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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, 1,623 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 towards 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.

Keywords

DNA methylation; Brain; Gene expression; Lead; Heavy metals

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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 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; Needleman *et al.* 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 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) is 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 diseaserelated 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.

MATERIALS AND METHODS

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 postnatal 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.

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 minutes for cortex and hippocampus respectively.

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).

Cluster generation and HiSeq sequencing

Equal amounts of four uniquely indexed libraries were pooled to fill one lane of the flow cell for clustering in the cBot system (Illumina, San Diego, CA). The pooled libraries at a final concentration of 15 pM were clustered onto a flow cell using Illumina's TruSeq PE Cluster kit v3, and sequenced for paired-end 2×100 cycles using TruSeq SBS kit v3 on Illumina HiSeq system according to the manufacturer's recommended protocol. All library preparation, clustering and sequencing steps were performed by the Genomics, Epigenomics and Sequencing Core of the University of Cincinnati.

Statistical differential methylation analysis

Methyl-seq data was demultiplexed and converted to fastq files using Illumina's CASAVA 1.8. The sequencing quality was assessed by FastQC software (Andrews 2014). Paired-end

sequence was aligned to UCSC mm10 mouse genome using Bismark (Krueger and Andrews 2011) with default parameters. Bismark was then used to extract methylation status from each read. This resulted in counts of methylated and un-methylated reads at each CpG site in every sample. In order to identify differentially methylated CpG sites caused by lead exposure, the male and female lead-exposed samples were separately compared to control samples of the same sex. The beta-binomial test from ibb R package (Pham *et al.* 2010) was used to test for differential methylation on the counts. Basically, the count of methylated C of a CpG site was distributed according to a binomial distribution with success probability p in n total reads of the site, and p was modeled as a random variable from a beta distribution. Since the differential methylation analysis was performed on raw counts, no filtering on coverage was performed. A Manhattan plot was used to visualize the distribution of differentially methylated CpG sites on the genome. Significant differentially methylated CpG sites were selected with FDR adjusted p-values of <0.001, <0.01, or <0.1. Two adjacent significant CpG sites were combined into one significant region if they were less than 1000 bp apart from each other. The significant region was extended until there were no more significant CpG sites within 1000 bp from the current region. The closest genes of significant sites and regions were identified using Bioconductor package VariantAnnotation (Obenchain *et al.* 2014).

Reverse transcription and real-time PCR

Total RNA was isolated from the tissue samples by using the RNeasy Mini kit (Qiagen). Reverse transcription was performed using random hexamer primers and SuperScript III transcriptase (Invitrogen) as indicated previously (Wang *et al.* 2010). Real-time qPCR was performed to quantify the expression levels of different genes which were normalized to *Gapdh* mRNA. Supplemental Table S1 shows a list of primers used for each gene. Raw data was analyzed using the $2^{\Delta\Delta Ct}$ method and it is shown as the $\log_2$ of the normalized level.

RESULTS

Blood lead levels in PND60 mice

We exposed dams to 0, 3, or 30 ppm lead acetate in drinking water for a minimum of 2 months prior to mating and maintained these conditions through weaning, while 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 given drinking water without lead for the duration of the experiment. At PND60, the time when they were subjected to DNA methylation analyses, we found no significant difference in blood lead values among the three groups of pups. Blood lead levels in pups (n=16 per group) of control, 3 ppm- and 30 ppm-exposed dams were 1.2 ± 1.1, 0.9 ± 0.8 and 1.3 ± 3.1 μg/dl, well below the current action level of 5 μg/dl, in good agreement with values observed in unexposed mice fed a normal diet (Ercal *et al.* 1996; Iavicoli *et al.* 2003) and consistent with prior observations by others (Virgolini *et al.* 2008; Widzowski *et al.* 1994).

CpG site coverage

Bismark alignment was performed after bisulfite sequencing to analyze the coverage of CpG data reads obtained. The number of CpG sites covered was higher in cortex than in

hippocampus samples, ranging between 1.5 million for males and 1.2 million for females in both groups of lead exposure. In the female hippocampus, sequencing covered slightly more than 0.8 million CpG sites for mice exposed to 3 ppm and 1.5 million CpG sites when exposed to 30 ppm. In the male hippocampus, the coverage was 0.8 million CpG sites for both lead doses (Supplemental Table S2).

Comparison of DMR methylation levels in exposed and unexposed mice

CpG site methylation in cortex and hippocampus at various lead doses relative to the unexposed control groups showed a large number of differentially methylated sites at *p*-values between 1×10^{-5} and 5×10^{-8} and below, the later corresponding to the Bonferroni correction for an approximate one million multiple tests at $p\leq0.05$. The distribution of differentially methylated sites appears to be fairly homogeneous across all chromosomes, except for the X chromosome, which as expected is significantly hypomethylated in males compared to females (see comparison of male *vs* female data for cortex in Supplemental Fig. S1). Using $p\leq5\times10^{-8}$ as the threshold for statistical significance, no significant hyper- or hypomethylated sites were detected in the hippocampus (Fig. 1) or cortex (Fig. 2) of male mice exposed to either 3 (panels A and C) or 30 (panels B and D) ppm lead doses. In contrast, three prominent hypermethylation regions were found in the hippocampus of females exposed to both 3 ppm (Fig. 3A) and 30 ppm doses (Fig. 3B). These three DMRs mapped to *Rn4.5s* in chromosome 2, *Sfi1* in chromosome 11, and *Rn45s* in chromosome 17; of the three sites, *Sfi1* was also hypermethylated in cortex of female mice exposed to both 3 and 30 ppm of lead, albeit at a lower significance level (Fig. 4A and 4B). In contrast to the hypermethylation results, no lead dose induced local DNA hypomethylation (Figs. 1–4, panels C and D).

