Toxicology and Applied Pharmacology 256 (2011) 179–190

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Sex-based differences in gene expression in hippocampus following postnatal lead exposure

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ARTICLE INFO

Article history:
Received 12 April 2011
Revised 3 August 2011
Accepted 5 August 2011
Available online 12 August 2011

Keywords:
Rat
Lead
Hippocampus
mRNA
Sex

ABSTRACT

The influence of sex as an effect modifier of childhood lead poisoning has received little systematic attention. Considering the paucity of information available concerning the interactive effects of lead and sex on the brain, the current study examined the interactive effects of lead and sex on gene expression patterns in the hippocampus, a structure involved in learning and memory. Male or female rats were fed either 1500 ppm lead-containing chow or control chow for 30 days beginning at weaning.Blood lead levels were 26.7 ±2.1 μg/dl and 27.1 ± 1.7 μg/dl for females and males, respectively. The expression of 175 unique genes was differentially regulated between control male and female rats. A total of 167 unique genes were differentially expressed in response to lead in either males or females. Lead exposure had a significant effect without a significant difference between male and female responses in 77 of these genes. In another set of 71 genes, there were significant differences in male vs. female response. A third set of 30 genes was differentially expressed in opposite directions in males vs. females, with the majority of genes expressed at a lower level in females than in males. Highly differentially expressed genes in males and females following lead exposure were associated with diverse biological pathways and functions. These results show that a brief exposure to lead produced significant changes in expression of a variety of genes in the hippocampus and that the response of the brain to a given lead exposure may vary depending on sex.

Introduction

Despite major initiatives to reduce environmental sources of exposure, lead poisoning remains an important public health problem in the United States and around the world. Although the toxic effects of lead have been known for centuries, it is only relatively recently that the effects of childhood lead exposure have become an issue of public health concern (Pueschel et al., 1996). Based on epidemiological and experimental studies conducted over the past 50 years there has been a gradual decrease in what is generally accepted as a "safe" level of lead exposure. Using venous blood lead levels as an index, the upper acceptable blood lead limit for children in the early 1960s was 60 μg/dl; in 1970 it was lowered to 40 μg/dl; in 1975 to 30 μg/dl; in 1985 to 25 μg/dl and finally, in 1991, to 10 μg/dl. However, data obtained over the last decade suggest detrimental effects of lead on cognition and behavior at blood levels <5 μg/dl and that there may be no threshold or "safety margin at existing exposures" (Koller et al., 2004) for the detrimental effects of lead on the brain and behavior (Anon., 2002, 2004; Chiodo et al., 2004; Lanphear et al., 2000). In addition, childhood lead poisoning does not have a particular "behavioral signature" (Finkelstein et al., 1998; Lidsky and Schneider, 2000) and the idiosyncratic nature of lead-induced cognitive and behavioral impairments in children is believed to be due to differences in age at first exposure, maximum lead levels and duration of exposure.

The additional influence of sex as an effect modifier of childhood lead poisoning has received little systematic attention. Few clinical studies have directly examined the influence of sex on outcome following childhood lead exposure and in the ones that have, the findings were mixed. McMichael et al. (1992) found that the inverse relationships between average postnatal blood lead concentration and children's abilities on tests of neuropsychological development assessed at 2 and 4 years of age were stronger for girls than for boys, after adjustments for a number of other possible confounding factors. Rabinowitz et al. (1991) also reported a stronger negative correlation between dentine lead levels and intelligence scores in girls than in boys. In contrast, Dietrich et al. (1987) reported greater developmental neurobehavioral deficits in males during early infancy as a result of prenatal exposures, assessed by maternal blood lead levels. Bellinger et al. (1990) also reported that boys were less able than girls to overcome early cognitive deficits associated with higher prenatal lead exposures. Tong et al. (2000) reported that

Abbreviations: RMA, Robust Multichip Average; IPA, Ingenuity Pathway Analysis; RT-PCR, Real Time Polymerase Chain Reaction; MDD, Major Depressive Disorder; NMDAR, N-Methyl-D-Aspartate Receptor.

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doi:10.1016/j.taap.2011.08.008

girls were more sensitive to the effects of lead than boys, with the interaction between lifetime average blood lead concentration and IQ being more pronounced in girls (11–13 years of age) than in boys. Jedrychowski et al. (2009) assessed the relationship between very low-level prenatal lead exposure (<5 μg/dL in cord blood) and gender-specific cognitive development during the first 3 years of life and reported cognitive deficits in males at 3 years of age but not in females, suggesting different susceptibilities to prenatal lead exposure across sex groups. Recently, the relationship between childhood lead exposure and adult brain volume using magnetic resonance imaging was examined and region-specific reductions in adult gray matter volume were found, particularly in portions of the prefrontal cortex and anterior cingulate cortex and were associated with higher childhood blood lead concentrations (Cecil et al., 2008). These findings were more pronounced for males, suggesting that childhood lead exposure has a different impact on the male brain compared to the female brain.

Few animal studies have systematically studied the influence of sex on outcome from lead exposure. Leasure et al. (2008) showed that low-level gestational lead exposure in mice resulted in several permanent male-specific motor deficits as well as late-onset obesity in males. In another study, female rats exposed to lead through gestation and lactation were more impaired in reference memory than male rats with similar lead exposures (Jett et al., 1997). De Souza Lisboa et al. (2005) reported that exposure to lead during both pregnancy and lactation induced depressive-like behavior (detected in the forced swimming test) in female but not male rats. In another study (Soeiro et al., 2007), exposure of mice to 50 and 500 ppm lead acetate from weaning to adulthood induced what was described as an anti-depressant-like effect in both male and female mice, whereas exposure to 500 ppm lead acetate induced an anxiogenic effect only in male mice. The combined effects of lead and stress have been well-documented and are potentiated in female rats (Cory-Slechta et al., 2004).

