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Sex- and tissue-specific methylome changes in brains of mice perinatally exposed to lead

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ABSTRACT

Changes in DNA methylation and subsequent changes in gene expression regulation are the hallmarks of age- and tissue-dependent epigenetic drift and plasticity resulting from the combinatorial integration of genetic determinants and environmental cues. To determine whether perinatal lead exposure caused persistent DNA methylation changes in target tissues, we exposed mouse dams to 0, 3 or 30 ppm of lead acetate in drinking water for a period extending from 2 months prior to mating, through gestation, until weaning of pups at postnatal day-21, and analyzed whole-genome DNA methylation in brain cortex and hippocampus of 2-month old exposed and unexposed progeny. Lead exposure resulted in hypermethylation of three differentially methylated regions in the hippocampus of females, but not males. These regions mapped to *Rn4.5s*, *Sfi1*, and *Rn45s* loci in mouse chromosomes 2, 11 and 17, respectively. At a conservative fdr < 0.001, 1623 additional CpG sites were differentially methylated in female hippocampus, corresponding to 117 unique genes. Sixty of these genes were tested for mRNA expression and showed a trend toward negative correlation between mRNA expression and methylation in exposed females but not males. No statistically significant methylome changes were detected in male hippocampus or in cortex of either sex. We conclude that exposure to lead during embryonic life, a time when the organism is most sensitive to environmental cues, appears to have a sex- and tissue-specific effect on DNA methylation that may produce pathological or physiological deviations from the epigenetic plasticity operative in unexposed mice.

1. Introduction

Environmental signals enable organisms to react and adapt to changing living conditions. More than a filter that selects potential phenotypic variations, the environment is itself the source of the variation through cues that enable the developing organism to increase its fitness in that particular environment (West-Eberhard, 2005). Because embryonic life is a time when the organism is most sensitive to environmental signals (Yamazaki et al., 2003), this phenotypic plasticity is particularly critical during development. Developmental plasticity, however, is not always adaptive and often gives rise to maladaptive pathophysiological consequences either in the embryo or in later adult life, as is the case with the responses to lead exposure. There is a good agreement that the most important cognitive, behavioral and psychiatric health effects of lead exposure are manifest long after exposure has ceased (Wright et al., 2008; Yuan et al., 2006), suggestive of either a genetic (mutational) or an epigenetic component. However, the causes of the long-term morbidity associated with prenatal and early postnatal exposure to lead are poorly understood. The variability in genetic or epigenetic factors as exacerbating or protective agents of human neurodevelopmental morbidity has not been adequately examined in relationship to early exposure to lead. Studies linking attention deficits, aggressive and disruptive behavior, and poor self-regulation have shown that early exposure to lead results in an increased likelihood of engaging in antisocial behavior in later life (Dietrich et al., 2001; Needleman et al., 1996, 2002; Wright et al., 2008). Current debate centers on the identification of the developmental periods during which the organism is most vulnerable to the effects of lead and on the exposure level and duration that produce adverse effects. Risk

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factors and biomarkers are needed to identify individuals at high risk for lead-associated maldevelopment.

In humans, early life exposure to lead can produce persistent alterations in the brain structure of adults, including loss of gray matter in the cortex (Brubaker et al., 2009; Cecil et al., 2008), changes in myelin structure in white matter (Brubaker et al., 2009), and low level of activation in brain areas associated with language function, such as left frontal cortex and left middle temporal gyrus (Yuan et al., 2006). Additionally, mice exposed to lead *in utero* have also neurochemistry alterations in the hippocampus, including increment of myoinositol/creatine (Ins/Cr) and glutamine (Gln) (Lindquist, unpublished). Recently, gestational lead exposure in Wistar rats was shown to reduce the number of pyramidal cells in the hippocampus (Baranowska-Bosiacka et al., 2013). In addition, differentiation of embryonic stem cells into glutamatergic neurons in the presence of lead caused alterations in the expression of glutamate receptor subunits *Grin1*, *Grin2D*, *Grik5*, *Gria4*, and *Grm6* that were also observed in hippocampus and cortex of mice gestationally exposed to this metal (Sanchez-Martin et al., 2013). Using primary rat hippocampal cultures, lead was found to negatively modify important neuronal pathways implicated in synaptic plasticity, such as learning, memory, and cell survival (Guilarte and McGlothan, 2003; Neal et al., 2011). These *in vivo* and *in vitro* findings suggest that cortex and hippocampus are the key target tissues of lead toxicity in the brain.

