number of animal studies investigating the effects on behavior of early chronic low-level lead exposure yielding low BLLs. Our studies of minority children living in lower socio-economic conditions showed that 60% had BLLs ≥ 2.5 µg dl^{-1} as determined by ICP-MS (Sobin, Parisi, Schaub, Gutierrez, and Ortega, 2011b) and suggested that large numbers of lower-income minority children are likely to be exposed to low-level environmental

lead. Animal models are needed to understand how early chronic low-level lead exposure alters development, and behavioral tests sensitive to very low-level lead exposure are needed. The two previous mouse studies examining effects of early chronic low-level lead exposure suggested that early chronic low-level lead exposure reduced exploratory ambulation (open field task) in adult animals (Kasten-Jolly *et al.*, 2012; Leasure *et al.*, 2008). The lack of significant effects for exploratory ambulation in the present study of pre-adolescent animals suggested that some effects of early chronic low-level lead exposure might not emerge until adulthood. Additional studies are needed to determine whether behavioral differences observed at pre-adolescence are sustained into adulthood, whether early chronic low-level lead exposure disrupts pathways associated with exploratory activity at pre-adolescence and whether early exposure predisposes animals to poorer behavioral and brain resilience in late life. For these studies, we administered two standard measures of exploration, the open field task and the unbaited nose poke task (Wahlsten, 2011). These tasks are used together to differentiate exploratory ambulation (horizontal locomotion) from exploratory activity (head-dips in unbaited holes) (Hoffman, Hornig, Yaddanapudi, Jabado, and Lipkin, 2004). Exploratory animal behaviors are critical for survival, reflect adaptation, learning and memory, and provide the means by which animals acclimate to a new environment and monitor a known environment. Horizontal movement in an open arena provides a measure of the simplest type of exploration via ambulation. Complex exploratory activity such as head-dipping during ambulation represents the animal's behavioral resolution of an approach-avoidance conflict in a novel environment. Studies have shown that in a novel environment, animals simultaneously experience curiosity and fear (Hughes, 2007). The completion of a nose poke is interpreted as curiosity over-riding fear, and thus the animal's capacity for this to occur. In this study, early chronic exposure to low-level lead decreased this exploratory activity in pre-adolescent lead-exposed animals. To explain the observed association, we speculated that in pre-adolescent mice, lead exposure somehow disrupted the capacity for curiosity to override fear, at the level observed in the control animals. To explore this possibility, additional studies are needed to distinguish effects of low-level lead exposure on fear vs. curiosity. Interestingly, the significant inverse associations between BLL and exploratory activity were observed during minute 1 and minute 3, and not during minute 2 of the nose poke task. The amount of activity for control mice was consistent across all minutes of the task, and for all mice, consistent within minute 2. For this reason, we speculated that the results observed in minute 1 vs. minute 3 might reflect disruption of two distinct processes. As discussed above, during initial exploration of the novel space (task minute 1), less exploratory activity in lead exposed as compared with control animals could have resulted from fear predominating over curiosity, which resolved by task minute 2, as suggested by similar exploratory activity among all animals in this epoch. If fear in fact had resolved by task minute 2, then fewer nose pokes in lead-exposed animals observed during task minute 3, rather than indicating the predominance of fear over curiosity, may have indicated loss of curiosity relative to the control animals. This is of course speculative. As suggested above, studies are needed to tease apart the possible effects of low-level lead exposure on the fear vs. curiosity components of exploration. The C57BL/6J mouse was selected for these studies because this outbred strain is a well-characterized animal model for neuroscience and toxicology research. Studies of metal-binding proteins such as ALAD and metallothioneins that influence lead absorption, and thus its neurotoxicity, have been modeled in mice (Gonick, 2011). Furthermore, studies have suggested that metallothioneins and ALAD dynamics in mice are highly comparable to those in humans (Gonick, 2011; Kenaga, Cherian, Cox, and Oberdorster, 1996; Takahashi, 2012) suggesting the value of a mouse model for understanding the effects of chronic low-level lead exposure on behavior. For future studies, it may be useful to consider briefly a few possible mechanistic sources of the behavioral differences observed. It has been suggested that activation of amygdalar GABA neurons, which underlie the fear response, also influence head-dipping ("nose poke") behavior in novel environments (Takeda, Tsuji, and Matsumilla, 1998). Thus, it could be useful to investigate whether early chronic low-level lead exposure specifically targets amygdalar GABA pathways. In addition, acetylcholine pathways linking the hippocampus and entorhinal cortex support and influence differences in exploratory activity in a novel environment, and specifically, curiosity for a novel environment in the nose poke task (Brodkin, 1999; Crusio, 1995). Studies of these specific pathways could build on the current findings and contribute to the development of an animal model of early chronic low-level lead exposure. Perhaps consistent with these findings, memory impairments have been found in children with BLLs similar to those of the low-dose mice in this study (<5 μg dl^{-1}) (e.g., Min *et al.*, 2007; Surkan *et al.*, 2007). In addition, in brain studies of pre-adolescent C57BL/6J mice exposed to chronic low-level lead, abnormalities in hippocampus/dentate gyrus were identified (Sobin *et al.*, 2013). The results of these studies should be interpreted cautiously. These studies used unculled litters and the number of females across the exposure groups was unbalanced. Sex was included as a control factor in all models tested and the lack of effects observed for sex may be attributable to lower numbers of females with higher-level exposure. Additional studies with balanced numbers of males and females at higher levels of exposure are needed. In addition, the statistical model tested for Rotarod performance did not return an adequate fit suggesting that different tests of motor coordination may be needed. Whether chronic low-level lead exposure disrupts motor coordination cannot be determined from these studies. Finally, this study examined behavior at one time point (pre-adolescence). Additional studies are needed to replicate these findings, and to examine effects of early chronic low-level lead exposure on exploratory activity at different developmental stages, perhaps using observational measures of home-cage behavior for younger mice.

Conclusion

Early chronic low-level lead exposure yielding BLLs ranging from 1.98 to14.84 μg dl^{-1} disrupt exploratory activity in pre-adolescent mice. The effects of early chronic low-level lead exposure on pre-adolescent behavior can be meaningfully modeled in the C57BL/6J mouse.

Acknowledgments

The authors would like to acknowledge Mari Golub, Environmental Toxicology, UC Davis, for her assistance in the preparation of the final manuscript. This research was made

possible by grants from the National Institute of Child Health and Human Development (NICHD), National Institutes of Health, (R21HD060120, CS, PI); the National Center for Research Resources, a component of the National Institutes of Health (5G12RR008124); the Center for Clinical and Translational Science, The Rockefeller University, New York, New York; the Paso del Norte Health Foundation, El Paso, Texas; and from funding sources at the University of Texas, El Paso, including the Border Biomedical Research Center (BBRC); the University Research Institute (URI); and by funds provided from the J. Edward and Helen M.C. Stern Endowed Professorship in Neuroscience (CS). The funders had no role in the design, collection, data analyses and interpretation, implementation and manuscript preparation for this study, or the decision to submit the article for publication.

Conflict of Interest

The Authors did not report any conflict of interest.

References

Azzaoui FZ, Ahami AO, Khadmaoui A. 2009. Impact of lead sub-chronic toxicity on recognition memory and motor activity of Wistar rat. *Pak. J. Biol. Sci.* **12**:173–177.

Bates D, Maechler M, Bolker B. 2011. lme4: Linear mixed-effects models using S4 classes R package version 0.999375-42.

Bellinger DC, Needleman HL. 2003. Intellectual impairment and blood lead levels. *N. Engl. J. Med.* **349**:500–502.

Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White JS. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* **24**:127–135.

Brodkin J. 1999. Assessing memory in mice using habituation of nose-poke responding. *Behav. Pharmacol.* **10**:445–451.

Burnham KP, Anderson DR. 2002. *Model Selection and Multi-model Inference: A practical information-theoretic approach*. Springer-Verlag: New York.

Canfield RL, Henderson CR Jr, Cory-Slechta DA, Cox C, Jusko TA, Lanphear BP. 2003. Intellectual impairment in children with blood lead concentrations below 10 micrograms per deciliter. *N. Engl. J. Med.* **348**:1517–1526.

Centers for Disease Control and Prevention. 2005. A review of evidence of health effects of blood lead levels < 10 micrograms per deciliter in children. Retrieved from http://www.cdc.gov/nceh/lead/ACCLPP/meetingMinutes/lessThan10MtgMAR04.pdf

Centers for Disease Control and Prevention. 2012. CDC response to advisory committee on childhood lead poisoning prevention recommendations in "low level lead exposure harms children: A renewed call of primary prevention." Retrieved from http://www.cdc.gov/nceh/lead/ACCLPP/Final_Document_030712.pdf

Crusio WE. 1995. Natural selection on hippocampal circuitry underlying exploratory behaviour in mice: Quantitative-genetic analysis. *Behavioural Brain Research in Naturalistic and Seminaturalistic Settings*. Kluwer Academic Press: The Netherlands; 323–342.

Ferguson SA, Bowman RE. 1990. Effects of postnatal lead exposure on open field behavior in monkeys. *Neurotoxicol. Teratol.* **12**:91–97.

Gilbert SG, Weiss B. 2006. A rationale for lowering the blood lead action level from 10 to 2 μg/dL. *Neurotoxicology* **27**:693–701.

Gonick HC. 2011. Lead-binding proteins: A review. *J. Toxicology.* **2011**:686050

National Research Council (U.S.), Institute for Laboratory Animal Research (U.S.), & National Academies Press (U.S.). 2011. *Guide for the care and use of laboratory animals*. National Academies Press: Wasingthon DC.

Hoffman KL, Hornig M, Yaddanapudi K, Jabado O, Lipkin WI. 2004. A murine model for neuropsychiatric disorders associated with group A beta-hemolytic streptococcal infection. *J. Neurosci.* **24**:1780–1791.

Hughes RN. 2007. Neotic preferences in laboratory rodents: Issues, assessment and substrates. *Neurosci. Biobehav. Rev.* **31**:441–464.

Jusko TA, Henderson CR, Jr, Lanphear BP, Cory-Slechta DA, Parsons PJ, Canfield RL. 2008. Blood lead concentrations < 10 μg/dL and child intelligence at 6 years of age. *Environ. Health Perspect.* **116**:243–248.

Kasten-Jolly J, Pabello N, Bolivar VJ, Lawrence DA. 2012. Developmental lead effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. *Neurotoxicology* **33**:1005–1020.

Kenaga C, Cherian MG, Cox C, Oberdorster G. 1996. Metallothionein induction and pulmonary responses to inhaled cadmium chloride in rats and mice. *Fundam. Appl. Toxicol.* **30**:204–212.

Landrigan PJ, Trasande L, Thorpe LE, Gwynn C, Lioy PG, Lipkind HS, … Susser E. 2006. The national children's study: A 21-year prospective study of 100 000 American children. *Pediatrics* **118**:2173–2186.

Lanphear BP, Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, … Roberts R. 2005. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. *Environ. Health Perspect.* **113**:894–899.

Leasure JL, Giddabasappa A, Chaney S, Johnson JE Jr, Pothakos K, Lau YS, Fox DA. 2008. Low-level human equivalent gestational lead exposure produces sex-specific motor and coordination abnormalities and late-onset obesity in year-old mice. *Environ. Health Perspect.* **116**:355–361.

Min J-Y, Min K-B, Cho S-I, Kim R, Sakong J, Paek D. 2007. Neurobehavioral function in children with low blood lead concentrations. *Neurotoxicology* **28**:421–425.

Pinheiro J, Bates D. 2000. *Mixed Effects Models in S and S-Plus*. Springer-Verlag: New York.

Reiter LW, Anderson GE, Laskey JW, Cahill DF. 1975. Developmental and behavioral changes in the rat during chronic exposure to lead. *Environ. Health Perspect.* **12**:119–123.

Schabenberger O. 2005. Introducing the GLIMMIX procedure for generalized linear mixed models. *Proceedings of the Thirtieth Annual SAS User's Group International Conference*; 196–305.

Sobin C, Parisi N, Schaub T, de la Riva E. 2011a. A Bland-Altman comparison of the Lead Care(R) System and inductively coupled plasma mass spectrometry for detecting low-level lead in child whole blood samples. *J. Med. Toxicol.* **7**:24–32.

Sobin C, Parisi N, Schaub T, Gutierrez M, Ortega AX. 2011b. delta-Aminolevulinic acid dehydratase single nucleotide polymorphism 2 and peptide transporter 2*2 haplotype may differentially mediate lead exposure in male children. *Arch. Environ. Contam. Toxicol.* **61**:521–529.

Sobin C, Montoya MG, Parisi N, Schaub T, Cervantes M, Armijos RX. 2013. Microglial disruption in young mice with early chronic lead exposure. *Toxicol. Lett.* **15**:00151–00153.

Surkan PJ, Zhang A, Trachtenberg F, Daniel DB, McKinlay S, Bellinger DC. 2007. Neuropsychological function in children with blood lead levels <10 μg/dL. *Neurotoxicology* **28**:1170–1177.

Takahashi S. 2012. Molecular functions of metallothionein and its role in hematological malignancies. *J. Hematol. Oncol.* **5**:41.

Takeda H, Tsuji M, Matsumiya T. 1998. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *Eur. J. Pharmacol.* **350**:21–29.

Wahlsten D. 2011. *Mouse Behavioral Testing: How to use mice in behavioral neuroscience*. Academic Press: New York.

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Olfactory recognition memory is disrupted in young mice with chronic low-level lead exposure

Mayra Gisel Flores-Montoya [a,b], Juan Manuel Alvarez [a,c], Christina Sobin [a,c,d,*]

[a] Border Biomedical Research Center, Toxicology Core, University of Texas, El Paso, USA
[b] Department of Psychology, University of Texas, El Paso, USA
[c] Department of Public Health Sciences, College of Health Sciences, University of Texas, El Paso, USA
[d] Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY, USA

HIGHLIGHTS

- Olfactory memory was examined in mice with and without early chronic lead exposure.
- Blood lead levels in exposed mice were 2.02–20.3 micrograms per deciliter.
- In males, as blood lead level increased olfactory memory decreased.
- In females, a non-linear effect was observed at lowest levels of exposure.

ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 9 April 2015
Accepted 24 April 2015
Available online 29 April 2015

Keywords:
Developmental lead exposure
Olfactory recognition memory
Dentate gyrus
Neurobehavioral toxicity

ABSTRACT

Chronic developmental lead exposure yielding very low blood lead burden is an unresolved child public health problem. Few studies have attempted to model neurobehavioral changes in young animals following very low level exposure, and studies are needed to identify tests that are sensitive to the neurobehavioral changes that may occur. Mechanisms of action are not yet known however results have suggested that hippocampus/dentate gyrus may be uniquely vulnerable to early chronic low-level lead exposure. This study examined the sensitivity of a novel odor recognition task to differences in pre-adolescent C57BL/6J mice chronically exposed from birth to PND 28, to 0 ppm (control), 30 ppm (low-dose), or 330 ppm (higher-dose) lead acetate (N = 33). Blood lead levels (BLLs) determined by ICP-MS ranged from 0.02 to 20.31 μg/dL. Generalized linear mixed model analyses with litter as a random effect showed a significant interaction of BLL × sex. As BLLs increased olfactory recognition memory decreased in males. Among females, non-linear effects were observed at lower but not higher levels of lead exposure. The novel odor detection task is sensitive to effects associated with early chronic low-level lead exposure in young C57BL/6J mice.

1. Introduction

The dangers of developmental lead exposure are well-documented and there is widespread recognition that even low-level exposure alters neurobehavior in young children. Child studies have suggested many neurobehavioral functions altered by early chronic low-level lead exposure. These include but are not limited to memory and learning, visual attention, abstract problem-solving, cognitive set-shifting, and motor dexterity (Bellinger and Needleman, 2003; Canfield et al., 2003; Franko et al., 2000; Gilbert and Weiss, 2006; Jusko et al., 2008; Landrigan et al., 2006; Lanphear et al., 1998, 2005; Needleman et al., 1990, 1996; Schnaas et al., 2000; Sobin et al., 2015; Wasserman et al., 2000). The mechanisms by which low-level lead disrupts neuro-development however are not yet known and few animal models of early chronic low-level lead exposure have been proposed. In order to advance knowledge in this area, neurobehavioral tests that are sensitive to the effects of early chronic low-level lead exposure in animals are needed.

Of the neurocognitive disruptions identified in low-level lead-exposed children, changes in memory may have the most profound implications for life-long brain health. The brain regions critical for memory and learning, in particular the hippocampus/dentate

* Corresponding author at: University of Texas, El Paso, 500 West University, El Paso TX, 79902, USA. Tel.: +1 915 747 7274; fax:+1 915 747 6553.
E-mail address: casobin@utep.edu (C. Sobin).

http://dx.doi.org/10.1016/j.toxlet.2015.04.013
0378-4274/

gyrus regions, overlap neurogenesis pathways. Early disruption of these regions and pathways has the potential to alter neural pathway formation (Schafer et al., 2012), memory function, learning during development (Schinder and Gage, 2004) and neurogenesis during adulthood and aging (Jessberger et al., 2009), perhaps increasing vulnerability to cognitive decline and dementia.

Only a few past studies have examined memory in rodents with early chronic low-level lead exposure. For example, in adulthood, BALB/c mice chronically exposed to 20 ppm lead acetate delivered in dam's drinking water had diminished memory (object recognition memory task) as compared to controls (Azzaoui et al., 2009). In a similar study, as compared to controls, adult Wistar rats chronically exposed to 20 ppm of lead in dam's drinking water had diminished spatial memory (water maze) (Kasten-Jolly et al., 2012) (blood lead levels were not reported). A recent study in our laboratory of pre-adolescent C57BL/6J mice showed that as blood lead level (BLL) increased, exploration of a novel environment decreased (Flores-Montoya and Sobin, 2014).

In the current study, the sensitivity of a novel odor detection paradigm to the effects of early chronic low-level lead exposure was examined in pre-adolescent C57BL/6J mice. It was hypothesized that from lowest to highest levels of lead exposure, as BLL increases, olfactory recognition memory decreases.

2. Method

2.1. Animals

All animal procedures had prior approval of the Institutional Animal Care and Use Committee (IACUC) and were carried out in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 2011). C57BL/6J mice were purchased from Jacksons Laboratories and housed in the Bioscience Research Facility at the University of Texas at El Paso (UTEP). Mice were group housed by sex in ventilated cages (22.22 cm × 36.83 cm × 13.97 cm) with *ad libitum* access to food and water. The animal holding room had a temperature of 20°–26°C, relative humidity of 30–70 percent, and a 12 h light-dark cycle.

Dams were mated beginning at post-natal day (PND) 40 using harem breeding. Two females were placed with one male, checked daily, and housed separately after vaginal plug was identified. Ten dams were mated with five sires. Nine of ten dams were successfully impregnated. Gestation durations were between 19 and 21 days. Prior studies suggested that early chronic low-level lead exposure may alter stress-responsive neuroimmune processes (Sobin et al., 2013) thus, to avoid stressing dams and pups, unculled litters were planned with sex and litter (as a random effect) controlled in all analyses. Seven dams produced litters ranging in size from 3 to 6 pups, N = 33, including 13 females and 20 males. Two remaining litters of one pup each were not included. Each litter was assigned to one of three lead treatments, either 0 ppm, control (n = 10, 2 females; 8 males), 30 ppm, low-dose (n = 10, 5 females and 5 males), and 330 ppm, higher-dose (n = 13, 6 females and 7 males). No animals died during the course of the study.

2.2. Lead exposure

Pups were exposed to lead via dams' milk. From PND 0 to PND 28 dams were given either lead-treated water (30 ppm or 330 ppm 99.4% lead acetate crystals, Sigma–Aldrich, St. Louis, MO) or sodium-treated water (30 ppm).

2.3. Behavioral testing

Recognition memory was tested at PND 28 with a novel odor recognition (NODR) task. The protocol was based on those used in previously published protocols (Bevins and Besheer, 2006; Simple Odor Recognition Protocol, 2011). This task was adapted from a novel object recognition memory task (NOR task) (Bevins and Besheer, 2006). The original task included a training phase and a testing phase. During the training phase, mice were placed in a square arena and allowed to explore two identical objects located in the upper corners of the arena. The testing phase then follows an inter-trial interval (ITI). A familiar object was replaced with a novel object. Mice were returned to the arena and allowed to freely explore the familiar and novel objects. Mice with intact memory spend more time exploring the novel as compared to the familiar object. For the current study, odors rather than objects were used to maximize possible group differences. The odors selected were those published in previous mouse behavioral protocols (Simple Odor Recognition Protocol, 2011).

All testing occurred between 10:00 a.m. and 1:00 p.m. Three identical square Plexiglas arenas (8 in × 8 in × 24 in) equipped with a timer were used for habituation (10 min), training (10 min), and testing (5 min) phases, with 5 min inter-trial intervals (ITI) between each phase. During the ITI, mice were returned to a holding cage with home bedding.

For the habituation phase, animals were placed in the empty arena and allowed to freely explore. For the training phase, animals were placed in the second arena with two identically scented vehicles. Orange or almond food-grade edible natural liquid flavors (McCormic®) were sprayed on 1" mouse-shaped felt objects positioned in the upper left and right arena corners approximately 4 cm from each wall. For the testing phase, the familiar scented object was replaced with a novel (orange or almond) scented object. Fixed visual cues in the testing room external to the testing arena were asymmetrical and to accommodate this, the location of the novel odor was fixed to the upper right corner; "familiar" and "novel" orange or almond odors were counterbalanced. All arenas were cleaned with 10% isopropyl alcohol after each trial. Each mouse was returned to the home cage when testing was completed.

Video cameras placed over the top of the arenas recorded all mouse activity during testing. Video recordings were later scored by four raters trained to reliability and blind to experimental condition. Exploration was recorded when the mouse nose was oriented towards and within a 2 cm proximity to the odor vehicle. Inter-rater reliability was determined after rater training and during and after test scoring. All post-training and scoring reliabilities exceeded 0.90.

2.4. Blood collection

Immediately after behavioral testing, mice were anesthetized with Avertin (5–10 mL). Animals were sexed and weighed after they were unresponsive to corneal touch and paw pinch tests. Heart blood was extracted (50 μL of blood per animal) via syringe puncture at the heart apex. Blood samples were refrigerated and processed for inductively coupled plasma mass spectrometry (ICP-MS) analysis within 72 h of sample collection.

2.5. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of blood lead

The detailed method for the measurement of BLL was previously described (Sobin et al., 2011). Briefly, an Agilent 7500ce ICP-MS with an octopole reaction system and a CETAC ASX520 autosampler was used. A Micro Mist U-series nebulizer

and a double-pass quartz spray chamber were used to introduce the samples into plasma. The instrument parameters were a carrier gas of 0.78 L/min, makeup gas of 0.15 L/min, RF power of 1420 W, and a spray chamber that was set to a temperature of 2 °C. Samples were processed by filling a propylene tube with 5.58 mL of water, 300 μL of blood, 60 μL of aqueous internal standard solution, and 60 μL of aqueous 10 ppm gold in 3% hydrochloric acid solution. The samples were vortexed, and centrifuged for one minute at 2000 rcf and the supernatant was analyzed by ICP-MS. BLLs were determined in micrograms per deciliter (μg/dL).

2.6. Statistical analyses

Data were entered, checked for accuracy, and examined for missing values, distribution properties, and outliers. No values were out of the range of plausible responses and all data were included for analyses. Data were analyzed with *SAS Version* 9.3. (SAS Institute Inc., Cary, North Carolina).

Preliminary analyses were conducted to determine whether exposure paradigm yielded differences between groups in blood lead and whether body weight was influenced by lead exposure. In the first preliminary analysis, BLL was predicted from group, sex, and group × sex, with litter included as a random effect. In the second preliminary analysis, body weight was predicted from BLL, sex and BLL × sex, with litter included as a random effect.

Primary analyses included tests of the main hypothesis and confirmatory tests of effects. The primary hypothesis for the study stated that, controlling for sex and litter, BLL predicted testing phase discrimination ratio such that as BLL increased the testing phase discrimination ratio (odor recognition memory) decreased. For confirmatory analyses, three additional models were tested to rule out the possibility that lead exposure induced differences that altered ambulatory exploration and thus the acquisition and/or recognition of odor information. In these models, associations were tested between BLL and training phase total exploration time, BLL and testing phase total exploration time, and BLL and training phase discrimination ratio (controlling for sex with litter as a random effect in all models).

An effect of BLL on total training phase and/or total testing phase exploration time would indicate that lead exposure somehow impacted ambulatory exploration required for information acquisition during the training or testing phase of the task, respectively. An effect of BLL on the training phase discrimination ratio would indicate that BLL somehow predisposed the animal to exploration of a left or right spatial location (of identical odors), perhaps biasing acquisition of information for one vs. the other spatial location.

Total exploration times for the training phase and the testing phase represented odor exploration according to the criteria stated in Section 2.3 above. Training phase discrimination ratio was calculated by dividing the time spent exploring the upper right odor by the total time spent exploring both odors [Tright/(Tright + Tleft)]. Testing phase discrimination ratio was calculated by dividing the time spent exploring the novel odor by the total time exploring the novel and familiar odors [Tnovel/(Tnovel + Tfamiliar)] (Bevins and Besheer, 2006).

Generalized linear mixed model analyses with maximum likelihood estimates (GLIMMIX procedure) were used for the preliminary and main analyses. Fixed effects for the preliminary analysis predicting BLL included group, sex and the interaction group x sex, with litter included as a random effect. Fixed effects for the preliminary analysis predicting body weight included BLL, sex and BLL × sex. Fixed effects for the four primary analyses predicting task behavioral outcomes included BLL, sex, and the interaction BLL × sex, with litter included as a random effect in all models. The Gaussian distribution with an identity link function was specified and all models were checked for convergence and the G matrix estimate. Model significance was evaluated by examining fixed effect Type III F-values and significance for one main effect (BLL) and one interaction (BLL × sex) controlling for litter. Parameter estimate significance values indicated difference from zero for continuous variables (i.e., BLL) or a significant difference between groups for the parameter estimates of the categorical variable (i.e., sex).

Significance for the Type III fixed effect tested whether the variable or interaction estimate differed significantly from zero and indicated the amount of model variance accounted for by a given (continuous or categorical) variable or interaction. When the fixed effect *F*-value was statistically significant, relevant post-hoc tests of least square means (for possible categorical effects) were evaluated; or regression coefficients (for the significant continuous predictor BLL or interaction) were determined and tested. Post-hoc comparisons of least square mean differences for categorical (sex) effects were calculated using the Tukey-Kramer adjustment for multiple comparisons. Least square means (LSM) reflected the mean of a variable after co-varying other model factors, i.e., BLL and litter. Adjusted alpha ≤0.05, and adjusted lower and upper 95% confidence intervals were used to evaluate all post-hoc comparisons.

3. Results

3.1. Subjects

All animals completed testing. No animals died during the study. No adverse physical or behavioral effects were observed in the lead exposed or control animals. Table 1 shows the means and standard deviations (SD) of BLL and body weight for males and females by exposure group. BLLs ranged from 0.02 to 20.31 μg/dL.

The first preliminary analysis predicted BLL from group, sex and group × sex interaction, with litter included as a random effect. Only group was a significant predictor of BLL ($t_{2/26} = 158.4$, $p < .01$). BLL was not predicted by sex ($t_{2/26} = 1.62$, $p =$ n.s.) or the interaction group × sex ($t_{2/26} = 1.04$, $p =$ n.s.).

The second preliminary analysis predicted body weight from BLL, sex and the interaction, with litter included as a random effect. Only sex approached significance with males weighing more than females ($t_{2/26} = 1.97$, $p = .06$) (LSmeans (SE) males = 14.23 (0.35), females = 12.52 (0.43)). BLL and the interaction of BLL × sex did not contribute to body weight variability.

3.2. Novel odor recognition task

The means and SDs for the training and testing phases of the task are shown in Table 2. Odor exploration times during the training phase (10 min exploration) were of longer duration than those observed during the testing phase (5 min exploration).

Table 1
Mean and SD of blood lead level and body weight for males and females in each exposure group following early chronic lead exposure, N = 33.

Lead exposure group	Blood lead levels (μg/dL)[a]		Body weight (grams)[a]	
	Males	Females	Males	Females
0 ppm	0.31 ± 0.02	0.02 ± 0.00	15.14 ± 1.09	14.33 ± 0.15
30 ppm	3.10 ± 0.57	2.63 ± 0.25	12.81 ± 0.90	11.82 ± 0.91
330 ppm	15.21 ± 3.86	12.92 ± 1.00	14.27 ± 1.59	12.46 ± 1.85

[a] In mixed model analyses with litter included as a random effect, BLL was predicted by exposure group, and not by sex or the interaction of group × sex; body weight was marginally predicted by sex ($t = 1.97$, $p = 0.06$), and not by BLL or the interaction of BLL × sex.

