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Early Chronic Low-Level Lead Exposure Reduced C-C Chemokine Receptor 7 in Hippocampal Microglia

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Abstract

Chronic low-level lead exposure reduces memory in children however the mechanisms mediating these deficits in the brain are not known. Previous studies in our laboratory showed that early lead exposure reduced the number of microglial cells in hippocampus/dentate gyrus of C57BL/6J mice. In the current study, C-C chemokine receptor 7 (CCR7) and major histocompatibility complex II (MHC II) were examined because these activation markers might promote microglia exit from hippocampus and antigen presentation in lymph nodes. Thirty-six C57BL/6J male mice were exposed to 0 ppm (controls, n = 12), 30 ppm (low-dose, n = 12), or 430 ppm (high-dose, n = 12) of lead acetate via dams' milk from postnatal day (PND) 0 to 28. Flow cytometry was used to quantify cell types and cell surface expression of MHC II and CCR7 in hippocampal and brain microglia. Non-parametric independent samples median tests were used to examine differences between groups. Statistical analyses revealed that the low-dose group had decreased CCR7 expression (measured as geometric mean fluorescence intensity, GMFI) in hippocampal microglia as compared to controls. The high-dose group had decreased frequency of CCR7⁺MHC II⁻ hippocampal microglia as compared to controls. Exploratory analyses revealed that the high-dose group had decreased frequency of CCR7⁺MHC II⁻ hippocampal macrophages as compared to controls but increased MHC II levels in MHC II⁺CCR7⁺ hippocampal macrophages as compared to controls. These studies indicated that low-level lead exposure reduced CCR7 in hippocampal microglia and suggested that microglial cell trafficking might be increased in low-level lead exposed mice. CCR7-induced tissue exit is a potential mechanism contributing to disrupted memory in children exposed to chronic low-levels of lead.

Keywords

Lead; Microglia; MHC II; CCR7; Flow cytometry

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⁶Conflict of interest statement

The authors have no conflict of interest to declare.

1. Introduction

Lead is a toxic heavy metal that alters a variety of body systems. It is widely accepted that there is no “safe” level of lead exposure during childhood (CDC, 2012). The central nervous system (CNS) and immune system are exquisitely sensitive to its effects (WHO, 2010) and children as compared to adults are far more vulnerable (CDC, 2013). Sources of exposure in United States (U.S.) include but are not limited to lead-emitting industries, contaminated soil, lead paint in old housing, lead pipes, children’s jewelry, toys, and cosmetics (EPA, 2006; EPA, 2015).

Clinical studies suggested that children chronically exposed to lead have diminished memory, poor cognitive-set shifting, and reduced fine-motor dexterity (e.g. Bellinger & Needelman, 2003; Canfield et al., 2003; Gilbert & Weiss, 2006; Jusko et al., 2008; Landrigan et al., 2006; Lanphear et al., 2005; Schnaas et al., 2000; Sobin et al., 2015; Wasserman et al., 2000). The effects of lead on memory are of special concern for learning. Child exposure to low-level lead is more likely to occur in lower socioeconomic neighborhoods (WHO, 2010) and recent events in Flint, Michigan are a stark reminder that environmental lead continues to pose a serious threat for children nationwide (Hanna-Attisha, LaChance, Casey Sadler, & Champney Schnepf, 2016). Studies in our laboratory have shown that 14% of children living in lower socioeconomic neighborhoods have BLLs $5 \mu\text{g/dL}$, and over 50% have BLLs between approximately 2.5 and $7 \mu\text{g/dL}$ (Sobin et al., 2015).

The hippocampus is primarily responsible for memory formation and retrieval (Jeffery, 2018, review). The hippocampal mechanisms that explain effects at levels of lead exposure shown to be common among children living in lower income neighborhoods are not understood, leaving very few options for intervention. Disruption of hippocampal function during early development has detrimental effects on memory that might be life-long. Animal models are needed in order to understand how low-level lead exposure alters the hippocampus and thus provide the groundwork for the development of more effective interventions.

In vitro and *in vivo* studies examining the effects in adult hippocampus following exposure to higher levels of lead provided the groundwork for this study examining effects at lower levels of exposure. Previous studies focused primarily on microglial cells in hippocampus because these cells assist pruning, neurogenesis, brain pathway development, and clearance of damaged neurons (Paolicelli et al., 2011; Ransohoff & Cardona, 2010; Schafer et al., 2012). For example, one study showed that in adult Wistar rats, chronic exposure to 100 parts per million (ppm) of lead increased microglial activation, reduced long-term potentiation (LTP), and increased neuronal death in the hippocampus (Liu 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). These results suggested that microglia are a target of lead toxicity and might be responsible for deficits in memory at high levels of lead exposure.

Our laboratory examined effects of low-level lead exposure on microglial cells and found reduced numbers of microglia in the dentate gyrus (DG) of hippocampus as compared to controls (Sobin et al., 2013). More recently, in a replication study, our laboratory found reduced numbers of microglial cells throughout the hippocampal structure (Dominguez et al., 2018). Altering the number or distribution of microglial cells as a result of low-level lead exposure would be expected to alter brain development and memory. Given that the CNS and immune system are now understood to be inter-dependent and interact via draining lymphatics (Clarkson et al., 2017; Louveau et al., 2015, review) it is plausible to suggest that effects identified in hippocampal microglia could be mediated by interactions between the CNS and the draining lymphatic system.

The discovery of the glymphatic system (Iliff et al., 2012) and more recently, the discovery of meningeal (dural) lymphatic vessels (Aspelund et al. 2015; Louveau et al. 2015) provided the foundation for new hypotheses regarding the possible trafficking and abnormal distribution of microglial cells following early chronic low-level lead exposure. For example, it might be that chronic low-level lead exposure promotes the trafficking of microglial cells to meningeal lymphatic vessels and deep cervical lymph nodes for antigen presentation. Microglia are considered to be the macrophages of the CNS and studies examining effects of lead in peripheral macrophages showed that although lead itself is not directly antigenic, exposure to the heavy metal increased major histocompatibility complex (MHC II) levels on the surface of macrophages and skewed T-cell responses towards a Th2 phenotype (and potentially antibody mediated immunity) (Dietert & Piepenbrink, 2006, review; Kasten-Jolly & Lawrence, 2014, review). In addition, in the periphery, up-regulation of MHC II has been associated with elevated antigen presentation on the surface of macrophages, and the upregulation of C-C chemokine receptor 7 (CCR7) has been associated with the trafficking of these cells into draining lymph nodes for antigen presentation to T-cells (Förster et al., 2008, review).

