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Gene Regulation and Epigenetic Effects of Lead Exposure

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Abstract

Embryonic life is a time when organisms are most sensitive to environmental signals, responding to cues with extreme phenotypic plasticity. Developmental plasticity however, often gives rise to maladaptive pathophysiological consequences in the embryo or in later adult life, as is the case with the responses to lead exposure. Developmental exposure to lead (Pb), an ubiquitous environmental contaminant, causes deficits in cognitive functions and IQ, behavioral effects, and attention deficit hyperactivity disorder. Long-term effects observed after early life exposure include reduction of gray matter, alteration of myelin structure, and increment of criminal behavior in adults. Despite growing research interest, the molecular mechanisms responsible for the effects of lead in the central nervous system are still largely unknown. We have developed an embryonic stem cell model of Pb exposure during neural differentiation that promises to be useful to analyze mechanisms of neurotoxicity induced by Pb and other environmental agents. We also used DNA methylation analyses to determine whether perinatal exposure to lead acetate in mice was associated with persistent DNA methylation changes. We find a highly significant sex- and tissue-dependent change in DNA methylation in the brains of exposed mice, negatively correlated with gene expression levels. Females showing greater hypermethylation than males. Lead exposure during embryonic life appears to have a sex- and tissue-specific effect that may produce pathological or physiological deviations from the epigenetic plasticity of unexposed mice. Further analyses to correlate DNA methylation and regulatory gene expression changes will be crucial to understand the mechanisms of lead neurotoxicity.

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

Introduction

Lead is a non-biodegradable metal and one of the most ubiquitous persistent toxicants present in the environment. Unlike other heavy metals, such as iron, copper or manganese, lead has no known biological functions (Marchetti and Gavazzo 2005), but, because of its physico-chemical properties of high malleability, ductility, softness, low melting point and resistance to corrosion, it has many industrial uses, including manufacturing of pipes, lead-based paints, ceramic glazes, batteries, pottery, and ammunition.

Lead (Pb) poisoning from occupational or accidental exposures has been described since the time of the ancient Romans (Flora *et al.* 2012; Riva *et al.* 2012), and is prevalent all over the world. Exposure during childhood is a world-wide health problem and a growing public health concern in West Africa (Adeniyi and Anetor 1999; Nriagu *et al.* 1996). Pb inhibits detoxification enzymes, heme synthesis, and cholesterol metabolism, causing liver damage, renal dysfunction, hypertension and encephalopathy (Degawa *et al.* 1995; Kojima *et al.* 2002; Piomelli 2002). Lead is also responsible for several reproductive system problems, including reduction of libido, delay in puberty and infertility (Flora *et al.* 2012; Rastogi 2008; Schwartz 1995). Chronic and low-dose exposure to Pb during prenatal life and early childhood damages the central nervous system. Solid epidemiological evidence has linked Pb exposure during early childhood 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). Importantly, early life exposure to Pb has remote effects later in life, producing persistent injury in adults, including cardiovascular disease (Ademuyiwa *et al.* 2005) and gray matter volume loss in prefrontal cortex (Brubaker *et al.* 2010; Cecil *et al.* 2011). Other long-term consequences of childhood lead exposure include changes of myelin structure in white matter (Brubaker

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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). Neurochemically, lead decreases the brain concentration of important metabolites, such as N-acetyl aspartate, cholines, creatinine, phosphocreatinine, and a composite of glutamate and glutamine (Cecil *et al.* 2011). Behaviorally, blood lead levels during childhood have been correlated to an increase of criminal behavior in adult life (Bellinger *et al.* 1994; Chen *et al.* 2007; Froehlich *et al.* 2009; Wright *et al.* 2008). Altogether, these data suggest that lead exposure during early life may produce irreversible neuronal dysfunction and reorganization that last into adult life.

Recent studies using primary neuronal cultures indicate that lead exposure during childhood may negatively modify important neuronal pathways implicated in the late effects observed during adult life, especially affecting pathways implicated in synaptic plasticity, learning, memory, and cell survival, including modification of the N-Methyl-D-Aspartate (NMDA) receptor architecture (Guilarte and McGlothan 2003; Neal *et al.* 2011), changes of the activity of Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), phosphorylation of transcription factor CREB, and expression and translocation of brain-derived neurotrophic factor (BDNF) (Stansfield *et al.* 2012; Toscano and Guilarte 2005). Pb exposure also represses the expression of presynaptic vesicular proteins implicated in neurotransmitter release, such as synaptobrevin (VAMP1) and synaptophysin (SYN), while it increases p75 neurotrophin receptor (p75(NTR)) levels and alters TrkB-p75(NTR) colocalization in glutamate synapses (Neal *et al.* 2010; Stansfield *et al.* 2012). 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.