In addition to the three prominent regions described above, a number of hyper- and hypomethylated sites were evident in the Manhattan plots in Figs. 1–4 with *p*-values comprised between the 1×10^{-5} and 5×10^{-8} thresholds indicated earlier. We set three different false discovery rate (fdr) values to categorize the significance of these sites and determine whether specific regions of the genome showed statistically significant changes in methylation patterns due to lead exposure. For this analysis, we considered that a group of contiguous sites plus 500 bp at either side constituted a genome region associated with its corresponding gene locus. At fdr≤ 0.1, most of the female genome showed methylation changes after exposure to 3 or 30 ppm of lead, with greater than 160,000 CpG sites, 60,000 genome regions and 16,000 genes differentially methylated in hippocampus. No significant number of sites were affected in the hippocampus of exposed males (Table 1). At this cut-off level, cortices in both sexes showed significant differential methylation, 10-times higher in females than in males (Supplemental Table S3).

At fdr≤0.01, we found 20,129 differentially methylated sites in female hippocampus, corresponding to 12,435 genome regions encompassing 6,180 genes (Table 1). At this cut-off value, neither the hippocampus of males nor the cortex of females exposed to lead presented significant sites with changes in the methylation pattern, but we found112 hypermethylated sites in the cortex of males exposed to 3 ppm and 181when exposed to 30 ppm (Supplemental Table S3).

At fdr≤0.001, we found 1,623 differentially methylated CpG sites in the hippocampus of exposed females only, and no significant sites in cortex of either sex. Of these 1,623 CpG sites, 322 corresponded to females exposed to 3 ppm and 1,537 (with some overlap with the previous dose) to 30 ppm exposure, encompassing 222 regions of the genome and affecting 117 unique genes (Table 1 and Supplemental Tables S3 and S4). Interestingly, most of the 1,623 significant CpG sites observed in the hippocampus at this cut-off were hypermethylated after lead exposure with little evidence of hypomethylation (Fig. 5 and Supplemental Table S4). In addition, data from male and female differential methylation clustered together for tissue and treatment dose, but separately for sex (Fig. 5), indicating that perinatal lead exposure induces differential methylation in male and female brain tissues, albeit at very different levels.

Gene expression analysis by real-time PCR

We used real-time PCR to analyze the expression of 60 of the 117 differentially methylated unique genes at fdr≤0.001 in the hippocampus of male and female mice exposed to 0 and 3 ppm lead. The selected genes had differentially methylated CpG sites with $p<0.05$ for females exposed to both lead doses, and the sites were located between −10 and +10 kb from the transcription start site (Supplemental Table S4). Expression of these genes in lead exposed mice, whether male or female, was very similar to their expression in control mice when the genes were analyzed individually (Supplemental Fig. S2); however, there was a trend towards a significant negative correlation between methylation change and mRNA level ($p_{slope}=0.083$; $r^2=0.327$) in the hippocampus of female mice exposed to 3 ppm lead (Fig. 6A) and not in the males ($p_{slope}=0.996$; $r^2=0.002$) (Fig. 6B). These data suggest that lead not only induces hypermethylation in the female hippocampus, but also that it may silence the expression of genes involved in brain function as a consequence of epigenetic modification.

DISCUSSION

In this study we show that *in utero* and lactational lead exposure induces persistent DNA methylation changes in hippocampus and cortex, two brain tissues that are lead targets in mice. The majority of changes result from DNA hypermethylation and are highly sex dependent, with female mice substantially more affected than males. Of the two brain tissues, the hippocampus had significantly higher levels of differentially methylated CpG sites than the cortex. At a restrictive p-value≤5×10^{-8}, only CpG sites in the *Sfi1* gene were hypermethylated in the female cortex at both exposure levels. In contrast, exposure led to high levels of hypermethylation in the hippocampus of females, that affected sites in three loci, *Rn4.5s*, *Sfi1*, and *Rn45s*, mapping to chromosomes 2, 11, and 17, respectively, showing a striking degree of DNA hypermethylation. At a conservative fdr≤0.001, we found 1,623 differentially methylated CpG sites in the hippocampus of exposed females, the majority (>90%) showing hypermethylation. These sites defined 222 regions of the genome, corresponding to an additional 117 unique genes. After analyzing the expression of 60 from this group, we found a trend towards a significant negative correlation between expression and methylation change in exposed female mice, but not males. This analysis is inherently limited for it examines only a single time point of expression and does not take into

consideration the timing of expression of the genes tested. These genes are distributed throughout the genome and do not appear to be related through either regulatory or functional connections, even though the methylation changes occur reproducibly in multiple mice at the same locations.