Considering the potential importance of the influence of sex on outcome following lead exposure and the paucity of information available concerning the interaction of lead and sex on the brain, the current study was performed to assess the effects of lead on gene expression patterns in one brain structure, the hippocampus, a region known to be sensitive to the effects of lead exposure and a brain region that has been well-studied for several decades in relation to effects of lead on its structure and function.

Materials and methods

Animals. The treatment of animals was in compliance with NIH guidelines for the care and use of laboratory animals and the study was approved by the institutional animal care and use committee at Thomas Jefferson University. Thirty-one Long Evans rats, 16 male and 15 female (Harlan Labs), were divided into two groups (lead exposed or control) of four per gender on postnatal day 25 (animals were weaned on postnatal day 21 at Harlan Labs). Animals received either lead-containing chow (Purina RMH 1000 compounded with 1500 ppm lead acetate) or control chow (Purina RMH 1000) ad libitum for 30 days prior to being euthanized. At the start of the exposure period the weight range for control animals was 42 g–53 g for males and 33 g–46 g for females; the weight ranges for animals assigned to lead exposure were 44 g–52 g for males and 33 g–47 g for females. All animals were exposed to a 12 h:12 h light:dark cycle for the duration of the experiment. Other than their differences in diet, all animals were housed and handled in exactly the same manner during the study and prepared as two independent cohorts with 4 animals per gender and experimental group in each cohort. Animals were euthanized on postnatal day 55 by decapitation and hippocampi were rapidly removed, flash frozen on dry ice and stored at −80 °C until processed.

Blood samples were collected at the time of euthanasia from all animals and analyzed for lead levels by graphite furnace atomic absorption with Zeeman background correction (ESA Labs, MA).

RNA extraction and processing. Total RNA was extracted from the hippocampus using the Qiagen RNeasy Kit according to the manufacturers protocol (Valencia, CA). Briefly, samples were homogenized in a micro-pestle and mortar (Kontes Inc) prior to being processed through spin columns. All samples were then assessed for quantity/quality and purity using both an Agilent 2100 Bioanlayzer and a GE Nanovue spectrophotometer prior to further processing. Acceptance criteria for RNA extraction was a 260 nm/280 nm ratio of 2.0 by UV analysis and a RNA integrity number of greater than 8.5 on the Bioanalyzer. RNA was amplified using the affymetrix (Santa Clara, CA) HT one-cycle target labeling kit using 1 μg total RNA to make 15 μg cRNA and the labeled RNA samples were hybridized to Affymetrix Rat Gene 1.0 ST RNA Arrays using standard methods according to Affymetrix at the Cancer Genomics Laboratory, Kimmel Cancer Center, Thomas Jefferson University.

Microarray analyses: normalization and outlier removal. The data normalization and statistical analysis to identify differentially expressed genes were performed using Partek Genomics Suite (Partek Inc., St. Louis, MO). The raw gene expression data was normalized using the standard Robust Multichip Average (RMA) approach (Irizarry et al., 2003). Principal Component Analysis revealed two of the female rat samples as outliers and these were excluded in further study. The RMA normalization was repeated for the remainder of the arrays after removal of the outlier samples.

Gender-dependent differential gene expression in control animals. The normalized data for the control male and female animals was analyzed using a two-tailed Student's T test with unequal variances for the two groups. The resulting p-values were corrected for multiple testing using a *q-value* approach using the *q-value* library implemented in the Bioconductor libraries (Gentleman et al., 2004) for the R Project for Statistical Computing (http://www.r-project.org). This approach estimates the proportion of non-differentially regulated genes and hence improves the sensitivity of the analysis (Storey and Tibshirani, 2003).

Differential gene expression in response to lead exposure. Normalized data were analyzed using a 3-way mixed effects ANOVA (implemented in Partek Genomics Suite, Partek Inc, St Louis, MO) that considered the following two variables and their interactions as fixed effects: (1) gender (male or female) and (2) lead exposure (0, 1500 ppm). The microarray batch (two separate runs, as reflected in the array scan date) was considered as the random effect to account for run-to-run differences across arrays. We analyzed the data using a Restricted Maximum Likelihood (REML) approach that is generally preferred for partially balanced or unbalanced experimental designs. Differentially expressed genes were identified based on statistically significant effects of lead exposure, gender or an interaction between these two factors. The raw *p-values* from ANOVA were corrected for multiple testing using the standard q-value approach (Storey and Tibshirani, 2003). A q-value threshold of 0.3 was used to identify differentially expressed genes. We chose a q-value of 0.3 in order to improve the sensitivity for detecting differential expression with significant interaction between Gender and Treatment factors. A q-value threshold of 0.3 yielded 33% more genes with interaction than at 0.2 threshold value.

Gene sets affected by lead exposure and visualization of sample groups. The list of differentially expressed genes from ANOVA was filtered based on a minimum fold change threshold of 1.3 up or down regulation in response to lead exposure in male or female animals. The fold-change filtered differentially expressed genes were considered further as three separate sets based on the ANOVA term that was statistically significant: