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Sex-Based Differences in Gene Expression in Hippocampus Following Postnatal Lead Exposure

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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 $\mu\text{g}/\text{dl}$ and 27.1 ± 1.7 $\mu\text{g}/\text{dl}$ 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 a variety of genes in the hippocampus and that the response of the brain to a given lead exposure may vary depending on sex.

Keywords

rat; lead; hippocampus; mRNA; 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

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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 60µg/dl; in 1970 it was lowered to 40µg/dl; in 1975 to 30µg/dl; in 1985 to 25µg/dl and finally, in 1991, to 10µg/dl. However, data obtained over the last decade suggest detrimental effects of lead on cognition and behavior at blood levels < 5µg/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 (Lanphear *et al.* 2000; 2002; 2004; Chiodo *et al.* 2004). 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 are 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 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 three years of life and reported cognitive deficits in males at 3 years of age but not in females, suggesting different susceptibility 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 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 42g - 53g for males and 33g - 46g for females; the weight ranges for animals assigned to lead exposure was 44g - 52g for males and 33g - 47g for females. All animals were exposed to a 12h:12h 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 Bioanalyzer and a GE Nanovue spectrophotometer prior to further processing. Acceptance criteria for RNA extraction was a 260nm/280nm 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), (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: (Set 1) Treatment alone, (Set 2) both Treatment and Gender: Treatment interaction and, (Set 3) Gender:Treatment interaction alone. In the heat map visualizations of these data sets, hierarchical clustering using complete linkage and Pearson correlation similarity metric was performed using the MeV software (Howe *et al.* 2010). Clusters of genes were identified based on the expression pattern across biological replicates of male and female samples. The differential gene expression data were then used in an established multidimensional scaling approach (Cox and Cox 2001) employing the *isoMDS* function in the *MASS* library and the *Dist* function in the *amap* library implemented in the Bioconductor libraries (Gentleman *et al.* 2004) for the R Project for Statistical Computing (<http://www.r-project.org>). The samples were grouped based on the 'canberra' distance metric, which is computed as the sum of scaled absolute differences in gene expression changes. The samples were colored based on a combination of Gender and Treatment, yielding four distinct colored groups. The objective of the multidimensional scaling approach employed here was to position the samples in the three dimensional space with the pair-wise Euclidean distances approximately equal to the 'canberra' dissimilarity metric computed. This permits a visual approach to investigating the similarities and differences between the gene expression responses across the biological samples.

Pathway Analyses

The lead-affected gene sets were analyzed for over-represented biological pathways, networks and other functional annotation using the DAVID software (Huang da *et al.* 2009) and Ingenuity Pathway Analysis (IPA: Ingenuity Inc., Redwood City, CA). The list of genes on the Affymetrix Rat Gene 1.0 array was used as the background reference in DAVID and IPA. The gene set clusters were considered separately in the DAVID analysis. We considered the default parameters of DAVID that considers all levels of Gene Ontology as well as KEGG and PANTHER pathways in identifying over-represented pathways, processes and functional annotations. In the IPA, a collated data set from the three lead affected gene sets was prepared to construct significantly enriched networks. The genes in the network were color coded based on differential gene expression data or the categorical cluster membership.

Quantitative RT-PCR

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm microarray results for a subset of selected genes from those fulfilling the criteria of significant ($P < 0.05$) expression differences. Reverse transcription for 2 μg of each sample was performed with Omniscript RT Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Real-time PCR was then performed using a LightCycler 480 (Roche Diagnostics, Ltd) according to the manufacturer's instructions with gene specific optimized primers generated as reverse phase HPLC purified oligonucleotides, designed to amplify 86–183 bp regions of the selected genes (Primer sequences are listed in Supplementary Table S1; SAB Biosciences, Inc) and LC480 SYBR Green I Master Mix (Roche Diagnostics, Ltd). A typical reaction took approximately 50 minutes to complete and included a 5 minute denaturation step at 95°C, followed by 45 cycles of 95°C for 5s (melting), 55°C for 5s (annealing) and 72°C for 5s (extension). To confirm specificity of amplification, the products were subjected to a melting curve analysis at the end of the final extension period. Standard curve amplification was performed using known amplicon dilutions ranging from 10^{-6} – 10^{-1} attomoles. The generated standard curve for each primer set was stored as a reference curve for use in future experiments and compared to standards in each run and against the unknown samples.