Early life exposure to lead may have toxic effects in the developing brain. Lead exposure during early childhood has been linked to deficits in cognitive functions and IQ, behavioral effects, and attention deficit hyperactivity disorder (Bellinger *et al.* 1994; Chen *et al.* 2007; Froehlich *et al.* 2009), suggesting that lead neurotoxicity may result from the alteration of mechanisms like DNA methylation that regulate transcriptional pathways contributing to synapse function, neurogenesis and ultimately, expression of memory-related genes. Lead has recently been proposed to act in a locus-specific way on the epigenome, depending on the genomic features in which affected CpG sites are located (Faulk *et al.* 2013; Faulk *et al.* 2014). Our current results are in conceptual agreement with this hypothesis.

Lead exposure alters the expression of genes involved in DNA methylation, such as methyl-cytosine-phosphate-guanine binding protein-2 (MeCP2) and DNA methyltransferases-1 and -3A (Bihaqi *et al.* 2011; Schneider *et al.* 2013; Wu *et al.* 2008). Exposure to lead from gestational day 13 to PND-20 resulted in significant changes of gene expression in cortical regions of mouse brain, at 20 and 700 days of age; these changes were correlated with changes in DNA methylation profile and repressed many genes normally up-regulated during normal aging, suggesting that early life exposure to lead disturbs developmental processes in the brain and compromises its ability to cope with adult challenges (Dosunmu *et al.* 2012). Hypermethylation of genes involved in neurogenesis signaling pathways has also been found in neuronal precursor cells derived from human embryonic stem cells chronically exposed to lead. These cells exhibit shorter neurites and less branching, as well as a significant decrease in the expression levels of the neural marker genes PAX6 and MSI1(Senut *et al.* 2014).

Methylation and expression show a strong sex-dependence, with changes more evident in females than in males. Similar sex-specific effects have been observed as the consequence of maternal separation, which caused repression of BDNF expression in hippocampus of C57BL/6J female mice but not males, and hypermethylation in males but not females (Kundakovic *et al.* 2013). Brain differences between males and females are a common phenomenon, since sexual differentiation in the brain takes place during a perinatal sensitive window as a result of gonadal hormone-induced developmental organization (Auger and Auger 2013; Chung and Auger 2013; Menger *et al.* 2010). Sex differences in gene expression patterns and in the regulation of genes coding for DNA methylases have been observed in hippocampus and frontal cortex of rats exposed to lead (Schneider *et al.* 2011; Schneider *et al.* 2012b), and shown to be differentially expressed depending on the developmental timing of the exposure (Schneider *et al.* 2012a). The effect of sex in the regulation of the genome and epigenome is largely unexplored. Sex influences genomic methylation status although the mechanisms underlying this effect are unknown (Liu *et al.* 2010). Possibly, interactions between sex hormones and lead exposure during development make the female hippocampus more susceptible to differentially methylation. In contrast to our results, observations in humans, have shown that early exposure to lead causes

neuropsychological alterations in mid-adolescent males not, so much in females (Ris *et al.* 2004). These epigenetic sex differences observed in mice may be independent consequences of exposure, underlining the complexity of the sexspecific response to early life adversities that can affect the epigenetic regulation of gene expression in males and females.

Rn4.5s codes for a 98-nucleotide nuclear RNA with unknown function that is transcribed by RNA polymerase III (Gogolevskaya *et al.* 2010) and *Rn45s* codes for the RNA precursor to 18S, 5.8S and 28S rRNA (Grozdanov *et al.* 2003); both of them show changes in methylation in the hippocampus of females exposed to 3 ppm of lead (Supplemental Figs. S3 and S4). Although neither of these two genes has been linked to metal toxicity or lead neurotoxicity previously, their hypermethylation by lead exposure may compromise ribosome structure or overall protein synthesis capacity in the hippocampus. Changes in expression of *Sfi1* have been observed in a genetic mouse model of neurodevelopmental disorder, being up-regulated in young brain and down-regulated in older brain (Trent *et al.* 2014). This gene has two regions that are differentially methylated in the hippocampus of females exposed to 3 ppm of lead (Supplemental Fig. S5) but its expression is not changed in either sex (Supplemental Fig. S2) possibly because the gene is already highly methylated and changes in these two regions are not enough to alter the expression levels.

The promoter of the *Dynlt1b* (dynein light chain Tctex-type 1B) gene is hypermethylated in the hippocampus of females exposed to 3 and 30 ppm of lead (Supplemental Fig. S6). In the dentate gyrus of the hippocampus, new neurons continue to be generated throughout life from progenitor cells at the subgranular zone (Eriksson *et al.* 1998; Kornack and Rakic 1999). These newborn neurons serve not only to maintain the pool of neurons, but also to build memory (Ernst *et al.* 2014; Nakashiba *et al.* 2012). *Dynlt1b* transcription is high in these cells (Dedesma *et al.* 2006), and acts as a regulator for the genesis of neurons (Gauthier-Fisher *et al.* 2009; Tseng *et al.* 2010) and possibly for neurite outgrowth and axon formation as well (Sachdev *et al.* 2007).