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.

Regulation of Gene Expression by Lead

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 (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). Similar results have been observed in the cortical region of rodents, in which early-life Pb exposure altered gene expression patterns and global methylation profiles (Basha *et al.* 2005; Dosunmu *et al.* 2009), and in zebrafish (*Danio rerio*), where Pb altered neurological development pathways and caused neurotoxicity (Dou and Zhang 2011; Peterson *et al.* 2011).

In our lab, we have initiated a test of the hypothesis that the Pb-induced alterations that occur during neural development may be responsible for the behavior and cognitive impairment observed in adult life. We exposed mice to Pb *in utero* and during weaning and examined gene expression in brains at postnatal day 60. In these studies, we found that mice perinatally exposed to Pb show gene expression changes in adult life many of which can also be found in Pb-treated neurons differentiated *in vitro* from mouse ES cells. We followed the expression of genes coding for neural markers, neurotrophins, transcription factors, and glutamate-receptors at postnatal day 60 in three different brain regions, namely hippocampus, cortex, and thalamus. Among the three brain areas analyzed, hippocampus was the one to show more Pb-induced gene expression changes for the gene tested (*Hud*, *Syn1*, *Htt*, *Vamp1*, *Ngn1*, *NeuroD1*, *Sox3*, *Bdnf* exon IX, *Ngf*, *Creb1*, *Creb2*, *Crel*, *NFκβ2*, *Rela*, *Relb*, *Grin1*, *Grin2A*, *Grin2D*, *Grin3B*, *Grik2*, *Gria1*, *Gria4*, *Grm4*, *Grm6*, and *Grm8*), followed by thalamus (*Nes*, *Vamp1*, *Ngn1*, *Sox3*, *Ngf*, *Nt4*, *Rela*, *Relb*, *Sp1*, *Grin2A*, *Grin2B*, *Grin2C*, *Grin2D*, *Grin3A*, *Grik2*, *Grik3*, *Grik5*, *Gria1*, *Gria2*, *Gria3*, *Gria4*, *Vglut1*, and *Grm6*) and cortex (*Reln*, *Bdnf* exon IX and IV, *Creb5*, *NFκβ2*, *Sp1*, *Grin3B*, *Grik1*, *Grik2*, *Grik3*, *Gria1*, *Grm4*, and *Grm6*). After a side-by-side comparison of the effects of Pb on the gene expression in ESC-derived neurons and brain tissues, we observed that *Ngn1* was up-regulated and *Grin1*, *Grin2D* and *Grm6* were down-regulated *in vitro* and in hippocampus, being *Ngn1* also up-regulated in thalamus. *Bdnf* exon IV and *Grik5* were down-regulated in vitro and in cortex and thalamus, respectively. Finally, *Gria4* was repressed in vitro, in hippocampus, and thalamus.

The ionotropic receptors NMDA, kainate, and AMPA, and the G-protein-coupled metabotropic receptor for glutamate are involved in excitatory transmission with important effects on synaptic plasticity that are implicated in neuronal processes such as learning and memory (Nakanishi and Masu 1994). Recently, chronic exposure to Pb of primary hippocampal neurons has been shown to cause modifications on NMDA receptor composition as a result of decreases in the expression of GRIN1, GRIN2A, and GRIN2B (Guilarte and McGlothan 2003; Neal *et al.* 2011). Our results suggest that Pb alters the expression of the different glutamate receptors subunits and that this effect may cause an imbalance of calcium homeostasis responsible for the impairment of other signaling pathways (Sanchez-Martin *et al.* 2013). In our *in vivo* studies, *Grin2A* was up-regulated in hippocampus and thalamus of mice treated gestationally with Pb, but we did not observe any expression changes in cortex or in cultures of glutamatergic neurons. Additionally, *Grin1* was down-regulated in hippocampus and ESC-derived neurons exposed to Pb. Calcium conductance increases when the kainate receptors contain the *Grik5* subunit, while when they contain *Grik1* and *Grik2* they are less permeable to calcium (Smith *et al.* 1999; Swanson *et al.* 1996). Although there were more alterations in gene expression in other subunits of NMDA, kainate, AMPA, and metabotropic receptors by Pb in both models, *Grik5* was down-regulated in both ESC-derived neurons treated with Pb and in thalamus, and *Grik2* was up-regulated in cortex and thalamus of mice treated gestationally with Pb. If as a consequence of Pb treatment the glutamate receptors are reorganized, their functional properties may be sufficiently affected as to disrupt calcium homeostasis and signaling.