Table 2
Exploration times (s) during training and testing phases of the novel odor recognition task in C57BL/6J mice at pre-adolescence, N = 33.

	Mean ± SD
Training phase	
(Two identical odors placed equidistant from upper left and right corners of arena)	
Left odor exploration	13.8 ± 9.4
Right odor exploration	17.1 ± 13.6
Odor exploration training total	30.9 ± 22.1
Testing phase	
(Left/familiar odor remains and the right familiar odor is replaced by the novel odor[a])	
Left/familiar odor exploration	3.4 ± 2.9
Right/novel odor exploration	4.2 ± 3.7
Odor exploration testing total	7.6 ± 5.2

[a] Familiar and novel odors were counterbalanced (half of the animals in each exposure group had orange as the familiar odor and almond as the novel odor; half had almond as the familiar odor and orange as the novel odor).

3.2.1. Primary analysis

The primary model predicting testing phase discrimination ratio from BLL and sex controlling for litter was tested. Convergence criterion for the model was reached. The Type III fixed effect solutions and parameter estimates for the main effects of BLL and sex, and the interaction of BLL × sex are shown in Table 3. Only a significant interaction effect of BLL × sex was observed for the novel odor recognition testing phase. Tests of the regression coefficients for males (est = −0.024, SE = 0.01, df = 28, $t = -3.06$, $p < 0.01$) and for females (est = 0.01, SE = 0.01, df = 28, $t = 1.22$, $p = 0.23$) showed that the effect was significant only for males. Among males, for every 1 unit increase in BLL, testing phase discrimination ratio decreased by 0.024. The effects in males and in females are separately illustrated in Fig. 1, A and B.

3.2.2. Confirmatory analyses

Three additional models were calculated to test whether lead exposure had altered exploratory ambulation in males and/or females. If so, these possible effects would have been expected to alter information acquisition during the learning phases, and thus confound the primary outcome. The three confirmatory models tested whether BLL, sex and/or BLL × sex, with litter included as a random effect, predicted total exploration time during the training phase, total exploration time during the testing phase, and/or training phase discrimination ratio. As shown in Table 3, neither BLL nor the interaction of BLL × sex were predictive of total exploration time during the training phase, total exploration during the testing phase, or training phase discrimination ratio (calculated according to the formula provided in Section 2.6).

4. Discussion

The NODR task was sensitive to the linear effects of chronic developmental lead exposure on olfactory memory in young C57BL/6J mice. In males, as BLL increased, the amount of time spent exploring the novel as compared with the familiar odor decreased. Effects among females were suggested only at lowest levels of exposure (Fig. 1) but were not linear and were not

Table 3
Parameter estimates showing effects of BLL, sex, and BLL × sex on novel odor exploration in C57BL/6J mice, controlling for litter (as a random effect).

Significant type III fixed effect			Solutions for fixed effects					
	F	*p*		Est	SE	DF	*t* value	*p*
Training Phase Discrimination Ratio			Intercept*	0.5	0.03	28	15.96	<0.01
BLL	1.31	0.26	BLL	0.01	0.01	28	1.13	0.27
Sex	3.5	0.07	Sex male	0.08	0.04	28	1.87	0.07
BLL × Sex	0.57	0.46	Sex female	0	–	–	–	–
			BLL × Sex male interaction	−0.01	0.01	28	−0.75	0.48
			BLL × Sex female interaction	0	–	–	–	–
Testing Phase Discrimination Ratio			Intercept*	0.51	0.07	28	7.48	<0.01
BLL	0.37	0.58	BLL	0.01	0.01	28	0.58	0.23
Sex	1.04	0.32	Sex male	0.05	0.09	28	0.62	0.32
BLL × Sex	7.16	0.01	Sex female	0	–	–	–	–
			BLL × Sex male interaction	−0.04	0.01	28	−2.68	0.01
			BLL × Sex female interaction	0	–	–	–	–
Training Phase Total Odor Exploration (s)			Intercept*	42.2	13.97	28	3.17	<0.01
BLL	0.45	0.51	BLL	1.57	1.37	28	1.41	0.26
Sex	0.2	0.65	Sex male	−3.65	8.07	28	−0.69	0.65
BLL × Sex	1.74	0.2	Sex female	0	–	–	–	–
			BLL × Sex male interaction	−1.69	1.28	28	−1.36	0.2
			BLL × Sex female interaction	0	–	–	–	–
Testing Phase Total Odor Exploration (s)			Intercept*	7.25	1.51	28	4.81	<0.01
BLL	0.11	0.74	BLL	0.01	0.27	28	0.02	0.99
Sex	0.07	0.8	Sex male	0.5	1.93	28	0.26	0.8
BLL × Sex	0.13	0.72	Sex female	0	–	–	–	–
			BLL × Sex male interaction	−0.11	0.32	28	−0.37	0.72
			BLL × Sex female interaction	0	–	–	–	–

*BLL values were centered for these analyses and the intercepts are directly interpretable.

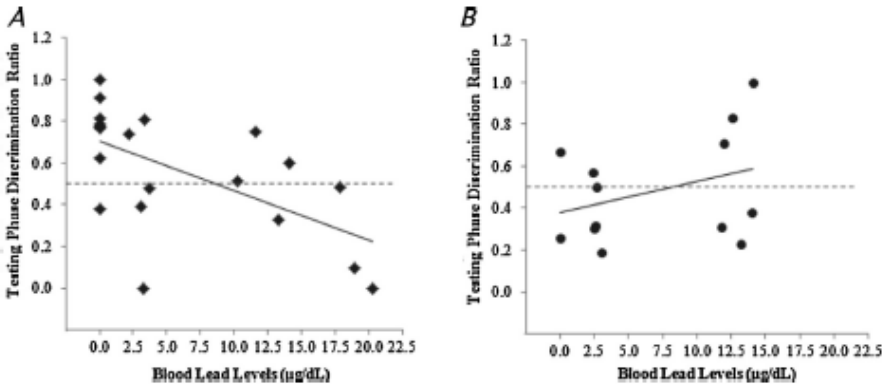


Fig. 1. Regression lines showing the association between blood lead level and test phase discrimination ratio for males (Fig. 1A) and females (Fig. 1B). The *y*-axis shows the amount of time subjects explored the novel as compared to novel and familiar odors (discrimination ratio). Males (A) and females (B) are graphed separately. Subjects above the dashed line explored the novel odor for more time than the familiar odor; subjects below the dashed line explored the familiar odor for more time than the novel odor. A linear effect of BLL on discrimination ratio was found only for males. Among females, a linear effect was not found, however at lowest levels of exposure 5 of 7 female subjects performed at or below chance. The dashed lines shown on the graphs indicate equal amounts of time spent exploring the novel and familiar odor.

statistically significant in the tests conducted. (Results in females are discussed below).

Broadly consistent with these findings, previous studies of adult mice with chronic lead exposure yielding low BLLs (discussed in Section 1) reported deficits in object recognition (Azzaoui et al., 2009) and in spatial memory (Kasten-Jolly et al., 2012), similar to those found in the current study. Object recognition and spatial memory are sub-served by brain regions that overlap olfactory recognition memory pathways including the prefrontal, entorhinal and perirhinal cortices, and the hippocampus/dentate gyrus (Jessberger et al., 2009; Parron and Save, 2004; Rinaldi et al., 2007; Winters and Bussey, 2005). Given the current findings, future studies could examine whether object recognition and spatial memory deficits also emerge before adulthood in pre-adolescent mice with early chronic low-level lead exposure.

This study included confirmatory analyses to test whether the effects of BLL on exploratory ambulation may have accounted for the association between BLL and testing phase discrimination ratio. This is an important step for the interpretation of findings. Apparent effects on memory might be accounted for by differences in, for example, lead-induced deficits in motor function. In this study, no additional effects were identified. These tests provided some assurance that any apparent effects of BLL on the testing phase discrimination ratio would not be attributable to lead-induced differences in "familiar" odor exploration time, odor location preference, or differences in the patterns of left-right exploration. Furthermore, the validity of the NODR task was suggested by a significant increase in the discrimination ratio during the testing phase as compared with the training phase among control animals. In other words, control animals remembered the familiar odor and recognized the novel odor. This provided evidence that the NODR task is a valid test of olfactory recognition memory in young C57BL/6J mice.

Among females at lowest levels of exposure, memory deficits were perhaps apparent, i.e., the majority of the females did not discriminate the familiar from the novel odor. At the same time, performance among low-level lead exposed females did not appear to be linearly related to blood lead. It is possible that the limited variability in BLL at lower levels of exposure limited the detection of a linear effect among females. To address this issue, future studies examining effects on memory of early chronic low-level lead exposure should include a moderate exposure group to achieve a broader distribution of BLLs from lowest to higher values. Among other things, these findings suggested that the effects of lower and higher levels of lead exposure may be qualitatively different among males and females; and further, that future studies should be designed to ensure that this interaction is characterized.

Another methodological issue concerned overall exploration times. The testing phase exploration times were notably shorter than the exploration times recorded during the training phase and a restricted range of values may limit the detection of linear effects. For future studies, modifications to the protocol here described could include longer ITI periods which may result in increased exploration times during the testing phase of the task.

In this study, linear regression analyses suggested that among males, as BLLs increased, males spent greater amounts of time exploring the familiar as compared with the novel odor. An obvious conclusion is that lead exposure impaired the ability of mice to remember the familiar odor, resulting in continued exploration of the familiar odor rather than the novel odor. It was noted however that among lead exposed mice, testing phase discrimination ratios did not cluster near 0.50 but instead dropped to lower levels suggesting perhaps a preference for the familiar odor. Thus, another interpretation may be suggested. Past studies have suggested that exploratory behavior represents the predominance of curiosity over fear (Hughes, 2007). It is possible that the effects of lead exposure on brain resulted in decreased curiosity and/or increased fear of the novel odor. Future behavioral studies could use tests that separately challenge fear and curiosity in non-memory tasks, to determine whether low-level lead exposure disrupts learning by altering the balance of fear and curiosity.

The results of the present study suggested that chronic low-level lead exposure diminished olfactory recognition memory in pre-adolescent male mice. For future mechanistic studies, it may be useful to briefly consider possible molecular pathways underlying these observed effects. The ability of mice to detect and remember novel and familiar odors is dependent on the integrity of olfactory recognition memory pathways. When healthy mice are exposed to a novel or familiar odor, G-protein coupled receptors located in the epithelium of the nose detect odors and transmit information to the main olfactory bulb (Imai et al., 2006). The olfactory bulb has major projections to the piriform cortex which in turn projects to the thalamus, orbitofrontal cortex, hypothalamus, entorhinal cortex, and hippocampus (Sanchez-Andrade et al., 2005) ultimately allowing the detection, storage,

and retrieval of olfactory information. Future studies could investigate whether low-level lead exposure disrupts odor detection, storage and/or retrieval by impairing the transmission of olfactory information via one or more of these pathways.

4.1. Strengths and limitations

The present study is the first to test memory in pre-adolescent mice with chronic low-level lead exposure, and is one of few animal studies that examined effects on cognitive behavior following early chronic low-level lead exposure in animals with BLLs <5 μg/dL. These animal studies were an extension of the current child literature showing memory deficits in preadolescent children with BLLs ≤5 μg/dL (Chiodo et al., 2004, 2007; Min et al., 2007; Sobin et al., 2015; Surkan et al., 2007) and suggested that a C57BL/6J mouse model of early chronic low-level lead exposure can be useful for understanding neurobehavioral effects in young human populations. The relatively short exploration times during the testing phase and the unbalanced numbers of males and females were weaknesses in the present study and the results may not fully characterize sex differences across groups.

5. Conclusion

The NODR task detects memory deficits associated with early chronic low-level lead exposure in pre-adolescent C57BL/6J male mice. Future studies could use a battery of tasks that attempt to differentiate deficits in fear and/or curiosity that may contribute to the observed effects on odor recognition memory, and that assesses and compares deficits in recognition memory for odors, objects, and spatial location. The findings may suggest novel mechanisms of action for future molecular studies.

Acknowledgments

The authors would like to acknowledge Mari Golub, Environmental Toxicology, UC Davis, for her assistance in the preparation of the final manuscript. This research was made possible by grants from the National Institute of Child Health and Human Development (NICHD), National Institutes of Health, (R21HD060120, CS, PI); the National Center for Research Resources, a component of the National Institutes of Health (5G12RR008124); the Center for Clinical and Translational Science, The Rockefeller University, New York, New York; the Paso del Norte Health Foundation, El Paso, Texas; and from funding sources at the University of Texas, El Paso including, the Border Biomedical Research Center (BBRC); the University Research Institute (URI); and by funds provided from the J. Edward and Helen M.C. Stern Endowed Professorship in Neuroscience (CS). The funders had no role in the design, collection, data analyses and interpretation, implementation, manuscript preparation for this study, or the decision to submit the article for publication.

References

Azzaoui, F.Z., Ahami, A.O., Khadmaoui, A., 2009. Impact of lead sub-chronic toxicity on recognition memory and motor activity of Wistar rat. Pak. J. Biol. Sci. 12, 173–177.

Bellinger, D.C., Needleman, H.L., 2003. Intellectual impairment and blood lead levels. New England J. Med. 349, 500–502.

Bevins, R.A., Besheer, J., 2006. Object recognition in rats and mice: a one-trial nonmatching-to-sample learning task to study 'recognition memory'. Nat. Protocols 1, 1306–1311.

Canfield, R.L., Henderson Jr., C.R., Cory-Slechta, D.A., Cox, C., Jusko, T.A., Lanphear, B. P., 2003. Intellectual impairment in children with blood lead concentrations below 10 micrograms per deciliter. New Engl. J. Med. 348, 1517–1526.

Chiodo, L.M., Jacobson, S.W., Jacobson, J.L., 2004. Neurodevelopmental effects of postnatal lead exposure at very low levels. Neurotoxicol. Teratol. 26, 359–371.

Chiodo, L.M., Covington, C., Sokol, R.J., Hannigan, J.H., Jannise, J., Ager, J., Delaney Black, V., 2007. Blood lead levels and specific attention effects in young children. Neurotoxicol. Teratol. 29, 538–546.

Flores-Montoya, M.G., Sobin, C., 2014. Early chronic lead exposure reduces exploratory activity in young C57BL/6J mice. J. Appl. Toxicol. 12 (epub ahead of print).