Although the expression level is not changed at PND60 after early life exposure to lead (Supplemental Fig. S2), it is plausible that prenatal and early postnatal exposure to lead may inhibit the formation of new neurons, decrease the total pool of neurons, and eventually compromise memory formation. Although the use of lead has been reduced in the last few decades, exposure is still an important concern due to its non-biodegradable nature and its ubiquitous presence, which poses a potential health risk as a result of the increased sensitivity of children to lead toxicity (Markowitz 2000). During the gestational period, lead crosses the placenta and blood-brain barrier reaching the developing fetal brain (Hu *et al.* 1998). It is becoming increasingly evident that early-life exposure to lead may produce enduring changes in the epigenetic mechanisms that regulate gene expression in the brain, contributing to pathological and physiological outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Perinatal lead exposure caused persistent DNA methylation changes in brain
- Lead exposure caused hypermethylation in the hippocampus of females, but not males
- 1,623 CpG sites, corresponding to 117 unique genes, were differentially methylated
- 60 of these genes showed negative correlation between mRNA expression and methylation
- Developmental lead exposure has sex- and tissue-specific effects on DNA methylation

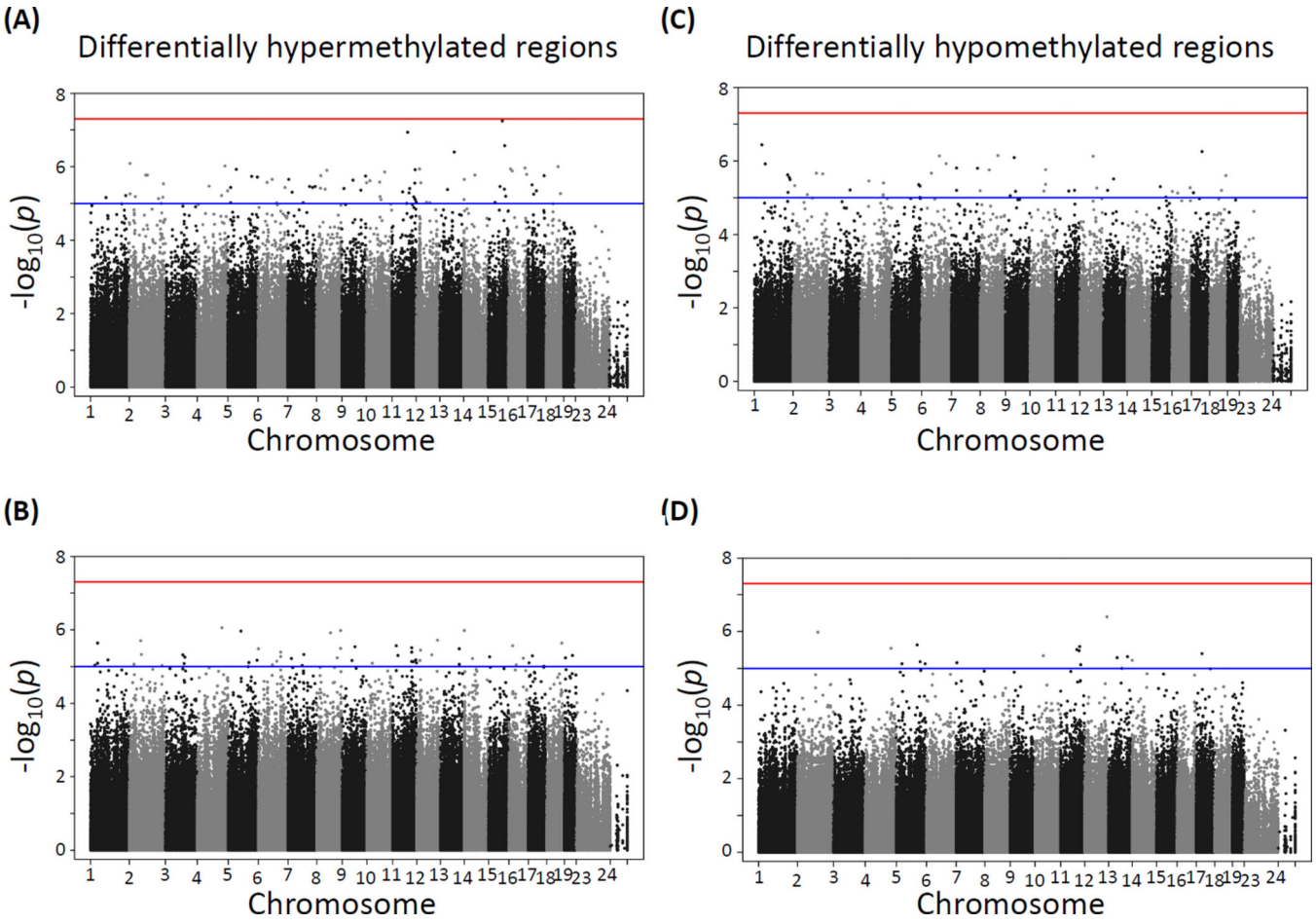

Figure 1. Manhattan plot of −log$_{10}$ *p*-values of differential methylation across the mouse genome induced by lead acetate in the hippocampus of male mice

(A) and **(B)**: differentially hypermethylated regions in mice exposed to 3 ppm **(A)** and 30 ppm **(B)** of lead. **(C)** and **(D)**: **d**ifferentially hypomethylated regions in mice were exposed to 3 ppm **(C)** and 30 ppm **(D)** of lead.

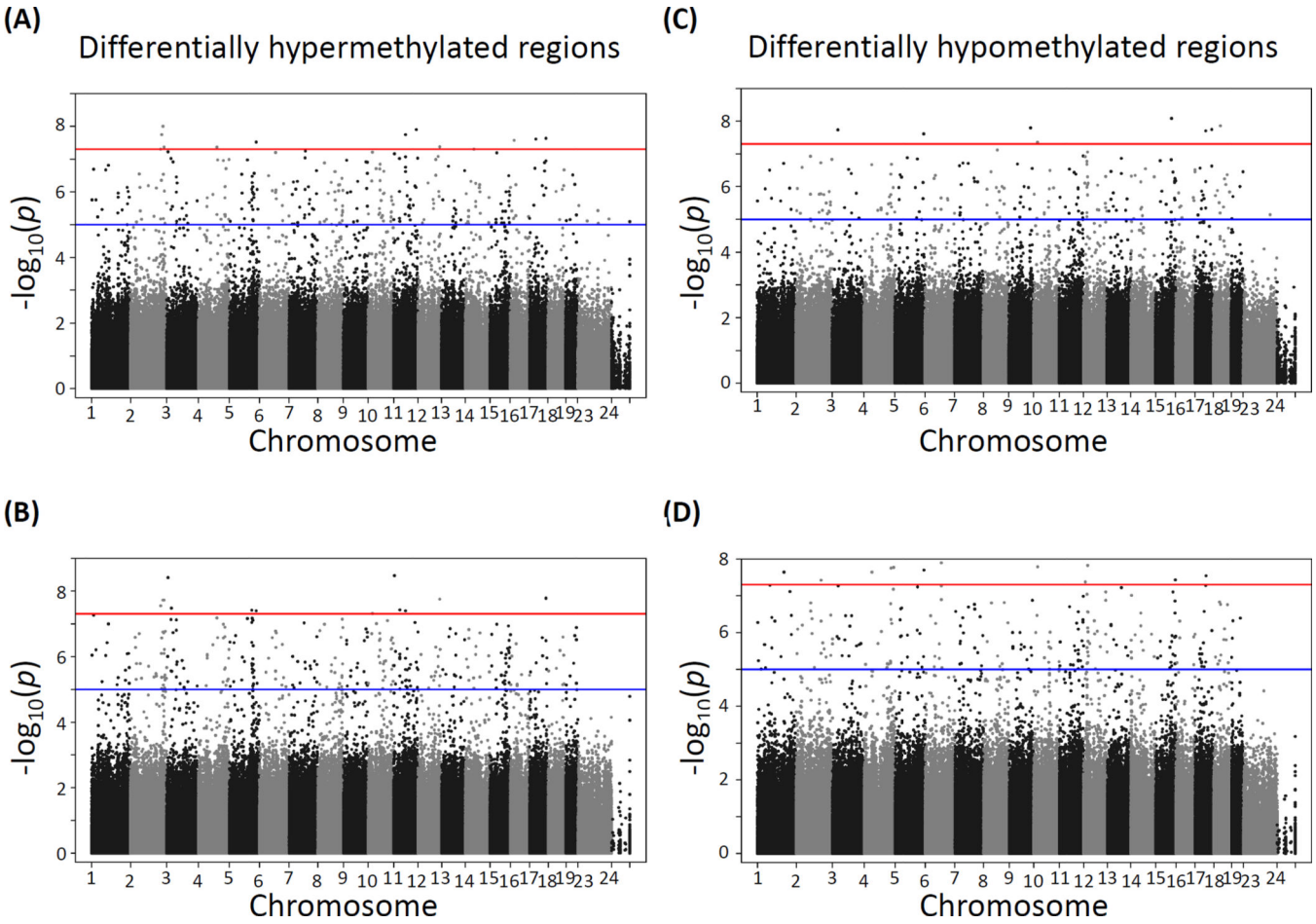

Figure 2. Manhattan plot of −log$_{10}$ *p*-values of differential methylation across the mouse genome induced by lead acetate in the cortex of male mice

(A) and **(B)**: differentially hypermethylated regions in mice exposed to 3 ppm **(A)** and 30 ppm **(B)** of lead. **(C)** and **(D)**: **d**ifferentially hypomethylated regions in mice were exposed to 3 ppm **(C)** and 30 ppm **(D)** of lead.

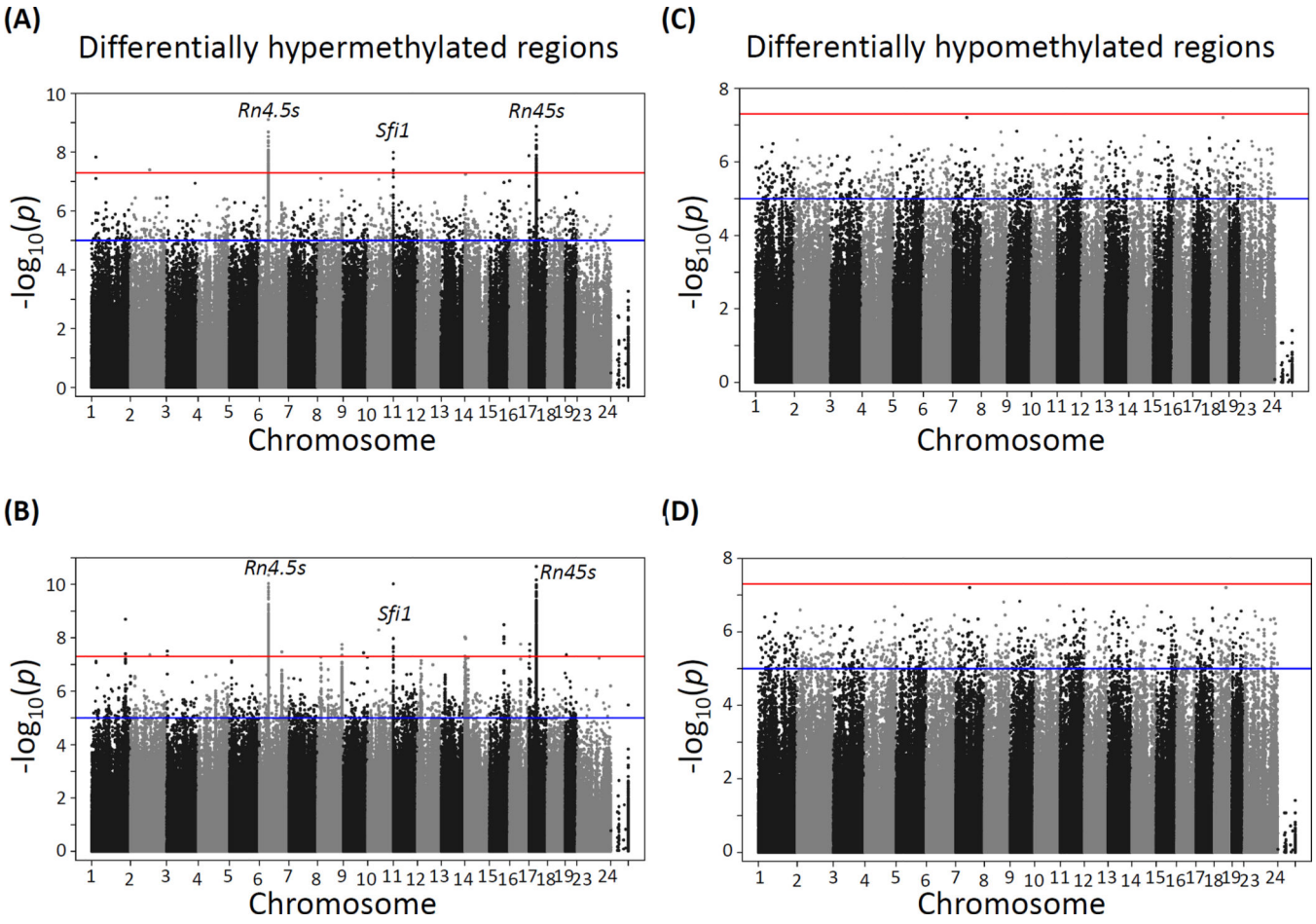

Figure 3. Manhattan plot of −log$_{10}$ *p*-values of differential methylation across the mouse genome induced by lead acetate in the hippocampus of female mice

(A) and **(B)**: differentially hypermethylated regions in mice exposed to 3 ppm **(A)** and 30 ppm **(B)** of lead. **(C)** and **(D)**: **d**ifferentially hypomethylated regions in mice were exposed to 3 ppm **(C)** and 30 ppm **(D)** of lead.