Results

Animal Characteristics

At the end of the study, there were no significant differences in body weight between female lead exposed animals and control animals (female lead $156.0 \pm 1.6\text{g}$ and female control $163.3 \pm 3.0\text{g}$, respectively, $t = 2.12$, $p > 0.05$) or between male lead exposed animals and control animals (male lead $197.4 \pm 8.5\text{g}$ and male control $209.0 \pm 6.4\text{g}$, respectively, $t = 1.09$, $p > 0.05$). There were significant differences in weight between control male and female animals ($t = 6.49$, $p < 0.0001$) and between male and female lead exposed animals ($t = 4.78$, $p > 0.001$).

The mean blood lead level for female and male control animals was $< 1.0 \mu\text{g/dl}$. In contrast, the mean blood lead level for females was $26.69 \pm 2.06 \mu\text{g/dl}$ and for males was $27.14 \pm 1.71 \mu\text{g/dl}$, a difference that was not statistically significant ($t = 0.17$, $p > 0.05$).

Gene Expression Profile in the Hippocampus in Control Male and Female Rats

The expression of 263 probes (175 unique genes of 193 annotated probes) was differentially regulated between control male and female rats at an average fold change > 1.3 and a multiple testing corrected q-value < 0.3 (Figure 1). Two hundred and four probes (165 unique genes of 183 annotated probes) showed a greater expression in females than in males

while only 59 probes (10 annotated genes) showed a greater expression in males than in females. Based on DAVID analysis, the major cell processes that showed a relatively higher expression in females than in males are concerned with signaling, ion binding and transport, and membrane receptor expression (Table 1). DAVID bioinformatics analysis did not reveal any particular biological functions or annotations containing significant numbers of differentially expressed genes for the small gene set with higher expression in males.

Effects of Lead on Gene Expression Patterns in Male and Female Rats

A total of 423 probes (167 unique genes, of 183 annotated probes) were differentially expressed in response to lead exposure in either males or females, at an average expression ratio > 1.3 fold up or down regulation. A significant gender-dependent effect of lead exposure dominated the gene expression response. Approximately 40% of lead exposure-dependent expression changes were distinct between males and females, indicating a strong gender-driven effect on the transcriptional response to lead exposure. In order to understand the impact of the gender-dependent gene expression changes and biological variability on sample grouping, the data were visualized using an established multidimensional scaling approach (Cox and Cox 2001). This analysis revealed a key feature of the gene expression response: male and female sample groups occupy separate, diagonally opposite segments. This indicates a significant gender-specific response that is in opposite directions in male vs. female animals (Figure 2). A consistent separation of sample groups was obtained despite biological response variability, providing confidence in the significance of the overall patterns of gene expression changes.

In order to gain further insight into the observed gender-specific response to lead, the differentially expressed gene set was divided into groups based on the statistical significance of corresponding factors in ANOVA, as described below:

Set 1—In this set, lead exposure had a significant overall effect without a significant difference between male and female responses (249 probes: 77 unique genes, of 77 annotated probes, Figure 3A). While the male vs. female differences in response were statistically insignificant in this group, examination of average gene expression changes revealed that a fraction of the responses occurred in a gender-dependent manner with only one of the groups (male or female) showing an average change in gene expression levels (Figure 3B). Multi-dimensional scaling analysis of the data in Figure 3A revealed the gender-independent sample group segregation based on lead exposure as well as the variability in the response (Figure 3C). This set included key genes such as *Ephx2* (epoxide hydrolase 2; role in blood pressure regulation with potential anti-hypertensive, anti-inflammatory roles and role in neuronal survival after ischemic injury (Koerner *et al.* 2007)), *Rtn4* (NOGO; inhibits neurite extension and axonal regeneration (Meier *et al.* 2003)), *Mdk* (midkine, neurite growth promoting factor-2; promotes neurite outgrowth (Nakamura *et al.* 1998) and possesses neurotrophic and neuroprotective properties in the central nervous system (Kim *et al.* 2010)), *Mmp2* (matrix metalloproteinase 2; functions in hippocampal synaptic plasticity (Szklarczyk *et al.* 2002)), *Lama3* (laminin, alpha3; role in axonal guidance (Hagg *et al.* 1997)), *Glycam1* (glycosylation dependent cell adhesion molecule 1; functions in cell adhesion, may be associated with stress (Bergstrom *et al.* 2007)), *Hspb1* (heat shock 27kDa protein 1; involved in protein folding and quality control, implicated in memory consolidation (Sirri *et al.* 2010)), *Igf2* (insulin-like growth factor-2; stimulates proliferation of neuronal and glial cells (Konishi *et al.* 1994)), promotes survival of several neuronal cell types (Haselbacher *et al.* 1989) and potentiates ACh release), *Sfrp1* (secreted frizzled-related protein 1; implicated in regulating synapse number and function in the hippocampus (Miquelajauregui *et al.* 2007)), *Ldha* (lactate dehydrogenase A; a glycolytic enzyme important for energy metabolism (Gomez Ravetti *et al.* 2010)), *Hsp40* (heat shock

protein 40; part of the cytoplasmic stress response system (induced by transient cerebral ischemia and potentially a molecular mechanism promoting recovery of cells from stress (Paschen *et al.* 1998)).

Set 2—In this set, lead exposure had a significant overall effect with differences in male vs female response (120 probes: 71 unique genes, of 75 annotated probes, Figure 4A). A majority of the genes in this set were responsive to lead exposure either in males or females, but not in both (Figure 4B). Multi-dimensional scaling analysis of the data in Figure 4A revealed the gender-dependent sample group segregation based on lead exposure, albeit with variability within each group (Figure 4C). This set included key genes such as *Gsta4* (glutathione S-transferase alpha 4; role in disposal of exogenous toxic compounds and the adaptive, antioxidant response to reactive oxygen species (Bjork *et al.* 2006)), *Acs3* (acyl-CoA synthetase medium-chain family member 3; may be involved in stress responses (Ising and Holsboer 2006)), *A2m* ($\alpha 2$ macroglobulin; a zinc binding protein with roles in protection against stress and inflammation (Mocchegiani and Malavolta 2007)), *Htr2c* (5-hydroxytryptamine (serotonin) receptor 2C; polymorphisms associated with attention deficits, hyperactivity and obsessive compulsive behavior (Orabona *et al.* 2009)), *SLC6A2* (thyroid hormone monocarboxylate transporter; role in thyroid hormone transport across the blood brain barrier, inactivation of which leads to psychomotor and cognitive dysfunction (Roberts *et al.* 2008)), *Cldn1* (claudin1; involved in blood brain barrier development and function (Liebner *et al.* 2008)), *Stra6* (stimulated by retinoic acid gene 6; potential role in synapse development and function (Chen *et al.* 2008)), *Trpv4* (transient receptor potential vanilloid 4; role in the temperature-dependent regulation of neural activity; physiological core body temperature-evoked *Trpv4* activation affects neural firing through NMDA receptor activation (Shibasaki *et al.* 2007)), *Prlr* (prolactin receptor, may specifically mediate pheromone-induced neurogenesis in the dentate gyrus, particularly in females (Mak *et al.* 2007)), *Enpp2* (eatonucleotide pyrophosphatase/phosphodiesterase 2; an estrogen-responsive gene and an NF- κ B target gene that may play a role in neurite remodeling, axon growth, neural responses to injury (Bergstrom *et al.* 2007)), *Yap1* (yes-associated protein 1; a transcriptional cofactor, mediates the expression of cell death-promoting genes, and induces apoptosis (Hoshino *et al.* 2006)).

Set 3—In this set, a total of 53 probes (30 unique genes, of 31 annotated probes) were differentially expressed in the hippocampus in opposite directions in males vs. females in response to lead exposure (Figure 5A and B). Thirty-one of these 53 probes had annotation information available and are listed in Table 2 along with fold changes. Multi-dimensional scaling analysis of the data in Figure 5A revealed the gender-dependent sample group segregation based on lead exposure, and shows the variability within each group (Figure 5C). The apparent sample outliers correspond to those samples with outlier expression for a subset of genes as shown in the heat map (Figure 5A). The affected genes clustered into biological pathways associated with plasma membrane, ion and anion transport, metal/zinc binding, extracellular region and glycosylation. A few of these genes (4 of 30) were expressed at a lower level in males than in females while the majority (27 of 30) were expressed at a lower level in females than in males. This set included key genes such as *Gstt3* (glutathione S-transferase, theta 3; glutathione S-transferase (Gst) enzymes are instrumental in protecting cellular macromolecules against electrophiles and products of oxidative stress, determine a tissue's ability to handle certain forms of chemical stress (Knight *et al.* 2007)), *Fgf1* (fibroblast growth factor 1; may protect against hippocampal excitotoxic injury (Hossain *et al.* 1998)), *Pon1* (paraoxonase 1; plays a role in pesticide metabolism (Costa *et al.* 1999)), *Sod3* (extracellular superoxide dismutase 3; important part of the cellular antioxidant defense mechanism (Freiberger *et al.* 2004)), *Igfbp1* (insulin-like growth factor binding protein-like 1; inhibits *Igf1* actions and may have a detrimental effect

on oligodendrocyte survival and proliferation and myelination (Ye *et al.* 1995; Ye *et al.* 1995)), Steap1 (six transmembrane epithelial antigen of the prostate 1; functions as a metalloredoxase in the brain to influence cellular metal homeostasis (Knutson 2007)), Folr1 (folate receptor 1; can influence psychomotor development and myelination (Labrie *et al.* 2009)), Slc4a5 (solute carrier family 4, sodium bicarbonate cotransporter; may play a role in depression (Andrus *et al.* 2010)), Ace (angiotensin I converting enzyme (peptidyl-dipeptidase A) 1; part of the 'renin-angiotensin system that modulates vascular homeostasis and the amyloid β pathway, involved in memory and cognition, polymorphisms may be involved in risk for Alzheimer's disease (Sleegers *et al.* 2005)), K1 (Klotho; variation in the Klotho gene is a possible contributor to cognitive differences in humans: an association between variants in the Klotho gene and various cognitive abilities in youth and old age (Deary *et al.* 2005)), Mfrp (membrane frizzled-related protein; may play a role in the response to stress (Bergstrom *et al.* 2007)).

Functional Analysis of Processes Affected by Lead Exposure

A subset of 183 probes contained annotation that permitted the subsequent pathway and functional analysis. Analysis using the DAVID bioinformatics tool (Huang da *et al.* 2009) revealed multiple biological functions that were over-represented in the three gene sets. These results did not reveal any particular biological functions or annotations containing significant numbers of differentially expressed genes (> 5) and genes significantly over-represented with a multiple testing corrected p-value less than 0.3. The highest ranked functional annotation groups included glycoprotein, glucose metabolic process, and response to estrogen stimulus, each containing less than 5 of the above-discussed genes, and were not analyzed further due to the small size of the annotation groups.

Pathway Analysis Revealed Two Distinct Networks Affected by Lead

We analyzed for networks of known interactions that were enriched in the gene expression response using IPA (Ingenuity Inc., Redwood City, CA). Our analysis revealed two distinct networks (Figures 6 and 7). Network 1 included 50 differentially expressed genes with almost all of these located in the periphery with limited direct connectivity to other genes (Figure 6). In contrast, Network 2 included 40 differentially expressed genes with several nodes such as CDKN1A, MMP2, MMP14, A2M, FGF1, IGF2, PRLR, located more centrally in the network with extensive connections to other genes (Figure 7). Notably, there were several distinct central, highly connected nodes between the two networks that may represent independent mechanisms by which the corresponding genes were regulated in response to lead exposure, e.g., Network 1 - HNF1A, HNF4A, SREBF1, MYC; Network 2 - NF κ B, MAPK, JNK, AP-1, PI3K, Pdgf, PON1, CDKN1A, A2M, MMP2, MMP14, PRLR. TGF beta and CREB were the highly connected nodes common to both the networks. Although the functional significance of these networks and findings is unclear at this time, this may be significant since there may be sex-related influences of CREB on cognitive functioning (Hedba-Bauer *et al.* 2007) and genetic and pharmacological studies in rodents suggest that CREB is required for a variety of complex forms of memory, including spatial learning and memory (Silva *et al.* 1998), processes known to be disrupted by lead exposure (ex., (Moreira *et al.* 2001)). Mapping the direction of gene expression change to these networks revealed a subset of highly connected nodes (A2M, MMP2, FGF1) in Network 2 as oppositely regulated in male vs. female animals in response to lead exposure. A2M, MMP2 and FGF1 are all complex cognition-related genes that may be related to synaptic plasticity, memory, and brain development (Mocchetti *et al.*, 2007; Reynolds *et al.*, 2007; Mizoguchi *et al.* 2010).