Microglial cells are functionally similar to macrophages and are capable of upregulating MHC II (Beyer et al., 2000; Harms et al., 2013) and CCR7 (Dijkstra et al., 2006). Given that meningeal lymphatic vessels lie adjacent to the brain and drain immune cells into deep cervical lymph nodes it was hypothesized that chronic low-level lead exposure would cause microglia to upregulate MHC II and CCR7 (which could in turn result in microglial exit from hippocampus and drainage into deep cervical lymph nodes). In fact, a recent study showed that CCR7 upregulated dendritic cells (professional antigen presenting cells similar to microglia (Derecki et al., 2013)) migrate from hippocampus and other brain regions into deep cervical lymph nodes perhaps via meningeal lymphatics (Clarkson et al., 2017). An increase in immune cell trafficking is a potential mechanism that might explain why microglial cell number were reduced from DG and hippocampus at lowest levels of lead exposure (reference).

The goal of this study was to examine the effects of low and high-level early chronic lead exposure on MHC II and CCR7 expression in hippocampal microglia in young mice. It was hypothesized that as compared to pre-adolescent controls, pre-adolescent C57BL6/J mice exposed during development to low or high levels of lead would have increased hippocampal microglial activation as measured by MHC II (MHC II⁺) and CCR7 (CCR7⁺) as quantified

by multi-color flow cytometry. In exploratory analyses, similar effects were examined in hippocampal and brain macrophages and undifferentiated immune cells which included lymphocytes (T- and B- cells) and myeloid cells (e.g. macrophages, microglia, and monocytes); and in microglia, macrophages and undifferentiated immune cells in brain sections that did not include hippocampus.

2. Materials and Methods

2.1. Animals

The procedures used for this study were approved by the Institutional Animal Care and Use Committee (IACUC, protocol #A201403-1) and were conducted in compliance with the US Public Health Service Policy and Human Care and Use of Laboratory Animals (National Research Council, 2011). C57BL/6J mice were purchased from Jackson Laboratories, 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) with *ad libitum* access to food and water. The room temperature was 20°-26° C, with relative humidity of 30%, and a 12-hour light-dark cycle.

At PND 60, 18 dams were mated with nine sires by harem breeding (two females per male). Dams were checked daily and pregnancy was detected via vaginal plug presence. When pregnancy was detected dams were separated from sires and housed individually. Dams gave birth to litters of five to nine pups. At PND 7, litters were culled randomly to five pups. Males, but not females were tested because past studies have shown that effects of chronic low-level lead exposure in males and females differ (Flores-Montoya et al., 2015; Kasten-Jolly & Lawrence, 2017; Leasure et al., 2008; Singh et al., 2018, review). Future studies will examine effects in females.

2.2. Lead Exposure

The lead exposure methods were previously shown by our laboratory to produce low and high BLLs in C57BL/6J mice (Flores-Montoya & Sobin, 2014; Flores-Montoya et al., 2015). Lead acetate treated water (99.4% pure, Sigma Aldrich, St. Louis, MO) was given to dams' when litters were born, and pups were exposed to one of three possible exposure conditions via dams' milk. The exposure groups included 30 ppm lead (low-dose), 430 ppm lead (high-dose) or no lead (controls). Dams and pups were exposed from PND 0 to PND 21 (pre-adolescence). At PND 21, pups were weaned and continued treatment exposure via water bottles until euthanasia at PND 28. In previous studies in our laboratory these exposure protocol and levels produced BLLs of 4.24 ($\pm$ 0.61) μ g/dL in the 30 ppm group and 21.30 ($\pm$ 7.03) μ g/dL in the 430 ppm group as measured with inductively coupled plasma mass spectrometry (ICP-MS).

2.3. Procedure

At PND 28, thirty-six male mice (controls = 12, low-dose = 12, and high-dose = 12) were anesthetized with Avertin (5-10 ml, depending on body weight). When mice were unresponsive to paw pinch and corneal reflex, they were transcardially perfused with pH 7.4 ice cold PBS. Brains were immediately removed, left and right hippocampi were dissected

and separated from the remaining brain. Pilot studies showed that to ensure sufficient numbers of microglial cells for flow cytometric analyses, hippocampi of three mice from each treatment group would have to be combined, yielding a total of 4 combined samples per group (control combined samples = 4, low-dose combined samples = 4, and high-dose combined samples = 4) of hippocampi, and 4 combined samples per group of remaining brain sections.

2.3.1. Single cell suspensions—Hippocampi (4 combined samples per group) and brain (4 combined samples per group) were gently dissociated in 6 ml of 1 x PBS, 0.01 M HEPES, 3% FBS (immune cell media) using a 5 ml syringe plunger and 70 μ m screens (Corning® Cat# 431751). Immune cells were separated from debris, neurons, and myelin with 70% percoll (Cat# GE17-0891-01, Sigma-Aldrich, St. Louis, MO), washed with 2 ml of immune cell media and adjusted by hemocytometer to 200,000 cells/100 μ l. Cells were then blocked for 15 min with F_c blocker (1:100, Cat#101302, Biolegend, San Diego, CA), protected from light and labeled with a cocktail of antibodies for 30 min (at 4°C).

2.3.2. Antibody cell labeling—Cells were labeled following previously published methods (Cronk et al., 2015; Derecki et al., 2012; Louveau et al, 2015). The concentrations of antibodies were determined by titration using pilot hippocampi and brain tissue. For identification of microglial cells and macrophages, anti-mouse CD11b conjugated with PE (PE-CD11b) (1:100, Cat#12-0112-82, eBioscience, San Diego, CA) and anti-mouse CD45 conjugated with Alexa 700 (A700-CD45) (1:20, Cat#56-0451-82, San Diego, CA) were used. For detection of the markers of interest in microglial cells, anti-mouse MHC II conjugated with FITC (FITC-MHC II) (1:100, Cat#11-5321-82, San Diego, CA) and anti-mouse CCR7 conjugated with APC (APC-CCR7) (1:100, Cat#120108, Biolegend, San Diego, CA) were used.

2.4. Flow Cytometry

2.4.1 Apparatus—A Beckman Coulter Lifesciences (Indianapolis, IN) Gallios™ flow cytometer was used. The acquisition parameters were set to detect four fluorophores using one blue laser (excitation wavelength of 488 nm) and one red laser (excitation wavelength of 633 nm). The blue laser was set to detect FITC (peak wavelength of 519 nm) and PE (peak wavelength of 612 nm). The red laser was set to detect APC (peak wavelength of 660 nm) and A700 (peak wavelength of 719 nm). FlowJo *Version 10.30* was used for compensation of spectral overlap (“spillover”), gating cells of interest, and retrieving data for statistical analyses.

2.4.2. Gating strategy for analyzing activation markers—The gating strategy included 1) single cells; 2) undifferentiated immune cells including lymphocytes (T- and B-cells) and myeloid cells (e.g. microglia, macrophages, and monocytes) (Abbondanzon et al., 2014); 3) microglia and macrophages (Wlodarczyk et al., 2015); and 4) MHC II positive (MHC II⁺) and CCR7 positive (CCR7⁺) cells; MHC II “single positive” (MHC II⁺ CCR7⁻) or CCR7 “single positive” (CCR7⁺ MHC II⁻) cells; MHCII/CCR7 “double positive” cells (MHC II⁺ CCR7⁺). Fluorescence minus one (FMO) for FITC-MHC II and APC-CCR7 were used as negative controls to delimit gating boundaries for identifying MHC II⁺ and CCR7⁺

cells. Figure 1 illustrates the gating strategy for identifying microglia and macrophages. (Supplementary Figures 1-4 illustrate the gating strategies for MHC II⁺ and CCR7⁺, MHC II⁺ CCR7⁻ or CCR7⁺ MHC II⁻, and MHC II⁺ CCR7⁺ microglia and macrophages).

2.4.3. Outcome variables—Median values of gated cells were determined for percent of parent population (total hippocampal microglia); for percent of parent population, geometric mean fluorescence intensity (GMFI), and for coefficient of variation (total MHC II⁺ and CCR7⁺). Quadrant-specific median values were determined for frequency of parent population, GMFI, and for coefficient of variation (MHC II⁺ CCR7⁻ and CCR7⁺ MHC II⁻; and MHC II⁺ CCR7⁺ cells). To further explore possible influences of lead exposure on the neuroimmune system, the same outcome activation markers were analyzed for brain microglia, hippocampi and brain macrophages, and for frequency (calculated as percent) and count of undifferentiated hippocampi and brain immune cells.

2.4.4. Statistical analyses—Data were checked for accuracy, and examined for missing values, distribution properties, and outliers, and analyzed with SPSS *Version 23.0*. Tests of homogeneity of variance were conducted and revealed that data were non-normally distributed. Non-parametric independent sample median tests were run to examine possible differences between groups for each outcome variable. When significant differences were detected between groups, pairwise comparison tests were used to identify the sources of significant differences.

3. Results

3.1. Descriptive Statistics

3.1.1. Mouse body and brain weights—To determine possible weight differences between groups, total body and brain weight of lead-exposed mice as compared to controls were examined. Table 1 shows the sample characteristics at PND 28 (euthanasia) by exposure group for 36 male C57BL/6J mice. The animal brain and body weights at euthanasia following chronic exposure to lead were slightly lower in the low- and high-dose groups as compared to the control group. One-way ANOVA did not reveal significant differences between groups. Significant group differences in body weight were detected, $F(2, 33) = 3.96$, $p = 0.029$. Post-hoc analyses with least square differences revealed that the low- and high-dose groups (low-dose group $M\ diff = 1.56$, $p = 0.034$; high-dose group $M\ diff = 1.85$, $p = 0.013$) had lower body weights as compared to the control group. No significant differences between the low- and high-dose groups were detected. No mice perished during the exposure period and there were no observable changes in motor or behavioral function.

3.1.2. Frequency of cell type and cells with MHC II and/or CCR7—To determine if exposure to lead affected immune cell compartment and/or the frequency of activation markers, MHC II⁺, CCR7⁺, MHC II⁺ CCR7⁻, CCR7⁺ MHC II⁻, or MHC II⁺ CCR7⁺, the frequency of each cell type of interest in hippocampal and whole brain (without hippocampus), and expression of the markers of interest, were analyzed by flow cytometry.

The median values in the parent population of the activation markers were calculated (see Table 2).

In hippocampus, the low-dose group had decreased frequency of microglia (*Med* diff = 4.8 %) and decreased frequency of undifferentiated immune cells (*Med* diff = 13.3%), but increased frequency of macrophages (*Med* diff = 1%) as compared to the control group. The high-dose group had increased frequency of microglia (*Med* diff = 16.1%) and increased frequency of undifferentiated immune cells (*Med* diff = 10.25%), but decreased frequency of macrophages (*Med* diff = 1.1%) as compared to the control group. In brain, the low-dose group (*Med* diff = 2.65%) and high-dose group (*Med* diff = 7.15%) had lower frequency of microglia, but increased frequency of undifferentiated immune cells (low-dose *Med* diff = 1.55%; high-dose *Med* diff = 8.3%) as compared to the control group. Low- and high-dose groups had a similar frequency of macrophages. Additionally, the frequency of hippocampal microglia MHC II⁺ and CCR7⁺ in the low- and high-dose groups were consistently lower (for overall and quadrant-specific median values) as compared to controls. Median values for the rest of the outcome variables measured in hippocampus and brain differed between groups, but with less consistency with regard to the direction of the difference (increase vs decrease) (Table 2).

3.1.3. Geometric mean fluorescence intensity and coefficient of variation—To determine if lead exposure affected the GMFI (geometric mean fluorescence intensity) of two activation markers in microglia and macrophages, MHC II and CCR7 were examined on the cell surface. GMFI was examined because flow cytometric data follows a logarithmic (but not linear) distribution (Finak et al., 2010). Additionally, in order to understand the distribution of scores, the coefficient of variation of GMFI was measured (GMFI/CV) for the markers of interest in hippocampal and brain microglia and macrophages (see Table 3). In hippocampus, CCR7⁺ expression levels were consistently lower (for overall and quadrant-specific median values) for microglia and macrophages as compared to the control group. Also, microglia CCR7⁺ expression levels were consistently lower for the high-dose group (for overall and quadrant-specific median values) and macrophages (for overall and MHC II⁺ CCR7⁺ median values only) as compared to control and low-dose groups. Median values for expression levels for the rest of the outcome variables measured in hippocampus and brain were different but inconsistent with regard to the direction of the effect. These observed differences were tested statistically.

3.2. Statistical Analyses

Tables 4-6 show results of independent sample median tests and post-hoc pairwise comparison tests of possible group differences. A detailed description of the significant differences detected between groups is given below and Figures 2 through 9 show significant differences between groups confirmed with post-hoc analyses.