Calcium signaling through the glutamate receptor has been shown to increase the level of CREB-dependent *Bdnf* transcripts containing exon IV (Tao *et al.* 1998), an effect that is down-regulated by Pb without affecting the expression of other non-coding exons (Stansfield *et al.* 2012). In our experiments, *Bdnf* exon IX transcripts were repressed after Pb treatment in both hippocampus and cortex, being not affected in thalamus and increased in the neurons obtained from neural differentiation of mESCs. These discrepancies suggest that other non-coding exon(s) may play a role in the maintenance of *Bdnf* expression. The transcripts of the coding exon IV are down-

regulated in cortex and our *in vitro* model, and not affected in hippocampus and thalamus. BDNF expression is highly controlled by different regulatory mechanisms, including epigenetic mechanisms, and mitogen-activated protein kinases, phosphoinositide-3 kinase and phospholipase-ɣ pathways (Boulle *et al.* 2012). As the neurotrophins are implicated in cell survival, differentiation, axon growth and guidance, synapses formation, and memory formation, the outcomes observed after Pb treatment provide a putative mechanism by which this metal may induce long-term potentiation and spatial memory impairments. The role played by neurotrophins in such physiological events has recently been reinforced by the findings that antibodies against BDNF and NT4 blocked long-term recognition memory in rats (Callaghan and Kelly 2013). Interestingly, we find that *Bdnf* exon IV and *Grin1* are down-regulated in cortex and hippocampus of mice treated gestationally with Pb and in 3-DIV neurons exposed to Pb during the neural differentiation process, suggesting that these changes may extend into adulthood, beyond the time when the organism was exposed to the agent.

Pb toxicity has been associated with apoptosis (Lu *et al.* 2013) and shown to induce an increase of caspase-3 cleavage and a decrease of intracellular glutathione levels in human SH-SY5Y neuroblastoma cells (Chetty *et al.* 2005). In our hands, however, treatment of ES cell-derived neurons with 0.1 µM Pb reduced cell numbers after disaggregation without inducing concomitant cell death directly and without activating caspase-3. These results are remarkably similar to those described for low-concentration, long-term treatment of neural stem cells derived from E15 rat cortex, striatum, and ventral mesencephalon, and mouse bone marrow-mesenchymal stem cells, which show that cell proliferation is slightly altered at low Pb concentration and considerably affected at high concentration (Huang and Schneider 2004; Kermani *et al.* 2008). We tested treatment of ESC with different Pb concentrations in the range of 0.01 to 0.1 µM for 10 days and we did not observe any reduction of cell proliferation. Other i*n vivo* studies have also shown discrepancies on casape-3 activation due to long-term exposure, suggesting that Pb effects may be region-, time-, and concentration-specific (Baranowska-Bosiacka *et al.* 2013; Kiran *et al.* 2009). Figure 1 shows a summary of gene expression changes resulting from treatment of mouse embryonic stem cells differentiating as neurons in the presence of 0.1 µM Pb acetate.

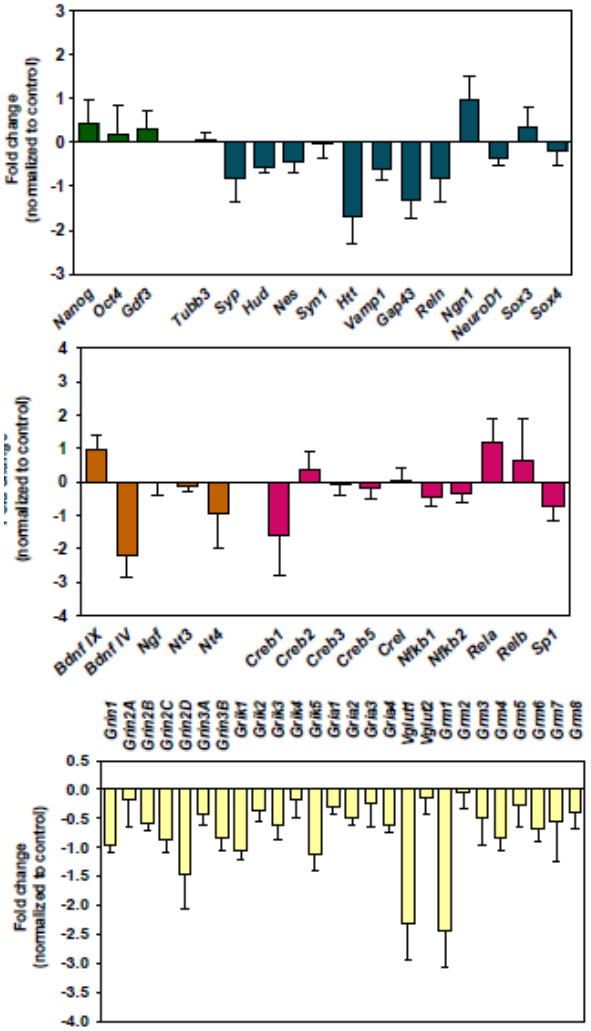

Figure 1: summary of gene expression changes resulting from treatment of mouse embryonic stem cells differentiating as neurons in the presence of 0.1 µM Pb acetate.

Epigenetic Effects of Lead

Early life exposure to lead may have toxic effects in the developing brain, suggesting that lead neurotoxicity may result from the long-term 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). 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). 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).

We have 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, including cortex and hippocampus, of exposed mice. We found a highly significant change in DNA methylation in both brain regions, with a trend to negative correlation to gene expression levels. The effect was sex- and tissue-dependent, with females showing greater hypermethylation than males, and more so in hippocampus than cortex. The majority of changes resulted from DNA hypermethylation and were 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 $\leq 5x10^{-8}$, 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 false discovery rate (fdr) ≤ 0.001, we found 1,623 differentially methylated CpG sites in the hippocampus of exposed females, the majority (>90%) of which showed hypermethylation. These sites defined 222 regions of the genome, corresponding to an additional 117 unique genes. After analyzing the expression of 60 genes 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. *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. Figure 2 shows massive hypermethylation of the *Rn45s* promoter in the hippocampus of mice exposed *in utero* to lead acetate in drinking water. 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 but its expression is not changed in either sex possibly because the gene is already highly methylated and changes in these two regions are not enough to alter the expression levels.