Franko, E., Palome, J., Brown, M., Kennedy, C., Moore, L., 2000. Blood lead levels in young children-United States and selected states, 1996–1999. Morb. Mortal. Weekly Rep. 49, 1133–1137.

Gilbert, S.G., Weiss, B., 2006. A rationale for lowering the blood lead action level from 10 to [mu]g/dL. Neurotoxicology 27, 693–701.

Hughes, R.N., 2007. Neotic preferences in laboratory rodents: issues: assessment and substrates. Neurosci. Biobehav. Rev. 31, 441–464.

Imai, T., Suzuki, M., Sakano, H., 2006. Odorant receptor-derived cAMP signals direct axonal targeting. Science 314, 657–661.

Jessberger, S., Clark, R.E., Broadbent, N.J., Clemenson Jr., G.D., Consiglio, A., Lie, D.C., Squire, L.R., Gage, F.H., 2009. Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Learn. Mem. 16, 147–154.

Jusko, T.A., Henderson Jr., C.R., Lanphear, B.P., Cory-Slechta, D.A., Parsons, P.J., Canfield, R.L., 2008. Blood lead concentrations < 10mg/dL and child intelligence at 6 years of age. Environ. Health Perspect. 116, 243–248.

Kasten-Jolly, J., Pabello, N., Bolivar, V.J., Lawrence, D.A., 2012. Developmental lead effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. Neurotoxicology 33, 1005–1020.

Landrigan, P.J., Trasande, L., Thorpe, L.E., Gwynn, C., Lioy, P.G., Lipkind, H.S., Susser, E., 2006. The national children's study: a 21-year prospective study of 100 000 american children. Pediatrics 118, 2173–2186.

Lanphear, B.P., Burgoon, D.A., Rust, S.W., Eberly, S., Galke, W., 1998. Environmental exposures to lead and urban children's blood lead levels. Environ. Res. 76, 120–130.

Lanphear, B.P., Hornung, R., Khoury, J., Yolton, K., Baghurst, P., Bellinger, D.C., Roberts, R., 2005. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. Environ. Health Perspect. 113, 894–899.

Min, J.-Y., Min, K.-B., Cho, S.-I., Kim, R., Sakong, J., Paek, D., 2007. Neurobehavioral function in children with low blood lead concentrations. Neurotoxicology 28, 421–425.

National Research Council (U.S.). Institute for Laboratory Animal Research (U.S.), & National Academies Press (U.S.). (2011). Guide for the care and use of laboratory animals. Washington, D.C.: National Academies Press.

Needleman, H.L., Schell, A., Bellinger, D., Leviton, A., Allred, E.N., 1990. The long-term effects of exposure to low doses of lead in childhood. An 11-year follow-up report. New England J. Med. 322, 83–88.

Needleman, H.L., Riess, J.A., Tobin, M.J., Biesecker, G.E., Greenhouse, J.B., 1996. Bone lead levels and delinquent behavior. Jama 275, 363–369.

Parron, C., Save, E., 2004. Comparison of the effects of entorhinal and retrosplenial cortical lesions on habituation: reaction to spatial and non-spatial changes during object exploration in the rat. Neurobiol. Learn. Mem. 82, 1–11.

Rinaldi, A., Mandillo, S., Oliverio, A., Mele, A., 2007. D1 and D2 receptor antagonist injections in the prefrontal cortex selectively impair spatial learning in mice. Neuropsychopharmacology 32, 309–319.

Sanchez-Andrade, G., James, B.M., Kendrick, K.M., 2005. Neural encoding of olfactory recognition memory. J. Reprod. Dev. 51, 547–558.

Schafer, D., Lehrman, E., Kautzman, A., Koyama, R., Mardinly, A., Yamasaki, R., Stevens, B., 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74, 691–705.

Schinder, A.F., Gage, F.H., 2004. A hypothesis about the role of adult neurogenesis in hippocampal function. Physiology 9, 253–261.

Schnaas, L., Rothenberg, S.J., Perroni, E., Martinez, S., Hernandez, C., Hernandez, R. M., 2000. Temporal pattern in the effect of postnatal blood lead level on intellectual development of young children. Neurotoxicol. Teratol. 22, 805–810.

Odor Recognition Protocol, 2011. Retrieved on August 26, 2014 from http://nbc.jhu.edu/behavioral_tasks/tasks/simple_odor_recognition_protocol.html

Sobin, C., et al., 2011. A Bland-Altman comparison of the Lead Care(R) System and inductively coupled plasma mass spectrometry for detecting low-level lead in child whole blood samples. J. Med. Toxicol. 7, 24–32.

Sobin, C., Flores, M.G., Parisi, N., Schaub, T., Cervantes, M., Armijos, R.X.M., 2013. Microglial disruption in young mice with early chronic exposure to lead. Toxicol. Letters 220, 44–52.

Sobin, C., Flores-Montoya, M.G., Gutierrez, M., Parisi, N., Schaub, T., 2015. Δ Aminolevulinic acid dehydratase single nucleotide polymorphism 2 (ALAD2) and peptide transporter 2*2 haplotype (hPEPT2*2) differently influence neurobehavior in low-level lead exposed children. Neurotoxicol. Teratol. 47, 137–145.

Surkan, P.J., Zhang, A., Trachtenberg, F., Daniel, D.B., McKinlay, S., Bellinger, D.C., 2007. Neuropsychological function in children with blood lead levels <10 [mu]g/Dl. Neurotoxicology 28, 1170–1177.

Wasserman, G., Liu, X., Popovac, D., Factor-Litvak, P., Kline, J.K., Waternaux, C., Graziano, J.H., 2000. The Yugoslavia Prospective Lead Study: contributions of prenatal and postnatal lead exposure to early intelligence. Neurotoxicol. Teratol. 22, 811–818.

Winters, B.D., Bussey, T.J., 2005. Transient inactivation of perirhinal cortex disrupts encoding retrieval, and consolidation of object recognition memory. J. Neurosci. 25, 52–61.

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in an object-in-place task in pre-adolescent C57BL/6J mice

Christina Sobin [a,b,c,*], Mayra Gisel Flores-Montoya [b,d], Juan Manuel Alvarez [a,b]

[a] Department of Public Health Sciences, College of Health Sciences, University of Texas, El Paso, USA
[b] Border Biomedical Research Center, Toxicology Core, University of Texas, El Paso, USA
[c] Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY, USA
[d] Department of Psychology, University of Texas, El Paso, USA

ARTICLE INFO

Article history:
Received 16 July 2016
Received in revised form 10 January 2017
Accepted 11 January 2017
Available online xxxx

Keywords:
Developmental lead exposure
Low-level lead exposure
Visual spatial memory
Visual object memory
Mouse behavior

ABSTRACT

The mechanisms by which early chronic low-level lead (Pb) exposure disrupts the developing brain are not yet understood. Rodent models have provided promising results however behavioral tests sensitive to effects at lowest levels of exposure during development are needed. Preadolescent animals (N = 52) exposed to low and higher levels of Pb via lactation from birth to PND 28 completed the Object-in-Place Task of visual spatial and visual object memory retrieval (at PND 28). Generalized linear mixed models were used, controlling for sex and litter as a random effect. As compared with controls, global vertical exploratory behavior (rearing) markedly increased during memory retrieval. The findings suggested that early chronic Pb exposure altered the development of critical exploratory functions needed for learning and survival. Behaviors exhibited in novel spatial and novel object zone perimeters suggested that the Object-in-Place task is a valid measure of visual spatial and visual object memory in pre-adolescent C57BL/6J mice. Additional studies are needed to understand how early chronic low-level lead exposure disrupts the trajectory and possible linkages of critical exploratory and perceptual systems during development.

1. Introduction

It is widely recognized that no level of lead (Pb) exposure is safe for the developing brain. Over fifty child clinical studies have provided evidence that chronic developmental exposure to Pb yielding blood Pb levels (BLLs) well below established thresholds, altered neurocognitive function, and specifically memory (Chiodo et al., 2004; Lanphear et al., 2000; Min et al., 2007; Sobin et al., 2015). Recent studies have suggested that as many as 50% of children living in lowest income neighborhoods may be chronically exposed to Pb yielding BLLs between, for example, 3 and 7 μg/dL (Sobin et al., 2009, 2011). How and why early chronic low-level Pb diminishes cognitive function is not yet understood. Source removal continues to be the only approach to prevention and can be difficult to accomplish at lowest levels of exposure, as demonstrated most recently in Flint, Michigan. Animal models are needed to guide new approaches for primary prevention and secondary intervention, and for those models, neurobehavioral tests specifically sensitive to early chronic lowest-level Pb exposure must be identified.

Relatively few animal studies have attempted to model the neurobehavioral effects of early chronic exposure yielding low BLLs similar to those reported in large numbers of low-income minority child populations (Sobin et al., 2011, 2015); even fewer have examined effects of early chronic low-level Pb exposure in preadolescent animals. For mechanistic developmental models it is necessary to characterize cognitive effects during development. Previously, we have examined performance in low-level Pb exposed preadolescent mice of ambulatory exploration (un-baited nose poke task) and two-choice odor recognition memory. In preadolescent C57BL6J mice chronically exposed to 30 ppm or 430 ppm Pb in dam's drinking water and yielding BLLs between approximately 2.5 and 15 μg/dL, both tests were shown to be sensitive to the effects of low-level Pb exposure (Flores-Montoya and Sobin, 2014; Flores-Montoya et al., 2015). More studies that measure behavioral differences at pre-adolescent developmental time points are needed to understand early effects in chronically exposed children and eventually, to identify mechanisms of action for novel early interventions.

The cognitive effects of low-level Pb exposure observed in young children coupled with knowledge regarding brain regions vulnerable to Pb exposure, guides the selection of tests for animal behavior models. A region central to the development of early cognitive function is the

* Corresponding author at: University of Texas, El Paso, 500 West University, El Paso, TX 79902, USA.
E-mail address: casobin@utep.edu (C. Sobin).

http://dx.doi.org/10.1016/j.ntt.2017.01.002
0892-0362/

dentate gyrus (DG) area of the hippocampus. With direct input from entorhinal cortex (Broadbent et al., 2004) and pathway connections to visual and other perceptual brain regions, segregated pathways in the DG (Kesner, 2007) are responsible for visual spatial (Burgess et al., 2002) and visual object (Eichenbaum et al., 2007) recognition memory. (Brain studies have begun to suggest specific mechanisms that could account for DG changes following chronic low-level Pb exposure (Liu et al., 2012; Sobin et al., 2013).) In C57BL6 mice, studies have suggested that major maturational events in neuronal differentiation and synapse formation occur between birth and PND 30 (Mody et al., 2001).

Developmental disruption of the DG may have multiple implications for early cognition and memory processes as well as brain health throughout the lifespan. Animal behavior studies of early chronic low-level Pb exposure have tested memory in adult rodents. For example, one study of BALB/c mice chronically exposed dams and pups to 20 ppm of Pb acetate delivered in dams' drinking water from gestational day 8 to post-natal day (PND) 21, and reported visual spatial memory deficits (Morris Water Maze) in adulthood (Kasten-Jolly et al., 2012). A second study of Wistar rats chronically exposed to 20 ppm Pb acetate delivered in drinking water for 90 days in early adulthood showed that low-level Pb exposure diminished object recognition memory as measured by a two-choice object paradigm (Azzaoui et al., 2009).

The construct of memory encompasses a complex process that in its simplest conceptualization includes three stages including acquisition, storage and retrieval. Different versions of the Object-in-Place (OIP) task have been used; the version reported by Di Viti et al. (2010) provides a single paradigm in which to assess spatial and object visual memory retrieval. In this version of the OIP task, acquisition and storage of spatial location or object information are accomplished during repeated learning trials. During the test trial memory retrieval is challenged by changing the location of familiar objects (first test trial) and by introducing a novel object (second test trial). Behaviors exhibited during the pretest and test trials are compared to assess behavioral changes in response to the spatial or object novelty.

The lack of cognitive tests validated for use in young rodents is a barrier to understanding early effects of Pb exposure in children. Valid cognitive tests for preadolescent animals can provide a means to quantify time-specific effects from toxicant exposure in juvenile animals; suggest specific mechanisms of action at particular developmental time points; and can provide the basis for characterizing a changing trajectory of effects throughout development in toxicant exposed animals. To the best of our knowledge, this version of the OIP task (Di Viti et al., 2010) has not been previously tested in pre-adolescent mice, with or without Pb exposure, thus these studies can also provide first evidence of whether this task is a valid test of memory retrieval during development.

The current study examined whether early chronic low-level Pb exposure altered visual spatial and/or visual object memory retrieval in pre-adolescent C57BL/6J mice. We predicted that, as compared to controls, animals with early chronic low- and higher-level Pb exposure would have diminished object and spatial memory retrieval, and that the effects would differ by dose.

2. Methods

2.1. Animals

The animal procedures used in this study were conducted in compliance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (2011) and were approved by the Institutional Animal Care and Use Committee (IACUC). C57BL/6J mice were purchased from Jackson Laboratories and housed in the Bioscience Research Facility at the University of Texas at El Paso (UTEP). Male and female mice were group-housed by sex in ventilated cages (22.22 cm × 36.83 cm × 13.97 cm) and with ad libitum access to food and water; with room temperature of 20–26 °C, relative humidity of approximately 30%, and a 12 hour light-dark cycle.

Dams were mated by harem breeding (two females per male) at PND 50–55. Female mice were checked daily. After detection of vaginal plug, females were moved to separate cages. Ten dams mated with five sires yielded nine impregnated dams and nine healthy litters, of 4 to 7 pups per litter, N = 52 (28 females/24 males). Litter stress was considered a potential confound for these studies and for this reason unculled litters were planned. Three litters per treatment were randomly assigned to either 0 ppm (controls, n = 18, 8 females; 10 males); 30 ppm (low-dose, n = 16, 10 females and 6 males); or 430 ppm (high-dose, n = 18, 10 females and 8 males). Litter-based analytic methods were used to control for litter variability; sex and litter (as a random effect) were controlled in all statistical models. All mice completed the study.

2.2. Pb exposure

Pb exposure was accomplished through treatment of cage water bottles with 30 ppm or 430 ppm 99.4% Pb acetate crystals (Sigma Aldrich, St. Louis, MO) dissolved with glacial acetic acid; controls were given water treated only with small amounts of glacial acetic acid (Flores-Montoya and Sobin, 2014; Flores-Montoya et al., 2015). Dams and pups were exposed from PND 0 to testing at PND 28.