Heavy metals such as lead elicit environmental signals that modulate epigenetic mechanisms often associated with regulation of gene expression, of which DNA methylation at CpG sites is the most common (Rountree et al., 2001). Expression and activity of DNA methyltransferases (DNMTs) are highly regulated in the central nervous system (CNS) (Feng et al., 2005). Important genes triggered during memory formation and synaptic plasticity, such as Reelin and brain-derived neurotrophic factor (BDNF), show dramatic changes in promoter methylation when DNMT activity is inhibited in hippocampus of young adult mice (Levenson et al., 2006), leading to the hypothesis that DNMT activity may be crucial to regulate brain function. Consistent with this hypothesis, 700-day old mice exposed during gestation to lead showed changes in the induction or repression of 150 genes that correlated with their DNA methylation profiles (Dosunmu et al., 2012). Strikingly, *Macaca fascicularis* monkeys exposed to lead during infancy showed epigenetic changes twenty-three years later that caused reduced levels of total DNA methylation, DNA methyltransferases-1 and -3A, methyl CpG binding protein-2, and modified histone marks critical for the regulation of gene expression. As a result of these changes, the aging brains of these monkeys showed elevated expression of Alzheimer disease-related genes, including β-amyloid precursor protein (APP) and β-site APP cleaving enzyme 1 (BACE1), as well as an increase of total amyloid plaques in the cortex (Bihaqi et al., 2011; Wu et al., 2008).

We used DNA methylation analyses to determine whether prenatal to early postnatal exposure to lead acetate were associated with persistent DNA methylation changes in the brain tissues of exposed mice. Our results show that, at a time point when blood lead levels of perinatally exposed and unexposed adult mice are undistinguishable from each other, there is a highly significant change in DNA methylation in the specific brain regions of the exposed mice, with a trend to be negatively correlated to gene expression levels. The effect is sex- and tissue-dependent, with females showing greater hypermethylation than males, and more so in hippocampus than cortex. Exposure to lead during embryonic life appears to have a sex- and tissue-specific effect that may produce pathological or physiological deviations from the epigenetic plasticity operative in unexposed mice. Further analyses to correlate DNA methylation and regulatory gene expression changes will be crucial to understand the mechanisms of lead neurotoxicity.

2. Materials and methods

2.1. Animals and lead exposure

C57BL/6 mice (Charles River) were housed in the Vivarium at the Cincinnati Children's Hospital Medical Center under standard conditions (10 h light/14 h dark) and given *ad libitum* access to food and water. The Animal Care and Use Committees of the Cincinnati Children's Hospital Medical Center and the University of Cincinnati approved all experimental procedures conducted with these mice. Mice were treated humanely and with regard for alleviation of suffering. Female mice were given drinking water containing 0, 3 (low dose), or 30 ppm (high dose) lead acetate (Sigma Aldrich, St. Louis, MO), approximately 60- and 600-fold, respectively the current action level, for a minimum of 2 months prior to mating to stabilize their circulating lead levels and were maintained on this water through weaning. Male breeding mice were exposed to leaded water only while they were with the females. Pups were weaned at post-natal day 21 and were put on normal drinking water for the duration of the experiment. Drinking patterns and water consumption showed no appreciable differences between the groups. One male and one female from each of four litters were evaluated at post-natal day 60 (±1 day) at a time when blood was collected for blood lead analysis and brain regions were collected for DNA methylation analyses. Blood and tissue samples were stored at −80 °C until analyzed.

2.2. Tissue collection and total DNA isolation

Brain cortex and hippocampus were collected by dissection using a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). Total DNA from the two brain areas was isolated using a DNeasy blood and tissue kit from Qiagen following the manufacturer's protocols. The incubation time for lysis was 60 and 150 min for cortex and hippocampus, respectively.

2.3. Global methyl-seq analysis

To prepare the sequencing library we used the SureSelect Methyl-Seq Target Enrichment System for mouse kit (Agilent, Santa Clara, CA). Following Agilent recommended conditions, we used 2–3 μg of high quality mouse genomic DNA sheared in a Covaris S2 focused-ultrasonicator (Covaris, Woburn, MA) to a size of 150–200 bp, as validated by 2100 Bioanalyzer (Agilent). The DNA fragments were end-repaired, 3′-end adenylated and ligated to the methylated adaptor. The size of the ligated libraries was validated in the Bioanalyzer, followed by hybridization with biotin-labeled RNA-baits to capture the regions where methylation impacts gene regulation, including CpG islands, CpG island shores, undermethylated regions, promoters, and differentially methylated regions. After hybridization, the libraries were captured with streptavidin beads, bisulfite-modified with the EZ DNA Methylation-Gold kit (Zymo, Irvine, CA), and enriched by 8 cycles of PCR. Individually amplified libraries were labeled with unique indices by 6 cycles of PCR and purified and size-selected using AMPure XP beads (Beckman Coulter, Indianapolis, IN). The quality and quantity of the libraries were assessed by the Bioanalyzer High Sensitivity DNA assay. To accurately quantify the library concentration for clustering generation, the libraries were diluted $1{:}10^6$ in a buffer containing 10 mM Tris–HCl, pH 8.0 and 0.05% Tween 20, and analyzed by qPCR using a Kapa Library Quantification kit (Kapabiosystem, Woburn, MA) in the ABI's 9700HT Fast Real-Time PCR System (Lifetech, Grand Island, NY).