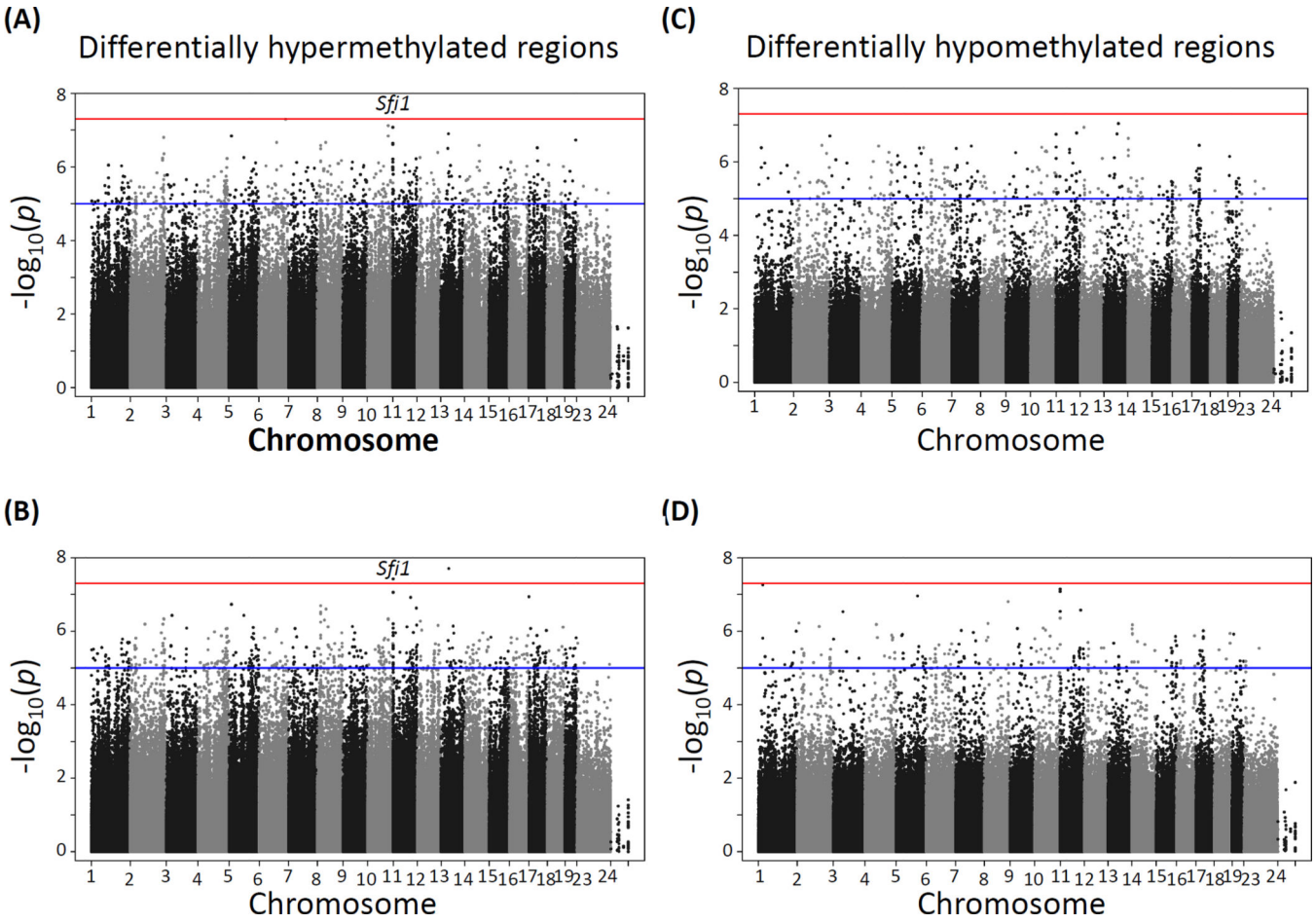

Figure 4. Manhattan plot of $-\log_{10}$ *p*-values of differential methylation across the mouse genome induced by lead acetate in the cortex of female mice

(A) and **(B)**: differentially hypermethylated regions in mice exposed to 3 ppm **(A)** and 30 ppm **(B)** of lead. **(C)** and **(D)**: **d**ifferentially hypomethylated regions in mice were exposed to 3 ppm **(C)** and 30 ppm **(D)** of lead.

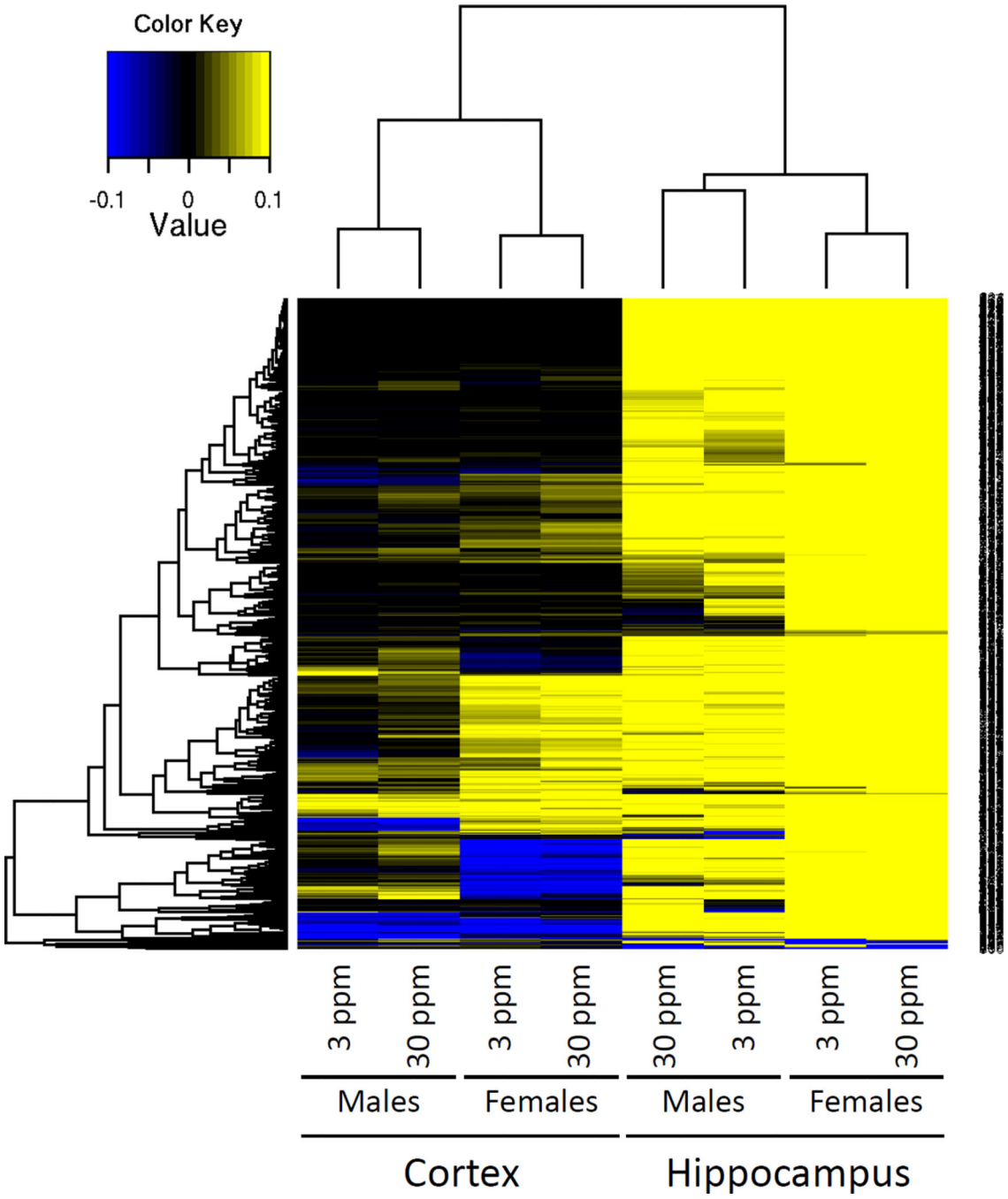

Figure 5. Heat map of significant CpG sites differentially methylated at a fdr≤0.001

The values shown for the females correspond to the normalized β-values calculated from the number of methylated counts divided by the number of methylated plus unmethylated counts for each one of the the1,623 CpG sites in the females. The male β-values are the corresponding values in the males.