2.3. Behavioral testing

At PND 28, pups completed the OIP task (Di Viti et al., 2010). The original task required 60 min per animal for completion. For the present study, the task was modified to 40 min per animal to reduce possible testing fatigue in young mice and increased testing efficiency of the pup sample. The OIP task included a spatial and an object recognition memory challenge test. The spatial memory test trial in this version of the OIP followed the methods of Di Viti et al. (2010) and altered the spatial environment in two ways, by moving a familiar object to a different familiar location, and by moving a familiar object to a novel location, providing two types of spatial novelty for comparison in Pb exposed and control animals. (The assumptions and interpretation of this spatial memory task are considered in Discussion.) The object memory test trial replaced a familiar object with a novel object in the same familiar location.

Testing was conducted in a round Plexiglas arena (70 cm × 29 cm; area = 3848 cm^2). An external orienting cue consisted of a 38 × 30 cm cardboard rectangle marked with vertical black and white stripes and was attached to the northwest external side of the arena. The positioning of the arena in the testing room remained unchanged throughout the duration of the testing period ensuring that other possible external cues and lighting sources were identical throughout all testing trials. All testing was conducted during the same range of daytime hours (10 am to 2 pm) to control for possible time of day effects.

For calculating zone-based behavior using discrimination ratios, the boundaries of six exploration zones were demarcated using the SMART software system (Harvard PanLab Systems, Cambridge, MA) for five object locations plus one novel location and included the area within 2 cm from the perimeter of each object (total area occupied by all objects including the 2 cm object zone was 552 cm^2, or approximately 14% of the total arena area). Mouse behavior was recorded via a digital image tracking system fixed above the center of the arena. Session recordings were later processed for analysis using the SMART software system.

Each mouse completed a total of 7 trials each of 4 min duration, separated by 2 min inter-trial intervals. During the inter-trial intervals animals were placed in individual cages and the arena and testing objects were cleaned. The complete task is depicted in Fig. 1.

Mice were habituated to the empty arena for 10 minute periods on the three consecutive days preceding testing. On Day 4 Trial 1, the mouse was placed in the empty Plexiglas arena and allowed to re-

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

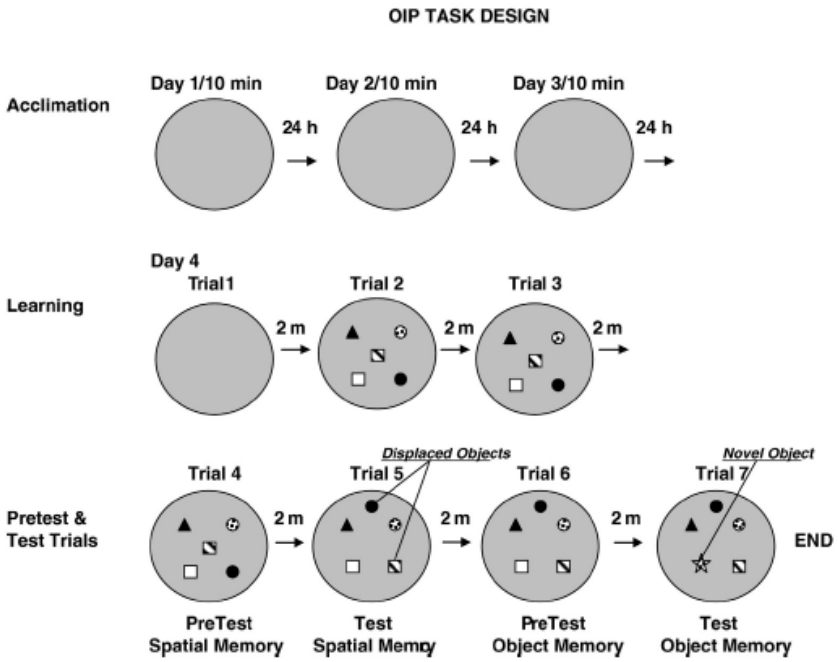


Fig. 1. OIP task design. Legend: Behavior was automatically recorded using the Harvard PanLab SMART Digital Video Tracking System. Global behavior and discrimination ratios were analyzed and compared for Trials 4 and 5, and for Trials 6 and 7. "Global behavior" was behavior that occurred anywhere in the arena during a given trial and included distance traveled, immobile time, and rearing. The "discrimination ratio" (DR) quantified the amount of a given behavior occurring within a 2 cm perimeter "zone of interest" relative to all zones (Trial 5, displaced object zones; Trial 7, novel object zone). Total area of the perimeter zones (only) included 8% of the total area of the arena (309 cm^2/3848 cm^2). DRs were calculated for perimeter zone entries, time in zone, immobile time in zone and rearing in zone.

acclimate to the space. During the first inter-trial interval, the researcher placed five objects within the demarcated zones. In Trials 2, 3, and 4 (the acquisition trials for the spatial memory challenge) the mouse was returned to the arena and allowed to explore the five objects. During the inter-trial interval following Trial 4, two of the five familiar objects were switched to new locations, resulting in one familiar object in a new familiar location, and one familiar object in a novel location, as illustrated in Fig. 1. Trial 5 was the spatial memory test trial. During this trial, if memory was intact, it was predicted that the mouse would spend a greater amount of time and exhibit more behaviors in the spatial location zones containing switched familiar objects.

In Trial 6, the learning trial for the object recognition memory test, objects remained in the same locations as in Trial 5. During the inter-trial interval following Trial 6, one novel object was placed in a familiar location. During Trial 7 object recognition memory was tested. During this trial, if memory was intact, it was predicted that the mouse would spend a greater amount of time or exhibit more behaviors in the zone where the novel object was placed.

Six behavioral outcome variables were quantified. Three variables quantified global behavior exhibited anywhere in the arena during a given pretest or test trial and included global distance traveled (cm), global immobile time (s) and global rearing (number).

Three variables quantified the ratio of behavior exhibited within "perimeter zone(s) of interest," that is, behavior exhibited within the 2 cm perimeter zone of an object in a novel spatial location (spatial location containing a displaced object), or the 2 cm perimeter zone of the novel object, for a given pretest and test trial. The total arena area occupied by (only) the 2 cm object perimeter zones was 8% (309 cm^2/3848 cm^2).

Discrimination ratios (DRs) were calculated for zone entries (number), total time in zone (s), and total rearing in zone (number). The DR formula (Di Viti et al., 2010) for the pretest and test trial of spatial memory retrieval was [displaced object zone behavior / displaced object zone behavior + non-displaced object zone behavior]. The DR formula for the pretest and test trial of object memory retrieval was [novel object zone behavior / novel object zone behavior + familiar object zone behavior]. Thus, for spatial memory retrieval DR ratios larger than 0.40 (2 zones/5 zones) indicated more time or behaviors displayed in the displaced object zones as compared to all zones. For object memory retrieval, DR ratios larger than 0.20 (1 zone/5 zones) indicated more time or behaviors displayed in the novel object zone as compared to all zones. For analyses comparing whether animals showed preference for the familiar object displaced to a different familiar location, or for the familiar object displaced to a novel location during the spatial memory test, DRs were calculated separately for each of type of spatial dislocation using the following formulas [displaced object familiar zone behavior / displaced object familiar zone behavior + non-displaced object zone behavior] and [displaced object novel zone behavior / displaced object novel zone behavior + non-displaced object zone behavior].

2.4. Statistical analyses

Data were analyzed with *SAS Version 9.3* for regression analyses (SAS Institute Inc., Cary, North Carolina) and *SPSS Version 23* for outlier analysis (IBM, Armonk, New York). Data were entered, checked for accuracy, and examined by group for missing values and distribution properties. The general hypothesis for this study was that Pb exposure diminished visual spatial memory retrieval and object memory retrieval at

preadolescence. Hypotheses for specific outcome variables were tested by comparing performance in the pretest and test trial (within subjects factor) by group (between subjects factor), controlling for sex with litter included as a random effect (Holson and Pearce, 1992). Generalized linear mixed model analyses were used (SAS GLIMMIX procedure). In each model, distributions were specified according to the outcome variable including Gaussian for continuous outcomes with identify link function; Poisson for count data with log link function. Fixed effects included trial (class), group (class), sex (class), and litter (class) as a random effect. All models tested the fixed effects trial, group, sex and the trial × group interaction. If sex was significant, the model was re-calculated to include the three-way interaction (trial × group × sex) (two of twelve models).

Type III sum of squares were requested for all models. Group and trial were coded such that the significance of parameter estimates for fixed effect solutions (Tables 4 and 5) tested the estimate difference from the pretest trial, and/or control group. As needed, additional post-hoc least square mean Tukey-Kramer corrected contrasts were calculated as needed to further understand significant effects. Alpha criterion (p) was ≤0.01. Significant effects for trial would indicate that all animals exhibited more time or more of a given behavior in the zone(s) of interest during the test as compared to pretest trial. Significant effects for group would indicate that Pb treated animals differed significantly from controls (regardless of trial). Significant interaction effects of group × trial would indicate that visual memory retrieval of Pb exposed animals (as measured by global behavior and/or DR) differed significantly from controls, and between test and pretest trial.

Litter was controlled for as a random effect in all models. While not the focus of the study, parameter estimate solutions for litter were calculated to determine whether litter significantly contributed to the variability of models. Litter sizes were unbalanced and litter was not nested within group. We also note that models were not re-calculated according to whether litter was found to contribute significant amounts of variance that is, litter as a random effect was not excluded from any model. Similarly, because testing the interaction term was the primary aim of the analyses, when the interaction term was not significant, we did not re-fit models with the interaction term removed.

All models converged. Significance was examined by evaluating the fixed effect Type III F-values for the main effects (group, trial) and the interaction (group × trial) controlling for sex and litter, and the parameter solutions for fixed effects and associated significance tests. The fixed effect parameter solutions given in Tables 4 and 5 represent the parameter estimate difference between the indicated category and the comparison group. For example, in Table 4 the DR zone entries parameter solution for the Test trial is 0.10, meaning that the DR zone entries ratio is estimated to be 0.10 greater than the value of the Pretest trial estimate, which is the intercept, in this case 0.42. The t-value (1.78) indicates whether the amount of difference was significantly greater than zero. In this case, it was not ($p = 0.079$, Table 4).

When sex was found to contribute significantly to a given model (in two of twelve models tested), the three way interaction (group × trial × sex) was added and the model was re-calculated (the two-way interaction was retained in models with the added three-way interaction). In addition to the model results provided in Tables 4 and 5, bar charts illustrate the significant three-way interactions (Figs. 2 and 3).

The object-in-place task used for this study tested spatial memory using two types of spatial displacement, that is, a familiar object moved to a different familiar location (DOF), and a familiar object moved to a novel location (DON). Using generalized linear regression models we calculated and compared DRs for DOF vs DON to determine whether groups showed preference for either type of spatial displacement. Also, to further probe the results showing increased global rearing, in exploratory analyses we tested the association between global rearing and time in zone DR controlling for group and sex, with litter as a random effect.

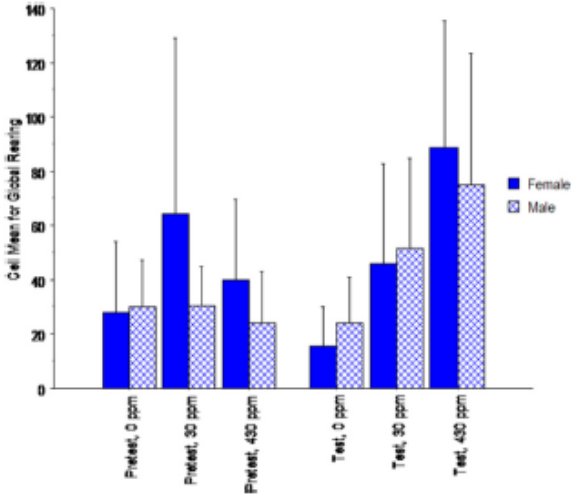


Fig. 2. Bar chart with standard deviations showing the 3-way interaction of trial × group × sex on global rearing during the spatial memory retrieval task (N = 52) (significant comparisons reported in Table 4).

3. Results

No animals died during the course of the study and all animals completed behavioral testing. Table 1 shows the mean BLLs and body weights at PND 28 averaged from four previously published studies using the current model (Flores-Montoya and Sobin, 2014; Flores-Montoya et al., 2015; Sobin et al., 2013; Basgen and Sobin, 2013). Pb exposed and control animals had no observable physical abnormalities. With regard to the random effect of litter, for six of twelve models, variability between litters was not statistically detectable; for the remaining models, the amount of variability contributed by litters was detectable but did not differ significantly from zero. Nonetheless, litter as a random effect was retained in all models for all analyses.

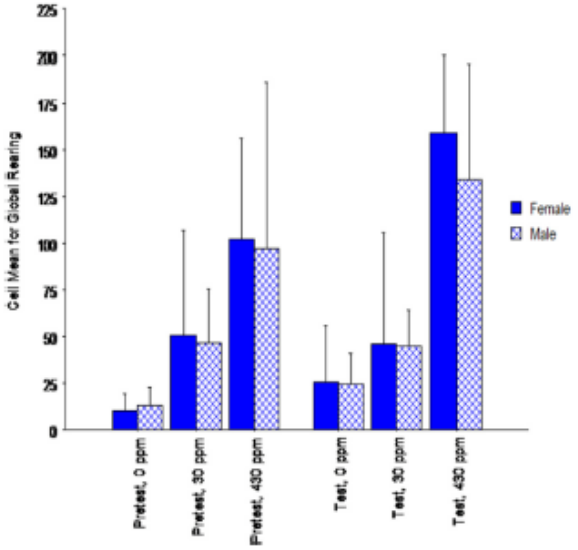


Fig. 3. Bar chart with standard deviations showing the 3-way interaction of trial × group × sex on global rearing during the object memory retrieval task (N = 52) (significant comparisons reported in Table 5).

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

Table 1
Dosing model arithmetic means and standard deviations (SD) for BLL determined by ICP-MS and body weight at PND 28 following chronic exposure to Pb via lactation from birth to PND 28 (N = 159, 91m/68f) averaged from previously published studies from our laboratory (citations given in text).