3.2.1. Hippocampal microglia

3.2.1.1. Overall analyses: Tests showed a significant difference between groups with regard to frequency of CCR7⁺ hippocampal microglia, *MEDCOMP test-statistic* (2) = 6.00,

$p = 0.05$. Post-hoc tests however did not confirm these differences (control $Med = 5.84\%$; low-dose $Med = 1.73\%$; high-dose $Med = 0.29\%$).

Tests also showed significant differences between groups with regard to CCR7 GMFI of hippocampal microglia, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc tests for CCR7 GMFI showed that contrary to what was hypothesized, the low-dose group ($Mdn = 4656 \pm 37$) had decreased CCR7 GMFI in hippocampal microglia as compared to the control group ($Mdn = 5604 \pm 38$), *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$ (see Figure 2). No significant difference was detected between the high-dose and control group, or between the high-dose ($Mdn = 3938 \pm 36$) and low-dose group.

3.2.1.2. Quadrant-specific analyses: Tests showed significant differences between groups with regard to frequency of CCR7⁺ MHCII⁻ hippocampal microglia, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc tests showed that the high-dose group ($Med = 0.15\%$) had a decreased frequency of CCR7⁺ MHCII⁻ hippocampal microglia as compared to the control group ($Med = 3.01\%$), *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$. No significant difference was detected between the low-dose group ($Med = 0.92\%$) and the control group or between the low-dose group and the high-dose group (see Figure 3).

Tests also showed significant differences between groups with regard to the frequency of MHCII⁺ CCR7⁺ microglia, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc tests however did not confirm significant differences between groups (control $Med = 1.40\%$; low-dose $Med = 0.29\%$; high-dose group $Med = 0.13\%$).

3.2.3 Brain microglia

3.2.3.1. Overall analyses: Tests showed significant differences between groups with regard to the frequency of MHCII⁺ brain microglia, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc tests showed that the low-dose group ($Med = 1.77\%$) had a decreased frequency of MHCII⁺ brain microglia as compared to the high-dose group ($Med = 4.22\%$). No significant difference was detected between the low-dose group and the control group ($Med = 2.86\%$), or between the high-dose group and the control group (see Figure 4).

3.2.3.2. Quadrant-specific analyses: Significant differences were found between groups with regard to the frequency of MHCII⁺ CCR7⁻ brain microglia, *MEDCOMP test-statistic* (2) = 8.00, $p = 0.02$. Post-hoc tests showed that the low-dose group ($Med = 1.28\%$) had decreased frequency of MHCII⁺ CCR7⁻ brain microglia as compared to the high-dose group ($Med = 2.91\%$). No significant differences were detected between the low-dose group and the control group ($Med = 2.20\%$) or between the high-dose group and the control group (see Figure 5).

3.2.4 Hippocampal macrophages

3.2.4.1. Overall analyses: Significant differences were found between groups with regard to the frequency of hippocampal macrophages, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc analyses however were not significant (controls $Med = 4.08\%$; low-dose $Med = 5.06\%$; high-dose $Med = 2.98\%$).

3.2.4.2. Quadrant-specific analyses: Significant differences were found between the groups with regard to the frequency of CCR7⁺MHCII⁺ hippocampal macrophages, *MEDCOMPS test-statistic* (2) = 8.00, $p = 0.02$. Post-hoc analyses indicated that the high-dose group ($Mdn = 0.32\%$) had decreased frequency of CCR7⁺MHCII⁺ hippocampal macrophages as compared to the control group ($Mdn = 5.21\%$), *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$. No significant difference however was detected between the low-dose group ($Mdn = 8.46\%$) and control group, or between the low-dose group and high-dose group (see Figure 6).

Significant differences between groups with regard to the levels of MHC II expressed on the surface of MHCII⁺ CCR7⁺ hippocampal macrophages were found, *MEDCOMP test-statistic* (2) = 6.00, $p = 0.05$. Post-hoc analyses indicated that the high-dose group ($Mdn = 29264 \pm 100$) had increased MHC II GMFI in hippocampal macrophages as compared to the control group ($Mdn = 21371 \pm 79$), *MEDCOMP test-statistic* = 8.00, $p = 0.01$ (see Figure 7). No significant differences were detected between the levels of MHCII expressed in high-dose and the low-dose group ($Mdn = 21720 \pm 49$), or between the low-dose and control group.

3.2.5. Brain macrophages

3.2.5.1. Overall analyses: No significant differences between groups were detected.

3.2.5.2. Quadrant-specific analyses: Significant differences between groups, *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$. Post-hoc analyses indicated that the low-dose group ($Mdn = 18752 \pm 104$) had decreased levels of MHC II expressed on MHCII⁺ CCR7⁺ brain macrophages as compared to the high-dose group ($Mdn = 25588 \pm 159$), *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$ (see Figure 8). No significant effects were found between the low-dose and control group ($Mdn = 24117$), or between the high-dose and control group.

3.2.6. Undifferentiated hippocampal immune cells—Significant differences between groups were found, *MEDCOMP test-statistic* (2) = 8.00, $p = 0.01$. Post-hoc analyses indicated that the low-dose group ($Mdn = 33.30\%$) had a decreased frequency of immune cells in hippocampus as compared to the high-dose group ($Mdn = 56.65\%$), *MEDCOMP test-statistic* = 8.00, $p = 0.01$. 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 (see Figure 9).

3.2.7. Undifferentiated brain immune cells—No significant differences between groups with regard to undifferentiated brain immune cells.

4. Discussion

4.1. Overview

The discovery of glymphatics (Illif et al., 2012) and meningeal lymphatic vessels (Aspelund et al., 2015; Louveau et al., 2015) has allowed for the development of new hypotheses with regard to how the CNS and immune system interact. Past studies in our laboratory showed that low-dose and high-dose lead exposed mice had reduced numbers of microglial cells in

the DG (dentate gyrus) (Sobin et al., 2013) and throughout the hippocampus (Dominguez et al., 2018) as compared to controls. Why microglial cells were missing from DG and throughout hippocampus is not known.

The present study was carried out to examine whether two activation markers, MHC II and CCR7, were upregulated by early chronic lead exposure in the hippocampal microglia of young mice. As described earlier, in previous studies following exposure to a foreign stimulus, antigen presenting cells such as macrophages and dendritic cells upregulate MHC II on their surface, which allows for presentation of foreign antigens to other immune cells (Förster et al., 2008, review). These cells also upregulate CCR7 on their surface which directs trafficking to lymphatics and draining lymph nodes guided by CCR7 ligands C-C chemokine ligand 19 (CCL19) and 21, (CCL21). It is within the lymph nodes that antigen presentation to T-cells and adaptive immune responses are initiated (Förster et al., 2008, review).

Microglia are the major antigen presenting cells of the CNS (Alomda et al., 2011) thus in this study, MHC II and CCR7 were examined in hippocampal microglia. These proteins were examined since they could contribute to the microglial cell exit from the CNS, and the loss from the hippocampus found following lead exposure in previous studies (reference?).

The results indicated that low-dose lead exposure (associated with blood lead concentrations often found in children living in lower socioeconomic neighborhoods) reduced CCR7 levels in hippocampal microglia. Additionally, it was found that high-dose lead exposure had significant effects on hippocampal microglia and macrophage MHC II and CCR7 membrane expression levels. These results suggested that low-dose chronic lead exposure had reduced numbers of hippocampal microglia. Table 7 summarizes and compares the significant effects in controls, low- and high-dose animals.

4.2. Low-dose mice as compared to controls had decreased levels of CCR7 in hippocampal microglia, and high-dose mice as compared to controls had decreased frequency of CCR7⁺ hippocampal microglia

Contrary to what was hypothesized, it was found that low-dose lead exposed mice had significantly reduced (rather than increased) levels of CCR7 in hippocampal microglia as compared to controls. Similarly, it was found that high-dose mice had markedly reduced frequency of CCR7 hippocampal microglia as compared to controls.

There are at least four possible explanations for these findings. It could be that in response to early lead exposure, microglia vary in the extent to which CCR7 is upregulated. If so, perhaps by PND 28 CCR7 activated microglia had already upregulated CCR7 and exited the brain, leaving behind microglia with limited CCR7 upregulation. Recent evidence supports this possibility. In one study, within seven days after injection into the brain, CCR7 promoted the exit of activated dendritic cells from hippocampus and into the deep cervical lymph nodes (Clarkson et al., 2017). Dendritic cells are phenotypically similar to other professional antigen presenting cells investigated (microglial cells and macrophages) in their expression of CD45, MHC II, and CCR7 (Clarkson et al., 2017; Derecki et al., 2012; Guillems et al., 2014; Merad et al., 2013).

Another possibility concerns the potential interaction of CCR7 with endogenous ligands, CCL19 or CCL21, found constitutively in cerebrospinal fluid and brain parenchyma (CCL19) (Krumbholz et al., 2007) and in meningeal lymphatic vessels (CCL21) (Louveau et al., 2015). Immune cells with upregulated CCR7 internalize CCR7 within 10 min after ligand binding; internalization is more likely with CCL19 as compared to CCL21 binding (Byers et al., 2008). Thus, significantly fewer CCR7⁺ microglia may reflect the consequences of this post-binding mechanism.

An additional possibility is that early chronic lead exposure disrupted the genetic expression of CCR7. Recent evidence showed that low-level lead exposure altered the transcription in whole brain of chemokine and chemokine receptor genes including CCL28, CCR2, and CCR8 (Kasten-Jolly et al., 2012). These results suggested that mechanisms typically associated with immune cell migration might be altered in brain. Future studies could examine whether changes in transcripts in these chemokines and chemokine receptors and in others including CCR7 specifically occur within hippocampal microglial cells of mice exposed chronically to low levels of lead.

Finally, reduced cell surface CCR7 could reflect incorrect protein folding. In order for proteins to be functional and upregulated on the surface of a cell they have to fold properly (Dong, Filipeanu, Duvernay, & Wu, 2007; Rothman & Shekman, 2011). A disruption in the normal folding of CCR7 could explain why lead-exposed animals had decreased upregulation of the receptor on the membrane surface of hippocampal microglia. An *in vitro* study provided support for this possibility. In this study, lead disrupted the spontaneous and chaperone-assisted (protein-assisted) folding of proteins in a bacterial model (Sharma, Goloubinoff, & Christen, 2008). Future studies could examine whether these effects occur in hippocampal microglia of chronic low-level lead exposed young mice.

4.3. High-dose mice as compared to controls had decreased frequency of CCR7⁺MHCII⁺ hippocampal macrophages, and increased levels of MHC II in MHCII⁺CCR7⁺ hippocampal macrophages

High-dose lead exposed mice had a notable decrease in the frequency of CCR7⁺MHCII⁺ hippocampal macrophages as compared to controls. However, high-dose mice had significantly *increased* levels of MHC II expressed on the surface of hippocampal MHCII⁺CCR7⁺ macrophages. The results suggested that by PND 28, hippocampal macrophages with upregulated CCR7 exited the brain to reach deep cervical lymph nodes and/or internalized their receptor after endogenous ligand binding in brain (CCL19/CCL21) while (fewer) MHC II-expressing macrophages remained in hippocampus and brain to continue neuroimmune surveillance. Alternatively, it could be that the genetic expression or molecular processing of MHC II and CCR7 were altered.

4.4. Low- as compared to high-dose lead exposed mice showed differences in brain microglia, brain macrophages and hippocampal undifferentiated immune cells

Low-dose as compared to high-dose lead exposed mice showed decreased frequency of MHCII⁺ and MHCII⁺CCR7⁺ brain microglia, decreased levels of MHC II in MHCII⁺CCR7⁺ brain macrophages, and an overall decrease in the frequency of undifferentiated

hippocampal immune cells. Significant differences were not detected however between low-dose lead exposed mice and controls, and high-dose lead exposed mice and controls, because effects in treated groups moved in opposite directions (non-linear effects). These findings are perhaps consistent with past studies in our laboratory that suggested qualitative differences between low- and high-dose lead exposed pre-adolescent mice (Sobin et al., 2013).

4.5. Peripheral effects on immune function provide hypotheses regarding effects of lead exposure in hippocampus and brain

New research demonstrating pathways by which mechanisms of the peripheral immune system directly impact the CNS and specifically brain, provide a basis for suggesting that changes in neuroimmune factors could mimic changes observed in the periphery. For example, high level lead exposure predisposed peripheral T-cells towards a Th2 phenotype (Dietert et al., 2006, review; Kasten-Jolly & Lawrence, 2014, review). Similarly, analyses of spleen immune cells following lower levels of lead exposure suggested increases in Th2 polarization (Kasten-Jolly et al., 2010). Interestingly, transcript analyses at lower levels of lead exposure showed decreased expression of CCR7 in spleen (Kasten-Jolly et al., 2010). Perhaps of particular relevance to the present study, data have shown that deficient peripheral CCR7 signaling can result in increased Th2 polarization (Moschovakis et al., 2012). Whether effects in peripheral immune cells and spleen provide a model for effects from low-level lead exposure in hippocampus and brain remains to be determined.