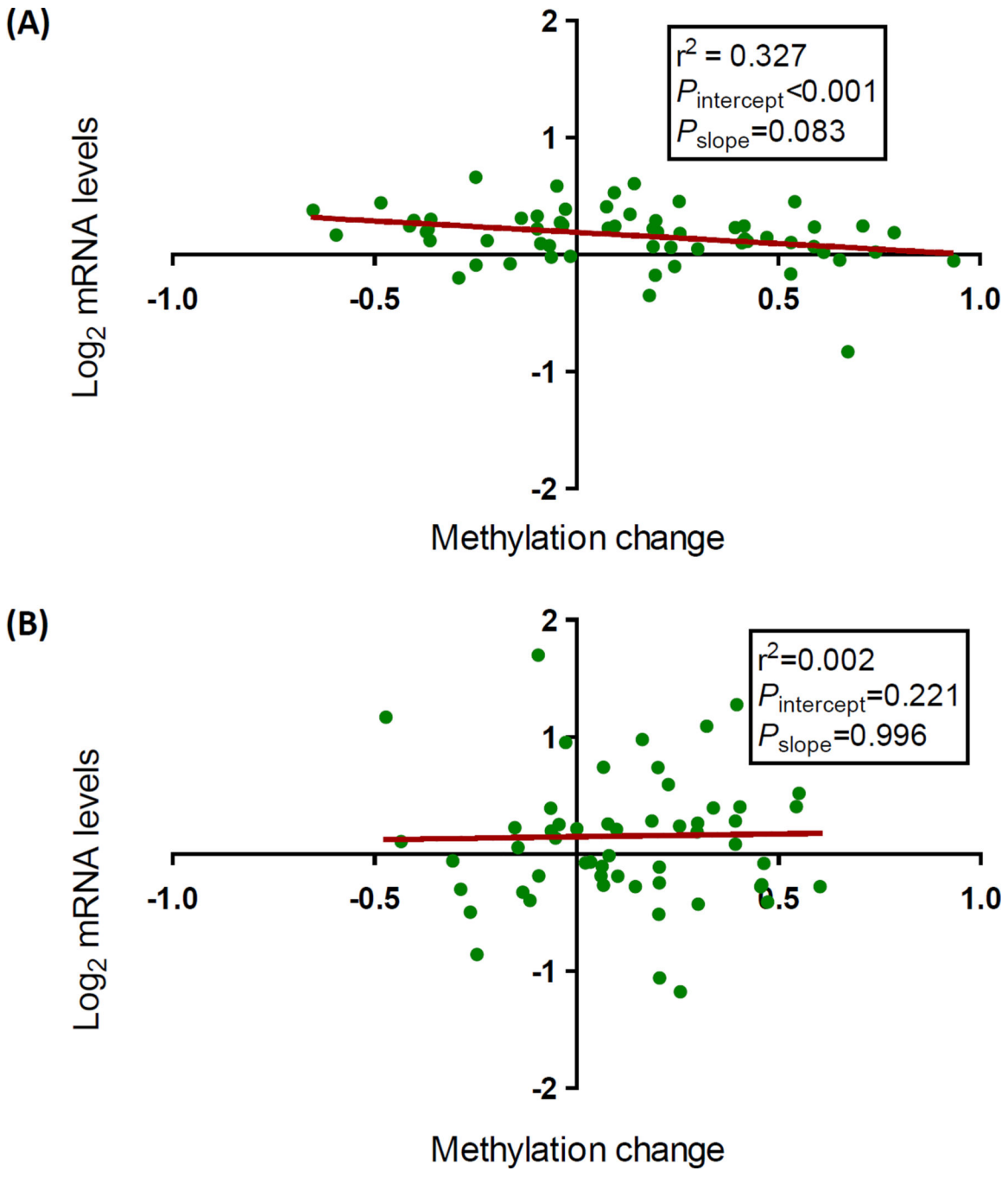

Figure 6. Analysis of the correlation between mRNA levels and methylation change in hippocampus of females (A) and males (B) exposed to 3 ppm of lead

The $\log_2$ mRNA levels determined by RT-PCR of the 60 genes tested (see Supplemental Fig. S2) is shown as a function of the corresponding methylation changes (Supplemental Table S4). Negative methylation change values indicate hypomethylation and positive, hypermethylation.

Table 1

Number of significant CpG sites, genome regions and genes affected by Pb exposure at different fdr cut-offs

FDR	CpG Sites	Genome Regions	Genes
0.001	1623	222	117
0.01	20129	12435	6180
0.1	159619	66539	16141