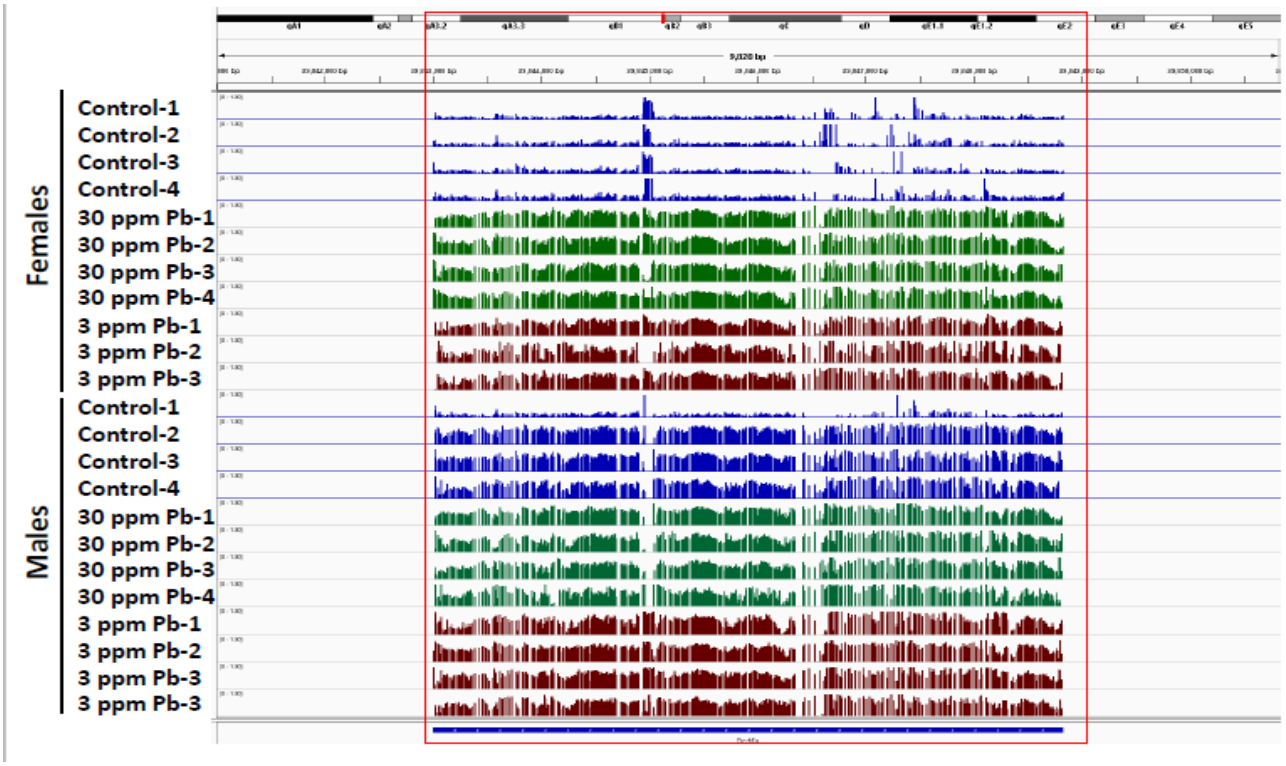

Figure 2: Massive hypermethylation of the *Rn45s* promoter in the hippocampus of mice exposed *in utero* to lead acetate in drinking water

We also found that the promoter of the *Dynlt1b* (dynein light chain Tctex-type 1B) gene was hypermethylated in the hippocampus of females exposed to 3 and 30 ppm of lead. *Dynlt1b* transcription is high in the dentate gyrus of the hippocampus (Dedesma *et al.* 2006) were 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), with DYNLT1B acting 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, 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.

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).

Conclusions

It is paramount to determine the molecular mechanisms by which Pb produces neurotoxicity. Although the use of lead has been reduced in the last few decades, its non-biodegradable nature and ubiquitous presence make it an environmental agent of general concern, posing a potential health risk as a result of the increased sensitivity of children to lead toxicity (Markowitz 2000). Developmental or early-life exposure to Pb may produce CNS disorders detectable later in adulthood. 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. The theory of the fetal

origin of adult disease states that during development, exposure to environmental agents such as heavy metals, or to stressful situations like poor nutrition, may adversely contribute to adult pathogenesis (Barker *et al.* 1989; Barker 1999), possibly by reprogramming specific gene expression patterns. Early-life exposure to lead may produce persistent changes in the mechanisms that regulate gene expression, contributing to adult neurological pathologies. Reduction of gray matter and alteration of the myelin structure and important metabolites are among the remote effects that early-life exposure to chronic and low-dose Pb produce in the CNS of adults (Cecil *et al.* 2011). Among its many other adverse effects, Pb is implicated in hepatic, renal, vascular, reproductive, and nervous diseases (Degawa *et al.* 1995; Flora *et al.* 2012; Rastogi 2008; Schwartz 1995). Additionally, lead accumulates in bones and blood, increasing the body burden and extending exposure well beyond the time of direct contact.

Our current studies have developed a suitable *in vitro* model that uses mouse ESC differentiation to recapitulate neural gestational exposure to lead, which may serve to characterize the molecular mechanisms of lead neurotoxicity. Further analyses to correlate DNA methylation and regulatory gene expression changes will be crucial to understand the mechanisms of lead neurotoxicity.

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Figure 1. Expression patterns of genes altered in ESC-derived neurons after Pb treatment. mESC were untreated or treated with 0.1 µM of Pb during the whole neural differentiation process. Total RNA was isolated from neurons obtained from mESC neural differentiation 3 days after CA disaggregation. Gene expression was normalized to *Gapdh* expression in each condition and expressed relative to the corresponding level in untreated controls. *Green bars* represent pluripotency markers; *blue bars*, neural markers; *orange bars*, neurotrophins; *pink bars*, transcription factors; and *yellow bars*, glutamate receptors subunits and vesicular glutamate transporters. The data shown are the mean ± SEM of three independent replicates. (*) $p < 0.05$.

F**igure 2. Integrative Genomic Viewer (IGV) view of the methylation status of the *Rn45s* gene.** The methylation status of the *Rn4.5s* gene in hippocampus of male and female mice exposed to 3 or 30 ppm of lead and their corresponding controls are shown. The promoter of the *Rn4.5s* gene is shown inside the red box.