	BLL (μg/dL)	Body weight (g)
Negative controls n = 51 (0 ppm)	0.2 (0.1)	14.6 (1.1)
Low dose n = 51 (30 ppm)	3.3 (0.7)	13.0 (1.2)
High dose n = 26 (230 ppm)	12.3 (2.6)	11 (1.7)
High dose n = 25 (330 ppm)	14.4 (2.6)	13 (1.6)

3.1. Spatial memory retrieval

Table 2 shows the arithmetic means and standard deviations for global behaviors during the spatial memory pretest and test trials. Differences and changes in global behavior mean values were observed (Table 2). Pretest values of global distance traveled were different for all groups. As compared to controls, total distance traveled by low dose animals was approximately 20% higher than controls; total distance traveled by high dose animals was approximately 20% less than controls. During the test trial, total distance traveled by treated groups was roughly similar to controls (within a range of approximately 8%). Change patterns from pretrial to trial however were qualitatively different across groups. As compared to pretest, during the test trial, controls increased distance traveled (by a relatively small amount). Starting from a higher distance traveled during pretest, during the test trial low dose animals decreased total distance traveled. High dose animals on the other hand started from a lower distance traveled at pretest, and increased distance traveled during the test trial, and the proportional change was approximately double that of controls. For all groups, global immobile time observed was approximately the inverse of patterns observed for total distance traveled.

With regard to global rearing, the amounts and change patterns from pretest to test were markedly different across groups. With regard to pretest rearing levels, controls and high dose animals were roughly equivalent; rearing in low dose animals during pretest was greater than controls and high dose animals by approximately 75%. During the test trial, rearing among controls decreased by approximately 30%; from a relatively high pretest level, rearing in low dose animals decreased by approximately 8%; in high dose animals, from a pretest similar to that of controls, rearing increased by approximately 150%.

Table 2 also shows the DRs for behaviors exhibited in spatial zones of interest (zones containing displaced objects). As shown in Table 2, as compared with the pretest mean DR values, mean DR values during the test trial for all groups increased, and all test trial mean DR values for all groups were >0.40, indicating that behaviors exhibited during the test trial in the zones of interest were above chance levels (explained in Methods). DR test trial increases ranged from 0.09 to 0.22. Observed differences for global behavior and for mean DR values were tested for statistical significance in generalized linear models.

Table 4 shows the fixed effect solutions, parameter estimate differences, and associated tests of significance. With regard to global behavior during spatial memory retrieval, observed differences for total distance and total immobile time were not statistically significant.

For global rearing, in the initial model with one interaction (not shown in Table 4), there were effects for trial ($F = 24.1$, $df_{1,91}$, $p < 0.001$), sex ($F = 93.2$, $df_{1,91}$, $p < 0.001$) and group × trial ($F = 158.3$, $df_{2,91}$, $p < 0.001$). Parameter estimate solution differences were significant for trial, sex and the trial × group interaction. A negative estimate for trial (est = −0.37; SE = 0.07; df_{91}; t = −5.44; $p < 0.001$) reflected a decrease in rearing in test vs pretest trials among controls and low dose animals. With regard to sex, overall rearing among females was significantly higher than rearing in males (diff est = 0.30; SE = 0.03; df_{91}; t = −9.65; $p < 0.001$). With regard to the trial × group interaction, in test as compared to pretest trials and as compared to controls, the amount of rearing was significantly greater among high dose animals (diff est = 1.30; SE = 0.08; df_{91}; t = 15.47; $p < 0.001$) and low dose animals (diff est = 0.30; SE = 0.08; df_{91}; t = 3.52; $p = 0.001$). The effect of group in the overall model was not statistically significant, which is consistent with the differing patterns of group effects described on page 12 above.

Given the significant effect of sex, the model for global rearing was re-calculated including the three-way interaction (trial × group × sex). The full model results are shown in Table 4 and the three-way interaction is illustrated in Fig. 2. For total rearing during spatial memory retrieval, effects included trial ($F = 40.08$, $df_{1,86}$, $p < 0.001$); sex ($F = 36.97$, $df_{1,86}$, $p < 0.001$), the trial × group interaction ($F = 142.08$, $df_{2,86}$, $p < 0.001$), and the trial × group × sex interaction ($F = 37.08$, $df_{5,86}$, $p < 0.001$).

The meaning of the individual effects and two-way interaction are best understood in the context of the significant three-way interaction (complete results provided in Table 4). As the parameter estimates show, during the test trial, females as compared to males had significantly greater rearing among high dose animals (est = 0.56, SE = 0.11) and low dose animals (est = 0.38, SE = 0.12). Among controls however females had significantly less rearing behavior as compared to males (est = −0.36, SE = 0.14). Differences between groups with regard to rearing during the pretest were also tested. During the pretest trial, as compared to males and to controls, females in both Pb exposed

Table 2
Spatial memory retrieval arithmetic means and standard deviations (SD) for global behaviors and DRs during the pre-test and test trials (N = 52).

Group	0 ppm		30 ppm		430 ppm	
	n = 18		n = 16		n = 18	
	Spatial memory pretest Mean ± SD	Spatial memory test Mean ± SD	Spatial memory pretest Mean ± SD	Spatial memory test Mean ± SD	Spatial memory pretest Mean ± SD	Spatial memory test Mean ± SD
Global behavior						
Distance (cm)	159 ± 46.4	171 ± 41.1	189 ± 46.6	179 ± 47.9	135 ± 55.6	156 ± 40.5
Immobile time (s)[b]	68.5 ± 26.4	61.8 ± 22.9	52.3 ± 17.9	59.4 ± 24.3	81.6 ± 33.0	64.3 ± 24.8
Rearing (number)[a,c,d]	29 ± 20	20 ± 16	52 ± 53	48 ± 35	33 ± 26	83 ± 47
DR						
Zone entries[a]	0.43 ± 0.19	0.52 ± 0.16	0.47 ± 0.08	0.57 ± 0.11	0.42 ± 0.23	0.58 ± 0.14
Time in zone (s)[a]	0.48 ± 0.20	0.63 ± 0.15	0.44 ± 0.16	0.57 ± 0.14	0.36 ± 0.24	0.57 ± 0.15
Rearing (number) in zone[a]	0.31 ± 0.32	0.53 ± 0.19	0.36 ± 0.24	0.47 ± 0.21	0.39 ± 0.28	0.56 ± 0.21

[a] Effect for trial (pretest, test) (Table 4).
[b] Effect for group (control, low-dose, high-dose) (Table 4).
[c] Effect for sex (male, female).
[d] Interaction effect (group × trial × sex) (Table 4).

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

groups had significantly more rearing behavior (high dose females, est = 0.90, SE = 0.13; low dose females, est = 1.26, SE = 0.13). An additional contrast suggested that during the pretest, the amount of difference in rearing between control males and females did not differ (diff est = 0.24; SE = 0.09; df_{86}, t = 2.60; adj p = 0.299). Thus, global rearing was sensitive to the effects of both levels of Pb exposure, and as compared to controls, rearing was significantly higher during the test trial, particularly in Pb exposed females.

With regard to the DRs, the fixed effect F-values for trial were significant for zone entries (F = 13.6, $df_{1,91}$, $p < 0.001$); time in zone (F = 21.3, $df_{1,91}$, $p < 0.001$); and rearing in zone (F = 12.1, $df_{1,91}$, $p = 0.001$). Full model solutions showed that the time in zone DR (est = 0.15, SE = 0.06) and rearing in zone DR (est = 0.20, SE = 0.07) increased significantly from pretest to test (Table 4); the DR parameter estimate difference for zone entries was not statistically significant (est = 0.10; SE = 0.05).

Thus, with regard to behavior during spatial memory retrieval, global rearing differed significantly across groups, and the three-way interaction revealed marked increases in rearing among Pb exposed animals, with special sensitivity to Pb-induced changes in rearing evident among females. With regard to zone-based DRs, two of three increased significantly from pretest to test trial.

3.2. Object memory retrieval

Table 3 shows the arithmetic means and standard deviations for the global behavior and discrimination ratio (DR) values for the pretest and test trial by group. As shown in Table 3, pretest global behavior mean values and changes patterns varied broadly across groups. With regard to total distance traveled, as compared with controls, pretest distance traveled was approximately 25% greater among low dose animals, and approximately 7% greater among high dose animals. During the test trial, as compared to pretest, distance traveled among controls increased by approximately 10%; among low dose animals, distance traveled decreased by approximately 6%; among high dose animals distance traveled increased by approximately 9%. Thus, controls and high dose animals were similar while low dose animals traveled a greater distance overall and decreased rather than increased travel during the cognitive challenge.

Unlike patterns observed during the spatial memory test, during the object memory test immobile time was not the approximate inverse of distance traveled. Very similar to controls, immobile time differences among low dose animals changed very little in the test as compared to pretest trial. Among high dose animals however, immobile time decreased by approximately 25% during the test trial.

With regard to rearing, broad differences in pretest and test levels, and changes from pretest to test were observed. During the pretest, as compared to controls, rearing among low dose animals was approximately 4-fold greater (400%), and rearing among high dose animals was approximately 10-fold greater (1000%). Very similar to rearing patterns observed during the spatial memory test, during the test trial, from a relatively high pretest level, rearing decreased slightly among low dose animals; among high dose animals, from a very high pretest level, rearing increased by approximately 50%.

As shown in Table 3, as compared with the pretest mean DR values, all mean DR values during the test trial for all groups increased, and all test trial mean DR values for all groups were > 0.20 indicating that behaviors exhibited during the test trial in the zones of interest were above chance levels. During object memory retrieval, DR test trial increases ranged from 0.22 to 0.50. Observed differences for global behavior and for mean DR values were tested for statistical significance in generalized linear models.

Table 5 shows the fixed effect solutions, and fixed effect parameter estimate differences for global behaviors and DRs. With regard to global behavior during object memory retrieval, observed differences for distance and immobile time were not statistically significant.

For global rearing, in the initial model with only one interaction (not shown in Table 5), there were effects for trial (F = 109.3, $df_{1,91}$, $p < 0.001$); group (F = 13.26, $df_{2,91}$, $p < 0.001$); sex (F = 30.5, $df_{1,91}$, $p < 0.001$); and group × trial (F = 45.8, $df_{2,91}$, $p < 0.001$). Fixed effect parameter solution estimate differences were significant for all of these effects. Rearing during the test trial as compared to pretest trial was increased (est = 0.76, SE = 0.08; df_{91}, t = 9.07, $p < 0.001$); as compared to controls, rearing in Pb exposed animals was increased (high dose animals, est = 2.20, SE = 0.40; df_{91}, t = 5.54, $p < 0.001$; low dose animals, est = 1.27, SE = 0.40, df_{91}, t = 3.18, $p = 0.002$); overall, as compared to males rearing in females was greater (est = 0.14, SE = 0.03, df_{91}; t = 5.52; $p < 0.001$); and the estimates for the interaction suggested that during the test trial as compared to controls, rearing was greater among high dose animals (est = 0.37, SE = 0.09, df_{91}, t = 4.15, $p < 0.001$); and low dose animals (est = 0.83, SE = 0.10, df_{91}, t = 8.49, $p < 0.001$). Given the significant effect of sex, this model was recalculated to include the three-way interaction (trial × group × sex, with the two-way interaction retained in the model).

The full model results are shown in Table 5 and the three-way interaction is also illustrated in Fig. 3. For total rearing during object memory retrieval, effects included trial (F = 107.83, $df_{1,86}$, $p < 0.001$); group (F = 12.13, $df_{2,86}$, $p < 0.001$); the trial × group interaction (F = 42.88, $df_{2,86}$, $p < 0.001$); and the trial × group × sex interaction (F = 6.96, $df_{5,86}$, $p < 0.001$). The meaning of the individual effects is best understood in the context of the significant three-way interaction (complete results provided in Table 5). As the parameter solution estimate differences for the three-way interaction show, during the test trial, as compared to males, rearing was greater in high dose females (est = 0.68,

Table 3
Object memory retrieval arithmetic means and standard deviations (SD) for global behaviors and DRs during the pre-test and test trials (N = 52).

Group	0 ppm		30 ppm		430 ppm	
	n = 18		n = 16		n = 18	
	Object memory pretest Mean ± SD	Object memory test Mean ± SD	Object memory pretest Mean ± SD	Object memory test Mean ± SD	Object memory pretest Mean ± SD	Object memory test Mean ± SD
Global behavior						
Distance traveled (cm)	138 ± 47.0	151 ± 42.4	172 ± 48.1	161 ± 52.6	147 ± 55.8	160 ± 56.1
Immobile time (s)	19.0 ± 9.3	18.5 ± 7.4	18.7 ± 9.0	21.9 ± 15.1	35.6 ± 26.3	26.3 ± 21.1
Rearing (number)[a,b,c,d]	12 ± 9.4	25 ± 22.8	49 ± 46.8	46 ± 47.4	100 ± 69.2	148 ± 51.4
DR						
Zone entries[a]	0.13 ± 0.11	0.42 ± 0.19	0.10 ± 0.06	0.38 ± 0.18	0.10 ± 0.08	0.36 ± 0.19
Time in zone (s)[a]	0.12 ± 0.14	0.49 ± 0.21	0.09 ± 0.08	0.42 ± 0.24	0.10 ± 0.11	0.37 ± 0.22
Rearing (number) in zone[a]	0.24 ± 0.30	0.74 ± 0.24	0.09 ± 0.11	0.44 ± 0.25	0.11 ± 0.09	0.33 ± 0.27

[a] Effect for trial (pretest, test) (Table 5).
[b] Effect for group (control, low-dose, high-dose) (Table 5).
[c] Effect for sex (male, female) (Table 5).
[d] Interaction effect (group × trial × sex) (Table 5).