4.6. Limitations

To ensure sufficient numbers of microglia, the hippocampi and brains of mice had to be combined, lowering the sample size from 12 per group to 4 per group, and reducing the statistical power to detect significant effects. The findings in this study may underestimate group effects.

5. Conclusions

At low and higher levels of early chronic lead exposure, CCR7 was reduced in hippocampal microglia. MHC II expression was increased only in high-dose lead exposed animals, and only in hippocampal macrophages. Unexpectedly, early chronic lead exposure reduced CCR7 in hippocampal macrophages. Studies are needed to determine when during development changes in CCR7 levels in microglia begin to occur and thus whether the observed effects are attributable to early microglial trafficking, increased receptor internalization, and/or disruptions in genetic expression or molecular processing of CCR7. Studies are also needed to examine whether activation of microglial cells as suggested by altered MHC II and/or CCR7 levels accounts for reduced numbers of microglial cells in hippocampus previously reported. These findings provide further support for the notion that early chronic lead exposure disrupts brain development by targeting neuroimmune mechanisms in hippocampal structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BLLs	blood lead levels
ICP-MS	inductively-coupled plasma mass spectrometry
µg/dL	micrograms per deciliter
MHC II	major histocompatibility complex II
CCR7	C-C chemokine receptor 7

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Figure 1. Representative images of gating strategy for identifying microglia and macrophages.
1a. Cells were plotted for forward scatter area (*x-axis*) and height (*y-axis*). Single cells that were closer together and formed an angle of approximately 45 degrees were selected. (Outlier cells were likely to represent doublets or cell clusters and were excluded.) **1b.** Single cells were plotted for forward scatter area (cell size, *x-axis*) and for side scatter area (cell complexity, *y-axis*) and gated based on the shape of the distribution. Remaining cells were likely dead or granulocytes (very small and/or high internal complexity), and excluded. **1c.** Immune cells (undifferentiated, e.g. T-cells, B-cells, monocytes, microglia, and macrophages) were plotted for PE CD11b (*x-axis*) and A700 CD45 (*y-axis*). 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 macrophages. The images shown are consistent with others in the literature illustrating the distributions of these cells (Włodarczyk et al., 2015).

Figure 2. Representative histograms showing significant effects of lead exposure on geometric mean fluorescence intensity (GMFI) of CCR7 hippocampal microglia.

The blue and red histograms show the fluorescence intensity of CCR7 hippocampal microglia in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples) and 30 ppm (low-dose group; n = 4 combined samples), respectively. The grey histogram shows the fluorescence intensity of APC CCR7 (FMO) of hippocampal microglia stained with all markers of interest except for APC CCR7 and was used as a negative control. Histogram shifts towards the right show a higher mean fluorescence intensity and thus increased CCR7. As shown, low-level lead exposed animals had decreased CCR7 (measured as GMFI) in hippocampal microglia as compared to the control group.

Figure 3. Frequency of CCR7⁺MHCII⁻ hippocampal microglia by exposure group.

The box plots show the frequency of CCR7⁺MHCII⁻ hippocampal microglia in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples), 30 ppm (low-dose group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples). Box plots show the interquartile-range (25-75th percentile); bars indicate the location of maximum fence values ($\pm 1.5 \times$ the IQR); central line shows the median; blue line shows the grand median. Comparison bars between groups show significant *p* values (***p* 0.01) from post-hoc independent sample median tests. As shown, the high-dose group had decreased frequency of CCR7⁺MHCII⁻ hippocampal microglia as compared to the control group.

Figure 4. Frequency of MHCII⁺ brain microglia by exposure group.

The box plots show the frequency of MHCII⁺ brain microglia in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples), 30 ppm (low-dose group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples). Box plots show the interquartile-range (25-75th percentile); bars indicate the location of maximum fence values ($\pm 1.5 \times$ the IQR); central line shows the median; blue line shows the grand median. Comparison bars between groups show significant *p* values (***p* < 0.01) from post-hoc independent sample median tests. As shown, the low-dose group had decreased frequency of MHCII⁺ brain microglia as compared to the high-dose group.

Figure 5. Frequency of MHCII⁺CCR7⁻ brain microglia by exposure group.

The box plots show the frequency of MHCII⁺CCR7⁻ brain microglia in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples), 30 ppm (low-dose group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples). Box plots show the interquartile-range (25-75th percentile); bars indicate the location of maximum fence values ($\pm 1.5 \times$ the IQR); central line shows the median; blue line shows the grand median. Comparison bars between groups show significant *p* values (***p* < 0.01) from post-hoc independent sample median tests. As shown, the low-dose group had decreased frequency of MHCII⁺CCR7⁻ brain microglia as compared to the high-dose group.

Figure 6. Frequency of CCR7⁺MHCII⁻ hippocampal macrophages by exposure group. The box plots show the frequency of CCR7⁺MHCII⁻ hippocampal macrophages in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples), 30 ppm (low-dose group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples). Box plots show the interquartile-range (25-75th percentile); bars indicate the location of maximum fence values ($\pm 1.5 \times$ the IQR); central line shows the median; blue line shows the grand median. Comparison bars between groups show significant *p* values (***p* 0.01) from post-hoc independent sample median tests. As shown, the high-dose group had decreased frequency of CCR7⁺MHCII⁻ hippocampal macrophages as compared to controls.

Figure 7. Representative histograms showing significant effects of lead exposure on MHC II geometric mean fluorescence intensity (GMFI) in MHCII⁺CCR7⁺ hippocampal macrophages The blue and red histograms show the fluorescence intensity of MHCII in MHCII⁺CCR7⁺ in young C57BL/6J mice exposed to 0 ppm (control group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples), respectively. The grey histogram shows the fluorescence intensity of FITC MHC II (FMO) of hippocampal microglia stained with all markers of interest except for FITC MHC II and was used as a negative control. Histogram shifts towards the right show a higher mean fluorescence intensity and thus increased MHC II. As shown, high-level lead exposed animals had increased MHC II (measured as GMFI) in MHCII⁺CCR7⁺ hippocampal macrophages as compared to controls.

Figure 8. Representative histograms showing significant effects of lead exposure on MHC II geometric mean fluorescence intensity (GMFI) of MHCII⁺CCR7⁺ brain macrophages. The light red and dark red histograms show the fluorescence intensity of MHC of MHCII⁺CCR7⁺ hippocampal microglia in young C57BL/6J mice exposed to 30 ppm (low-dose group; n = 4 combined samples) and 430 ppm (high-dose group; n = 4 combined samples), respectively. The grey histogram shows the fluorescence intensity of FITC MHC II (FMO) of hippocampal microglial cells stained with all markers of interest except for FITC MHC II and was used as a negative control. Histogram shifts towards the right show a higher mean fluorescence intensity and thus increased MHC II. As shown, low-level lead exposed animals had decreased levels of MHC II (measured as GMFI) in MHCII⁺CCR7⁺ hippocampal macrophages as compared to high-level lead exposed animals.

Figure 9. Frequency of hippocampal undifferentiated immune cells by exposure group. The box plots show the frequency of hippocampal undifferentiated immune cells (e.g. T-cells, monocytes, microglia, and macrophages) in young C57BL/6J mice exposed to 0 ppm (control group; $n = 4$ combined samples), 30 ppm (low-dose group; $n = 4$ combined samples) and 430 ppm (high-dose group; $n = 4$ combined samples). Box plots show the interquartile-range (25-75th percentile); bars indicate the location of maximum fence values ($\pm 1.5 \times$ the IQR); central line shows the median; blue line shows the grand median. Comparison bars between groups show significant p values (** $p < 0.01$) from post-hoc independent sample median tests. As shown, the low-dose group had a significantly decreased frequency of hippocampal undifferentiated immune cells as compared to the high-dose group.

Table 1.

Means and standard deviations for body and brain weight at PND 28 following chronic exposure to lead via lactation from birth to PND 28^{*}

	Exposure Group Mean $\pm$ (SD)		
	Controls	Low-Dose	High-dose
Body weight (grams)	15.90 (1.95)	14.34 (1.90)	14.05 (1.23)
Brain weight (grams)	0.46 (0.01)	0.44 (0.03)	0.43 (0.07)

^{*} N = 36 animals, controls n = 12, low-dose n = 12, high-dose n = 12.

Table 2.

Frequency of parent population median values measured in hippocampus and brain of young C57BL/6J mice with and without chronic lead exposure *

		% of Parent Population (Median)					
		Hippocampus			Brain		
		0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm
Microglia							
	<i>Overall</i>						
	Microglia	.4425	.3945	.6035	.8515	.8285	.7800
	MHC II	.1055	.0238	.0301	.0286	.0177	.0422
	CCR7	.0584	.0173	.0029	.0044	.0075	.0056
	<i>Quadrant</i>						
	MHC II	.0337	.0148	.0186	.0220	.0128	.0291
	CCR7	.0301	.0092	.0015	.0020	.0045	.0030
	MHCII ⁺ CCR7 ⁺	.0140	.0029	.0013	.0008	.0015	.0021
Macrophages							
	<i>Overall</i>						
	Macrophages	.0408	.0506	.0298	.0390	.0334	.0389
	MHC II	.5575	.5760	.7535	.3735	.3600	.4110
	CCR7	.4445	.2380	.2525	.1150	.0884	.1410
	<i>Quadrant</i>						
	MHC II	.3025	.4830	.5010	.2810	.3040	.2865
	CCR7	.0521	.0846	.0032	.0309	.0265	.0049
	MHCII ⁺ CCR7 ⁺	.2655	.1600	.2380	.0807	.0578	.1465
Undifferentiated Immune cells ^I		.4640	.3330	.5665	.7530	.7685	.8360

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

^I Lymphocytes (T-cells and B-cells) and myeloid cells (e.g. monocytes, microglia, and macrophages).

Table 3.

Geometric mean fluorescence intensity (GMFI) median values measured in hippocampus and brain of young C57BL/6J mice with and without chronic lead exposure *

Geometric Mean Fluorescence Intensity (GMFI/CV)**						
	Hippocampus			Brain		
	0 ppm	30 ppm	430 ppm	0 ppm	30 ppm	430 ppm
Microglia						
<u>Overall</u>						
MHC II	6341±117	4878±114	4988±69	5308± 93	4722±109	5371±67
CCR7	5604±38	4656±37	3938±36	4164±42	4279±50	4329± 41
<u>Quadrant</u>						
MHC II	12317±167	14529±89	12825±140	12056±120	13327±208	12056±126
CCR7	11115±18	10923±9	10768±24	11976±110	11845±137	11528±108
MHCII ⁺ CCR7 ⁺						
MHC II	14177±104	13105±10	13748±34	17879±98	18859±125	14471±72
CCR7	11752±16	10945±3	10783±7	11505±25	12038±20	11443±19
Macrophages						
<u>Overall</u>						
MHC II	10786±175	10920±247	15964±267	7940±275	7763±357	8946±311
CCR7	9695±247	7342±67	6969±165	5346±162	5315±284	5722±241
<u>Quadrant</u>						
MHC II	19555±181	17908±184	18879±288	19034±208	18678±274	20745±235
CCR7	20778±128	15338±24	27967±67	17357±170	19970±222	26494±189
MHCII ⁺ CCR7 ⁺						
MHC II	21371±79	21720±49	29264±100	24117±193	18752±104	25588±159
CCR7	14753±89	13880±51	13360±28	13388±52	13247±31	12446±47

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

** Geometric mean fluorescence intensity/coefficient of variation.

Table 4.

Hippocampal and brain microglia differences between groups with independent samples median tests in young C57BL/6J mice with and without chronic lead exposure.

		Test effects			Post-hoc effects				
	Variable	Test-statistic	df	p	Comparison	Test-statistic	df	p	
Hippocampus									
Overall Analyses									
	Microglia	%	2.00	2	0.37	.	.	.	
	MHC II	%	2.00	2	0.37	.	.	.	
	GMFI**		2.00	2	0.37	.	.	.	
	CCR7	%	6.00	2	0.05	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	2.00	2	0.47	
					30 vs 430 ppm	2.00	2	0.47	
	GMFI		6.00	2	0.05	0 vs 30 ppm	8.00	2	0.01
					0 vs 430 ppm	2.00	2	0.47	
					30 vs 430 ppm	2.00	2	0.47	
Quadrant Analyses									
	MHC II	%	0.00	2	1.00	.	.	.	
	GMFI		2.00	2	0.37	.	.	.	
	CCR7	%	6.00	2	0.05	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	8.00	2	0.01	
					30 vs 430 ppm	2.00	2	0.47	
	GMFI		2.00	2	0.37				
	MHCII+CCR7+	%	6.00	2	0.05	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	2.00	2	0.47	
					30 vs 430 ppm	2.00	2	0.47	
	MHC II	GMFI	0.00	2	1.00	.	.	.	
	CCR7	GMFI	2.00	2	0.37	.	.	.	
Brain									
Overall Analyses									
	Microglia	%	2.00	2	0.37	.	.	.	
	MHC II	%	6.00	2	0.05	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	2.00	2	0.47	
					30 vs 430 ppm	8.00	2	0.01	
	GMFI		2.00	2	0.37	.	.	.	
	CCR7	%	2.00	2	0.37	.	.	.	
	GMFI		0.00	2	1.00	.	.	.	
Quadrant Analyses									
	MHC II	%	8.00	2	0.02	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	2.00	2	0.47	
					30 vs 430 ppm	8.00	2	0.01	
	GMFI		2.00	2	0.37	.	.	.	

		Test effects			Post-hoc effects			
	Variable	Test-statistic	df	p	Comparison	Test-statistic	df	p
CCR7	%	2.00	2	0.37	.	.	.	.
	GMFI	2.00	2	0.37	.	.	.	.
					.	.	.	.
MHCII ⁺ CCR7 ⁺	%	2.00	2	0.37	.	.	.	.
	MHC II	GMFI	2.00	2	0.37	.	.	.
	CCR7	GMFI	2.00	2	0.37	.	.	.

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

** Geometric mean fluorescence intensity.

Table 5.

Hippocampal and brain macrophages differences between groups with independent samples median tests in young C57BL/6J mice with and without chronic lead exposure*

		Test effects			Post-hoc effects			
	Variable	Test-statistic	df	p	Group Comparison	Test-statistic	df	p
Hippocampus								
Overall Analyses								
Macrophages	%	6.00	2	0.05	0 vs 30 ppm	0.00	2	1.00
					0 vs 430 ppm	2.00	2	0.47
					30 vs 430 ppm	2.00	2	0.47
MHC II	%	2.00	2	0.37	.	.	.	.
	GMFI**	2.00	2	0.37	.	.	.	.
CCR7	%	2.00	2	0.37	.	.	.	.
	GMFI	2.00	2	0.37	.	.	.	.
Quadrant Analyses								
MHC II	%	6.00	2	0.05	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	0.00	2	0.47
					30 vs 430 ppm	0.00	2	1.00
CCR7	%	8.00	2	0.02	0 vs 30 ppm	0.00	2	1.00
					0 vs 430 ppm	8.00	2	0.01
					30 vs 430 ppm	2.00	2	0.47
MHCII+CCR7+	%	0.00	2	1.00	.	.	.	.
					.	.	.	.
					.	.	.	.
MHC II	GMFI	6.00	2	0.05	0 vs 30 ppm	0.00	2	1.00
					0 vs 430 ppm	8.00	2	0.01
					30 vs 430 ppm	2.00	2	0.47
CCR7	GMFI	0.00	2	1.00	.	.	.	.
Brain								
Overall Analyses								
Macrophages	%	2.00	2	0.37	.	.	.	.
MHC II	%	2.00	2	0.37	.	.	.	.
	GMFI	2.00	2	0.37	.	.	.	.
CCR7	%	2.00	2	0.37	.	.	.	.
	GMFI	0.00	2	1.00	.	.	.	.
Quadrant Analyses								
MHC II	%	0.00	2	1.00	.	.	.	.
	GMFI	2.00	2	0.37	.	.	.	.
CCR7	%	2.00	2	0.37	.	.	.	.
	GMFI	2.00	2	0.37	.	.	.	.
MHCII+CCR7+	%	2.00	2	0.37	.	.	.	.

		Test effects			Post-hoc effects			
	Variable	Test-statistic	df	p	Group Comparison	Test-statistic	df	p
MHC II	GMFI	8.00	2	0.01	0 vs 30 ppm	2.00	2	0.47
					0 vs 430 ppm	2.00	2	0.47
					30 vs 430 ppm	8.00	2	0.01
CCR7	GMFI	2.00	2	0.37	.	.	.	.

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

** Geometric mean fluorescence intensity.

Table 6.

Hippocampal and brain undifferentiated immune cells differences between groups with independent samples median tests in young C57BL/6J mice with and without chronic lead exposure^{*}

		Test effects			Post-hoc effects				
		Variable	Test-statistic	df	p	Group Comparison	Test-statistic	df	p
Hippocampus									
Undifferentiated Immune cells ^I	%	8.00	2	0.01	0 vs 30 ppm	0.00	2	1.00	
					0 vs 430 ppm	0.00	2	1.00	
					30 vs 430 ppm	8.00	2	0.01	
Brain									
Undifferentiated Immune cells	%	2.00	2	0.37	.	.	.	.	

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

^I Lymphocytes (T-cells and B-cells) and myeloid cells (e.g. monocytes, microglia, and macrophages).

Table 7.

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

	30ppm vs 0ppm		430ppm vs 0ppm		30ppm vs 430 ppm	
	%	GMFI	%	GMFI	%	GMFI
HIPPOCAMPUS						
<i>Microglia</i>						
CCR7		↓				
MHCII						
MHCII ⁺ CCR7 ⁻						
MHCII ⁺ CCR7 ⁺						
CCR7 ⁺ MHCII ⁻			↓			
<i>Macrophages</i>						
CCR7						
MHCII						
MHCII ⁺ CCR7 ⁻						
MHCII ⁺ CCR7 ⁺				↑ ^I		
CCR7 ⁺ MHCII ⁻			↓			
<i>Undifferentiated Immune Cells</i> ²					↓	
BRAIN (not including hippocampus)						
<i>Microglia</i>						
CCR7						
MHCII					↓	
MHCII ⁺ CCR7 ⁻					↓	
MHCII ⁺ CCR7 ⁺						
CCR7 ⁺ MHCII ⁻						
<i>Macrophages</i>						
CCR7						
MHCII						
MHCII ⁺ CCR7 ⁻						
MHCII ⁺ CCR7 ⁺					↓ ^I	
CCR7 ⁺ MHCII ⁻						

¹Indicates significant results for MHC II in MHC II⁺CCR7⁺ cells.

²Indicates an undifferentiated sample of immune cells including lymphocytes (T-cells and B-cells) and myeloid cells (e.g. monocytes, microglia, and macrophages).

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