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

Table 4
Effects on behavior during spatial memory retrieval in C57BL/6J mice and parameter estimate solutions for fixed effects (N = 52).

	df	F	p	Solutions for fixed effects					
					Est	SE	df	t	p
Global behavior									
Distance (cm)		n.s.							
Immobile time (s)		n.s.							
Rearing (number)				Intercept	3.35	0.37			
Trial	1,86	40.08	< 0.001	Test	−0.23	0.09	86	−2.70	0.008
				Pretest	0	.	.	.	.
Grp	2,86	1.22	(0.300)						
Sex	1,86	36.97	< 0.001	Females	−0.24	0.09	86	−2.60	0.011
				Males	0	.	.	.	.
Trial × group	2,86	142.08	< 0.001	Test/430 ppm	1.38	0.12	86	11.49	< 0.001
				Test/30 ppm	0.77	0.12	86	6.02	< 0.001
				Test/Ctrl	0	.	.	.	.
				Pretest/all groups	0	.	.	.	.
Trial × group × sex	5,86	37.08	< 0.001	Test/430 ppm females	0.56	0.11	86	5.24	< 0.001
				Test/430 ppm males	0	.	.	.	.
				Test/30 ppm females	0.38	0.12	86	3.24	0.002
				Test/30 ppm males	0	.	.	.	.
				Test/Ctrl females	−0.36	0.14	86	−2.53	0.013
				Test/Ctrl males	0	.	.	.	.
				Pretest/430 ppm females	0.90	0.13	86	7.06	< 0.001
				Pretest/430 males	0	.	.	.	.
				Pretest/30 ppm females	1.26	0.13	86	10.04	< 0.001
				Pretest/30 ppm males	0	.	.	.	.
				Pretest/Ctrl females	0	.	.	.	.
				Pretest/Ctrl males	0	.	.	.	.
DR									
Entries				Intercept	0.42	0.04			
Trial	1,91	13.6	< 0.001	Test	0.10	0.05	91	1.78	0.079
				Pretest	0	.	.	.	.
Time				Intercept	0.51	0.05			
Trial	1,91	21.3	< 0.001	Test	0.15	0.06	91	2.52	0.014
				Pretest	0	.	.	.	.
Rearing				Intercept	0.37	0.07			
Trial	1,91	12.1	0.001	Test	0.20	0.07	91	2.73	0.008
				Pretest	0	.	.	.	.

SE = 0.15); and in low dose females (est = 0.70, SE = 0.16). Among controls however rearing in males and females did not differ (est = 0.29, SE = 0.17).

Differences between groups by sex during the pretest were also tested. During the pretest trial, as compared to males and controls, Pb exposed females had significantly more rearing behavior (high dose animals, est = 0.55, SE = 0.15; low dose animals, est = 0.74, SE = 0.16). An additional contrast comparing male and female controls during the pretest was not significant (diff est = −0.46; SE = 0.14; df_{86}, t = −3.18; adj p = 0.081). These relationships are illustrated in Fig. 3.

With regard to the DRs, trial was significant for zone entries (F = 104.74, $df_{1,91}$, $p < 0.001$); time in zone (F = 85.84, $df_{1,91}$, $p < 0.001$); and rearing in zone (F = 56.79, $df_{1,91}$, $p < 0.001$). The group effect for rearing in zone was also significant (F = 5.41, $df_{2,91}$, p = 0.006). Full model parameter solutions for fixed effects showed that the DR parameter estimates for zone entries (est = 0.30, SE = 0.05), time in zone (est = 0.37, SE = 0.06) and rearing in zone (est = 0.45, SE = 0.07) were statistically significant (Table 5). The fixed effect parameter solutions for the group effect were not statistically significant (Table 5).

3.3. Secondary analyses

3.3.1. Do juvenile mice show preference for displaced familiar object in a familiar location vs displaced object in a novel location?

The version of the OIP task used in this study provided the opportunity to test spatial memory for two types of spatial change (see Methods). Prior to conducting the main analyses described above, DRs were calculated separately for the familiar location displaced object zone (DOF) and novel location displaced object zone (DON), and models were tested to determine whether the combined DR for zone entries, time in zone, or rearing in zone was predicted by dislocation type (DOF/DON), controlling for group, sex, or the dislocation type × group interaction. All models included litter as a random effect.

No significant effects were observed for zone entries DR or rearing DR. For time in zone DR, significant effects were found for DOF/DON (F = 13.98, $df_{1,91}$, $p < 0.001$), and the DOF/DON × group interaction (F = 6.65, $df_{1,91}$, p = 0.002) (neither group nor sex were significant predictors in this model). The fixed effect parameter solution for DOF/DON suggested overall preference for the familiar object in the novel location (est = 0.23, SE = 0.06, df_{91}, t = 4.04, $p < 0.001$), while the interaction revealed that among high dose males there was preference for the familiar object in the different familiar location (est = 0.28, SE = 0.08, df_{91}, t = 3.40, p = 0.001).

3.3.2. Might global rearing predict memory retrieval performance (DR)?

Main results revealed substantially altered vertical exploratory behavior (global rearing) distal to the altered spatial and object zones in Pb exposed animals; no group or group × trial effects for DR values were found. These results raised questions regarding whether and how increased vertical exploration might be related to memory retrieval processes among Pb exposed animals. For example, significant associations could suggest that global rearing somehow influenced, either positively or negatively, the amount of time an animal spent in areas proximal to the displaced objects or novel object. To explore this possibility, single-order correlations were examined (Table 6); to limit the total number of additional analyses, we narrowed these exploratory analyses to only the association between global rearing and time in zone DR. Three of four correlations from the spatial memory test trial were negative, that is, the greater the global rearing, the lower the time in zone DR. In contrast, during the object memory test trial, three

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

Table 5
Effects on behavior during object memory retrieval in C57BL/6J mice and parameter estimate solutions for fixed effects (N = 52).

	df	F	p	Solutions for fixed effects					
					Est	SE	df	t	p
Global behavior									
Distance (cm)		(n.s.)							
Immobile time (s)		(n.s.)							
Rearing (number)				Intercept	2.56	0.31			
Trial	1,86	107.83	<0.001	Test	0.64	0.11	86	5.83	<0.001
				Pretest	0	.	.	.	.
Grp	2,86	12.13	<0.001	430 ppm	1.97	0.43	86	4.63	<0.001
				30 ppm	0.92	0.43	86	2.14	0.035
				Ctrl	0	.	.	.	.
(Sex	1,86	0.78	0.379)						
Trial × group	2,86	42.88	<0.001	Test/430 ppm	−0.32	0.12	86	−2.68	0.009
				Test/30 ppm	−0.68	0.14	86	−4.93	<0.001
				Test/Ctrl	0	.	.	.	.
				Pretest/all groups	0	.	.	.	.
Trial × group × sex	5,86	6.96	<0.001	Test/430 ppm females	0.68	0.15	86	4.50	<0.001
				Test/430 ppm males	0	.	.	.	.
				Test/30 ppm females	0.70	0.16	86	4.23	<0.001
				Test/30 ppm males	0	.	.	.	.
				Test/Ctrl females	0.29	0.17	86	1.71	0.090
				Test/Ctrl Males	0	.	.	.	.
				Pretest/430 ppm females	0.55	0.15	86	3.63	0.001
				Pretest/430 males	0	.	.	.	.
				Pretest/30 ppm females	0.74	0.16	86	4.51	<0.001
				Pretest/30 ppm males	0	.	.	.	.
				Pretest/Ctrl females	0	.	.	.	.
				Pretest/Ctrl males	0	.	.	.	.
DR									
Entries				Intercept	0.09	0.04			
Trial	1,91	104.74	<0.001	Test	0.30	0.05	91	6.46	<0.001
				Pretest	0	.	.	.	.
Time				Intercept	0.08	0.05			
Trial	1,91	85.84	<0.001	Test	0.37	0.06	91	6.41	<0.001
				Pretest	0	.	.	.	.
Rearing				Intercept	0.23	0.07			
Trial	1,91	56.79	<0.001	Test	0.45	0.07	91	6.08	<0.001
				Pretest	0	.	.	.	.
Group	2,91	5.41	0.006	430 ppm	−0.24	0.10	91	−2.35	0.021
				30 ppm	−0.12	0.11	91	−1.09	0.028
				Ctrl	0	.	.	.	.

of four correlations were positive (the fourth was positive but near zero), that is, as global rearing increased, time in zone DR increased, suggesting that rearing distal to the novel object may have facilitated exploration of the novel object.

In generalized linear models controlling for sex, group and litter as a random effect the association between time in zone DR and global rearing was modeled and included the two-way interaction DR × sex, and the three-way interaction DR × sex × group (Table 7). In both models, time in zone DR was significantly associated with global rearing but in opposite directions, as suggested by the single order correlations.

For the spatial memory test trial, the parameter estimate solution for DR was negative, that is, during the spatial memory challenge, as global rearing increased, DR decreased (est = −0.91, SE = 0.38, df_{23}, t = −2.38, $p = 0.026$). There were also significant effects for sex (est = −0.64, SE = 0.19, df_{23}, t = −3.47, $p = 0.002$) and the DR × sex interaction which indicated that the DR association with global rearing was significantly higher in females (est = 1.03, SE = 0.35, df_{23}, t = 2.99, $p = 0.007$). The three-way interaction was not significant.

Table 6
Single order correlations between global rearing and time in zone DR during the spatial and object memory test trials in Pb exposed animals (N = 34).

	Spatial memory		Object memory	
	Females	Males	Females	Males
30 ppm	−0.27	0.41	0.67	0.44
430 ppm	−0.18	−0.73	0.50	(0.07)

For the object memory test trial, the parameter estimate solution for DR was positive, that is, during the object memory challenge, as global rearing increased, DR increased (est = 0.59, SE = 0.22, df_{23}, t = 2.71, $p = 0.12$). There were also significant effects for sex, group, and both interactions. The two- and three-way interactions together suggested that the association was strongest in low dose females.

4. Discussion

The goal of this study was to examine whether early chronic low-level Pb exposure altered visual memory retrieval in preadolescent mice. Because few studies have examined behavioral effects at pre-adult time points in Pb exposed animals, secondary analyses were also conducted to generate hypotheses for future studies. The low-level Pb dosage administered in this study (30 ppm) has been shown to produce blood Pb levels in young mice between approximately 2.0 and 5.0 μg/dL (Flores-Montoya and Sobin, 2014; Flores-Montoya et al., 2015), that is, levels that overlap those measured in approximately 50% of children living in some low-income neighborhoods (Sobin et al., 2011, 2015). To the best of our knowledge, this is the first study to examine visual spatial and visual object memory retrieval deficits in juvenile animals following early chronic low-level Pb exposure. For the OIP paradigm used in this study, in addition to behaviors occurring within the 2 cm perimeter of objects, which represented approximately 8% of the arena space, global behaviors were also measured.

Previous studies of memory in adult rodents with a history of early chronic low-level Pb exposure have shown visual spatial and visual

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

Table 7
Tests of association between time in zone DR and global rearing during test trials in Pb exposed animals controlling for sex and group with litter as a random effect (N = 34).

	df	F	p	Solutions for fixed effects					
					Est	SE	df	t	p
Spatial memory retrieval				Intercept	*4.20*	*0.37*			
DR time in zone	1,23	8.63	0.007		−0.91	0.38	23	−2.38	0.026
Sex	1,23	12.07	0.002	Female	−0.64	0.19	23	−3.47	0.002
				Male	0	.	.	.	.
Group	1,23	2.82	n.s.						
DR × Sex	1,23	20.08	<0.001	Female	1.03	0.35	23	2.99	0.007
				Male	0	.	.	.	.
DR × Sex × Group	2,23	15.38	<0.001	Female/430 ppm	0.12	0.39	23	0.31	n.s.
				Female/30 ppm	0	.	.	.	.
				Male/430 ppm	−0.83	0.39	23	−2.14	0.043
				Male/30 ppm	0	.	.	.	.
Object memory retrieval				Intercept	*3.19*	*0.28*			
DR time in zone	1,23	128.42	<0.001		0.59	0.22	23	2.71	0.012
Sex	1,23	50.16	<0.001	Female	−0.66	0.09	23	−7.08	<0.001
				Male	0	.	.	.	.
Group	1,23	25.53	<0.001	430 ppm	1.90	0.38	23	5.05	<0.001
				30 ppm	0	.	.	.	.
DR × sex	1,23	113.90	<0.001	Female	1.99	0.20	23	9.95	<0.001
				Male	0	.	.	.	.
DR × sex × group	3,23	6.74	0.005	Female/430 ppm	−0.74	0.24	23	−3.11	0.005
				Female/30 ppm	0	.	.	.	.
				Male/430 ppm	−0.83	0.23	23	−3.59	0.002
				Male/30 ppm	0	.	.	.	.

object memory deficits. In this study however, no differences in visual memory behaviors proximal to altered zones were observed, while broad differences in rearing behavior outside of the perimeter zones were found. At the same time, in previous studies of pre-adolescent mice using the same rodent dosing model as that used in the current study, olfactory memory deficits within an odor perimeter zone were found (Flores-Montoya et al., 2015). The combination of findings from developmental Pb exposure rodent studies showing olfactory memory deficits at pre-adolescence, disrupted global exploratory rearing behavior during visual memory challenge with no specific evidence of visual spatial or object memory deficits at pre-adolescence (the current study), and visual spatial and visual object memory deficits in adulthood, together may begin to suggest that effects of early chronic low-level Pb exposure on brain function follow a changing trajectory of deficits that involve early disruption of entorhinal/DG memory pathways, perhaps with later disruption of visual/DG memory pathways. Planned studies are needed to investigate this possibility. Whether development of visual system memory pathways is dependent on adequate stimulation of olfactory system memory pathways could also be examined. The marked sex differences in rearing behavior during the test trials also requires further examination in studies specifically designed to compare males and females.

Tactile, olfactory and visual exploratory behaviors are critical to learning and survival in developing rodents. (In infants and toddlers tactile, motor and sensory exploration is the foundation for higher cognitive development (Wadsworth, 2003).) For rodent models of early chronic Pb exposure, very little data are available regarding the development of central exploratory behaviors. Pb-exposed animals as compared to controls accomplished exploration in novel test trial environments very differently by exhibiting substantially more vertical exploratory activity than controls. Secondary analyses revealed that during the memory retrieval challenges, exploratory vertical behavior in Pb exposed animals was associated with less proximal exploration of the novel spatial arrangements, and greater proximal exploration of the novel object. Thus, in Pb-exposed animals, rearing appeared to disrupt the accomplishment of visual spatial memory retrieval but facilitated the accomplishment of visual object memory retrieval. More broadly speaking, the results suggested the importance of including in the conceptualization of "behavioral effects," not only precisely defined constructs but also characterization of how cognition is accomplished. Understanding how Pb exposure alters the trajectory of critical exploratory processes during development in rodent models may eventually suggest new behavioral indicators of how Pb exposure alters brain development in young children.

In this regard, differences in the patterns of global rearing behavior in Pb exposed as compared to control animals during memory retrieval were especially interesting. Recent work has suggested that rearing is an independent measure of a unique type of exploration associated with hippocampal function in rodents (Lever et al., 2006). During horizontal exploration, rodents have monocular 2D vision; in a hind leg rearing position head movement allows the animal to approximate binocular vision, changing the visual perspective from 2D to 3D. Substantial increases in vertical (rearing) exploration may have suggested that Pb exposure negatively impacted the visual processing system. Additional studies are needed to understand the sources of excessive rearing and whether it indicated perceptual confusion via disruption of the visual system.

In behavioral studies of adult rodents, in paradigms such as the OIP task, moving a familiar object to a different familiar location has been interpreted as a test of object discrimination, rather than a test of spatial memory. This interpretation assumes that spatial memory is context-free. In other words, if moving a familiar object to a different familiar location does not alter perception of and memory for the familiar spatial location, a reasonable interpretation of disproportionate exploration of the displaced familiar object would be that the animal is discriminating the object in the changed (familiar) location. In pre-adolescent mice however, the hippocampal region is still maturing and evidence has not yet been provided to suggest whether a young mouse continues to perceive a familiar location as "familiar" when it contains a different familiar object. In at least one regard, the object change renders the familiar location "novel" and alters the spatial landscape. Animal and human studies have demonstrated how visual memory is often context-dependent (Smith and Vela, 2001; Yeshenko et al., 2004), and little is currently known regarding visual spatial and visual object memory in juvenile mice. If it is not assumed that a young mouse perceives a familiar location with a different familiar object as "familiar," an alternative understanding of the task conditions can be that moving a familiar object to a different familiar location, and moving a familiar object to a novel location, are two ways to spatially manipulate the visual environment (using only familiar objects and familiar locations), and each represents

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

a different type of spatial re-arrangement. Presenting both in the same trial provides the opportunity to examine preference for one versus another type of spatial dislocation in young mice while combining exploration of both provides a measure of spatial memory.

Analyses in this study comparing preference for the familiar object in the different familiar location versus the familiar object in a novel location revealed that overall, animals tended to explore more the familiar object in the novel location, however high dose males had a strong preference for the familiar object in the different familiar location. This finding suggested interesting possibilities to investigate in future studies that could explore, for example, the intersection of spatial and object memory during development, and also how lead exposure may alter the perception of novelty salience, and what this might mean for cognitive deficits in children. Additional studies in pre-adolescent mice are also needed to continue testing different types of spatial and sequential memory.

The OIP task was selected for these studies because it provided a method for testing visual spatial and visual object memory retrieval in a single paradigm. As demonstrated in generalized linear mixed models controlling for sex and litter, discrimination ratios for zone-based exploratory behaviors significantly increased during the test trial. Thus, in response to the changed spatial locations or novel object, animals significantly increased the number of entries into the novel zone, and the time, and number of rears in the novel zone. These significantly increased zone-based behaviors suggested that overall, the animals correctly recognized as novel the changed spatial arrangement of objects, or the changed object. Moreover, the amount of increase for all test trials resulted in DR ratios that exceeded chance levels. In these ways the data suggested that the OIP provided a valid measure of DR-defined visual and object memory retrieval in preadolescent mice.

Two observations of data from the novel spatial as compared with novel object memory retrieval task may have suggested that visual spatial perception (as compared to visual object perception) was less well developed in these preadolescent animals. DR differences between pretest and test trials for spatial memory retrieval were smaller than those observed for object memory retrieval and did not differ by group. This could have suggested that the spatial memory task, while providing the opportunity to compare preferences, had greater complexity and perceptually overwhelmed the mice. Alternatively, this could have suggested that visual spatial memory was not as well developed as object memory at pre-adolescence. Second, as described above, among Pb exposed animals during spatial memory retrieval, excess vertical exploratory behaviors were associated with smaller DR for time in zone. For rodent models of early chronic Pb exposure, further studies of juvenile mice are needed to examine possible differences in the development of spatial and object memory.

Importantly, the findings showed that effects on developmental behaviors following early chronic low-level Pb exposure extended beyond a singularly defined construct. The lack of group effects for DR values in the context of remarkable differences in global rearing behavior raised fundamental questions regarding how best to define "cognitive deficit" for animal and translational models of early chronic low-level Pb exposure. The substantial differences in global behavior of Pb exposed preadolescent animals perhaps suggested major effects on the development of the visual processing system that could be incorporated into a conceptualization of how early chronic low-level Pb exposure alters the brain and thus behavior.

The limitations of these data should also be considered. With regard to the spatial memory task, we used a procedure that for adult animals has been interpreted as a measure of object discrimination rather than spatial memory (familiar object in a different familiar location). This interpretation however assumes spatial memory for location is context-free in juvenile mice. Studies are needed to examine whether spatial memory in juvenile mice is or is not context-dependent, and whether age influences context dependency. Also in this study, one test rather than a battery was used. Behavioral differences during the memory challenge could be attributable to disruptions in, for example, attention, or temporal memory, as well as to deficits in spatial and object memory. We note that the lack of group differences in DR values supports the notion that other cognitive deficits did not directly impact memory as measured by DRs in these young mice. It was also noted that neither total distance traveled nor immobility time differed between groups, suggesting that motor functioning during the pretest and test trials were approximately equivalent across groups. Nonetheless, for future studies, additional tests would undoubtedly help to parse the meaning of observed effects in tests of complex cognitive function such as memory, and whether effects observed might be attributable to other disrupted cognitive processes. Such batteries must balance multiple considerations in juvenile mice, for example, fatigue, order effects, and rapid aging, and the protocol design must ensure that the testing experience itself does not alter brain development and thereby confound results. Rodent studies that measure various functions during development are needed to promote the development of effective testing batteries for juvenile mice.

5. Conclusions

The OIP task is a valid measure of visual spatial and visual object memory retrieval in preadolescent C57BL6J mice. Early chronic low-level Pb exposure markedly altered vertical exploratory behavior during memory retrieval in preadolescent animals with no evidence of spatial or object memory deficit as measured with the OIP task. At the same time, markedly increased rearing appeared to disrupt novel spatial exploration but facilitated novel object exploration. These are among the first findings to report effects in juvenile mice of early chronic low-level Pb exposure and the findings require replication. Whether global rearing was compensation for perceptual confusion or disruptive effects of early chronic Pb exposure on the visual exploratory system require further study. Because previous findings have shown olfactory memory deficits in preadolescent animals with early chronic low-level Pb exposure, and visual spatial and visual object memory deficits in adult rodents with early chronic low-level exposure, we speculated that early chronic low-level Pb exposure may alter an intertwined developmental progression of these critical perceptual systems. Studies are needed to examine possible linkages and discontinuities between olfactory and visual memory systems during development, and how early chronic Pb exposure may disrupt their normal developmental trajectory.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

The authors would like to acknowledge Mari Golub, Environmental Toxicology, UC Davis, for her assistance in the preparation of the final manuscript, and Soyoung Jeon, UT El Paso, for statistical consultation. This research was made possible by grants from the National Institute of Child Health and Human Development (NICHD), National Institutes of Health (R21HD060120, CS, PI); the National Center for Research Resources, a component of the National Institutes of Health (5G12RR008124); the Center for Clinical and Translational Science, The Rockefeller University, New York, New York; the Paso del Norte Health Foundation, El Paso, Texas; and from funding sources at the University of Texas, El Paso including, the Border Biomedical Research Center (BBRC); the University Research Institute (URI); and by funds provided from the J. Edward and Helen M.C. Stem Endowed Professorship in Neuroscience (CS). The funders had no role in the design, collection, data analyses and interpretation, implementation, manuscript preparation for this study, or the decision to submit the article for publication.

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

References

Azzaoui, F.Z., Ahami, A.O., Khadmaoui, A., 2009. Impact of lead sub-chronic toxicity on recognition memory and motor activity of Wistar rat. Pak. J. Biol. Sci. 12, 173–177.

Basgen, J.M., Sobin, C., 2013. Early chronic low-level lead exposure produces glomerular hypertrophy in young C57BL/6J mice. Toxicol. Lett. 225:48–56. http://dx.doi.org/10.1016/j.toxlet.2013.10.11.1031.

Broadbent, N.J., Squire, L.R., Clark, R.E., 2004. Spatial memory, recognition memory, and the hippocampus. Proc. Natl. Acad. Sci. U. S. A. 101, 14515–14520 (Epub 12004 Sep 14527).

Burgess, N., Maguire, E.A., O'Keefe, J., 2002. The human hippocampus and spatial and episodic memory. Neuron 35, 625–641.

Chiodo, L.M., Jacobson, S.W., Jacobson, J.L., 2004. Neurodevelopmental effects of postnatal lead exposure at very low levels. Neurotoxicol. Teratol. 26, 359–371.

Di Viti, S., Martino, A., Musilli, M., Fiorentini, C., Diana, G., 2010. The Rho GTPase activating CNF1 improves associative working memory for object-in-place. Behav. Brain Res. 212:78–83. http://dx.doi.org/10.1016/j.bbr.2010.1003.1049 (Epub 2010 Apr 1011).

Eichenbaum, H., Yonelinas, A.P., Ranganath, C., 2007. The medial temporal lobe and recognition memory. Annu. Rev. Neurosci. 30, 123–152.

Flores-Montoya, M.G., Sobin, C., 2014. Early chronic lead exposure reduces exploratory activity in young C57BL/6J mice. J. Appl. Toxicol. http://dx.doi.org/10.1002/jat.3064 (n/a-n/a).

Flores-Montoya, M.G., Alvarez, J.M., Sobin, C., 2015. Olfactory recognition memory is disrupted in young mice with chronic low-level lead exposure. Toxicol. Lett. 236: 69–74. http://dx.doi.org/10.1016/j.toxlet.2015.04.013.

Guide for the Care and Use of Laboratory Animals: Eighth Edition. The National Academies Press.

Holson, R.R., Pearce, B., 1992. Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. Neurotoxicol. Teratol. 14, 221–228.

Kasten-Jolly, J., Pabello, N., Bolivar, V.J., Lawrence, D.A., 2012. Developmental lead effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. Neurotoxicology 33:1005–1020. http://dx.doi.org/10.1016/j.neuro.2012.1004.1017 (Epub 2012 May 1016).

Kesner, R.P., 2007. A behavioral analysis of dentate gyrus function. Prog. Brain Res. 163, 567–576.

Lanphear, B.P., Dietrich, K., Auinger, P., Cox, C., 2000. Cognitive deficits associated with blood lead concentrations <10 microg/dL in US children and adolescents. Public Health Rep. 115, 521–529.

Lever, C., Burton, S., O'Keefe, J., 2006. Rearing on hind legs, environmental novelty, and the hippocampal formation. Rev. Neurosci. 17, 111–133.

Liu, M.C., et al., 2012. Involvement of microglia activation in the lead induced long-term potentiation impairment. PLoS One 7:e43924. http://dx.doi.org/10.41371/journal.pone.0043924 (Epub 0042012 Aug 0043931).

Min, J.-Y., et al., 2007. Neurobehavioral function in children with low blood lead concentrations. Neurotoxicology 28, 421–425.

Mody, M., et al., 2001. Genome-wide gene expression profiles of the developing mouse hippocampus. Proc. Natl. Acad. Sci. U. S. A. 98, 8862–8867 (Epub 2001 Jul 8863).

Smith, S.M., Vela, E., 2001. Environmental context-dependent memory: a review and meta-analysis. Psychon. Bull. Rev. 8, 203–220.

Sobin, C., Gutierrez, M., Alterio, H., 2009. Polymorphisms of delta-aminolevulinic acid dehydratase (ALAD) and peptide transporter 2 (PEPT2) genes in children with low-level lead exposure. Neurotoxicology 30, 881–887.

Sobin, C., Parisi, N., Schauh, T., Gutierrez, M., Ortega, A.X., 2011. Delta-aminolevulinic acid dehydratase single nucleotide polymorphism 2 and peptide transporter 2*2 haplotype may differentially mediate lead exposure in male children. Arch. Environ. Contam. Toxicol. 61:521–529. http://dx.doi.org/10.1007/s00244-00011-09645-00243 (Epub 02011 Feb 00216).

Sobin, C., et al., 2013. Microglial disruption in young mice with early chronic lead exposure. Toxicol. Lett. 15, 00151–00153.

Sobin, C., Flores-Montoya, M.G., Gutierrez, M., Parisi, N., Schauh, T., 2015. δ-Aminolevulinic acid dehydratase single nucleotide polymorphism 2 (ALAD2) and peptide transporter 2*2 haplotype (hPEPT2*2) differently influence neurobehavior in low-level lead exposed children. Neurotoxicol. Teratol. 47:137–145. http://dx.doi.org/10.1016/j.ntt.2014.12.001.

Wadsworth, B.J., 2003. Piaget's Theory of Cognitive and Affective Development: Foundations of Constructivism. fifth ed. Pearson.

Yeshenko, O., Guazzelli, A., Mizumori, S.J., 2004. Context-dependent reorganization of spatial and movement representations by simultaneously recorded hippocampal and striatal neurons during performance of allocentric and egocentric tasks. Behav. Neurosci. 118, 751–769.

Please cite this article as: Sobin, C., et al., Early chronic low-level Pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in..., Neurotoxicol Teratol (2017), http://dx.doi.org/10.1016/j.ntt.2017.01.002

CURRICULUM VITA

Mayra Gisel Flores-Montoya was born in Chihuahua, Mexico. The second daughter of Rosa Maria Montoya Chavez and Victor Manuel Flores Hernandez. She completed her undergraduate and graduate degrees at the University of Texas at El Paso (UTEP). In August 2017, she completed a doctoral degree in Psychology with a concentration in Social, Cognitive, and Neuroscience at UTEP. She has six years of teaching experience and has taught courses in Introduction to Psychology, Statistics, and Experimental Psychology at UTEP and medical terminology in Spanish at Paul L. Foster School of Medicine, Texas Tech. She worked in the laboratory of Dr. Christina Sobin (Psychologist/Neuroscientist) from summer 2009 to August 2017 and in the laboratory of Dr. Charlotte Vines (Immunologist) from summer 2015 to August 2017. She is first author in two articles entitled "Early chronic lead exposure reduces exploratory activity in young C57BL/6J mice" (2014) and "Olfactory recognition memory is disrupted in young mice with chronic low-level lead exposure" (2015). She is second author in three articles entitled "Microglial disruption in young mice with early chronic exposure to lead" (2013), "Δ Aminolevulinic acid dehydratase single nucleotide polymorphism 2 (ALAD2) and peptide transporter 2*2 haplotype (hPEPT2*2) differently influence neurobehavior in low-level lead exposed children" (2015) and "Early chronic low-level pb exposure alters global exploratory behaviors but does not impair spatial and object memory retrieval in an object-in-place task in pre-adolescent C57BL/6J mice" (2017). She will continue her investigation of memory impairment and neuroimmune system disruption in mouse models of chronic low-level lead exposure and neuroimmune mechanisms underlying behavior in health and disease.

Permanent Address: Northfield, Minnesota
mflores@carleton.edu