Confirmation of Gene Expression Changes by Quantitative RT-PCR

We carried out quantitative RT-PCR confirmation of the microarray data using total RNA samples from all experimental groups. Primer pairs designed for these 8 genes are described in Supplementary Table S1. These 8 genes were chosen based on large differential fold changes in the microarray data between male and female lead-exposed animals and potential biological relevance of these genes. Analysis of the fold-change by both quantitative RT-PCR and microarray were similar in 14 of 16 analyses and array and PCR data were significantly positively correlated (Pearson Correlation Coefficient $r=0.9486$, $p < 0.0001$) (Table 3).

Discussion

The results of this study describe a significant effect of sex on gene expression changes in the hippocampus following post-weaning exposure to lead in rats. Although the expression of a number of genes in the hippocampus were affected in both male and female animals, of particular interest was the finding of a diverse group of genes that were differentially expressed in opposite directions in males compared to females in response to lead exposure.

Although the functional significance of the subset of genes that were highly differentially expressed in hippocampus of males and females following lead exposure is quite varied, a number of these genes (i.e., *F5*, *Aqp1*, *Mdk*, *Nt5dc2*, *Prlr*, *Enpp2*) have previously been shown to be differentially expressed (i.e., significantly down-regulated) in an endogenous depression model in rats (Andrus *et al.* 2010). In the present study, these genes were significantly down-regulated in female rats exposed to lead but not in male animals. This suggests the possibility that lead exposure may differentially modify molecular signatures associated with depressive behavior in males and females and may further suggest a differential risk of developing depression in males and females exposed to lead. In further support of this, CREB, one of the two highly connected nodes in the networks identified in Figures 6 and 7 showing sex-dependent differential gene expression, is also known to be involved in a sexually dimorphic molecular response to stress and to potentially play a differential role in stress-related depression in females compared to males (Lin *et al.*, 2009). TGF beta and CREB were the highly connected nodes common to both networks identified with differentially expressed genes (see Figures 6 and 7). Both TGF beta and CREB are influenced by environmental factors, including hormones, and may be involved in a sex-dependent modulation of neurogenesis. The connection between TGF beta and CREB may involve crosstalk with Wnt signaling, important for precursor cell proliferation and neurogenesis (Malaterre *et al.* 2007). This is of potential significance since neurogenesis is a possible substrate through which antidepressants alleviate symptoms of depression and antidepressant effects on depression appear to be at least in part sex-dependent (Hodes *et al.* 2009). Using data collected from the National Health and Nutrition Examination Survey (for 1999 to 2004) to investigate the relation between blood lead levels and the odds of developing major depressive disorder (MDD), a higher blood lead quintile was significantly associated with higher odds of meeting *DSM-IV* criteria for MDD. Women may have had higher rates of MDD and other psychiatric disorders than did men, despite having lower blood lead levels than did men, although a larger sample is necessary to verify this (Bouchard *et al.* 2009).

Abnormal N-methyl-D-aspartate receptor (NMDAR) function has long been associated with developmental lead exposure and has also been implicated in a variety of cognitive disorders including the pathophysiology of schizophrenia (Coyle 2006). Lead has potent inhibitory effects on the NMDAR (Alkondon *et al.* 1990; Guilarte 1997) that appear to be age and brain region specific (Guilarte 1997). The developing brain is more sensitive than the adult brain to lead-induced effects on the NMDAR and the hippocampus appears to be

particularly vulnerable (Guilarte and McGlothan 1998). Rats exposed to lead during development in particular have altered hippocampal NR1 and NR2A mRNA expression (Nihei and Guilarte 1999). Although different experimental studies have utilized a variety of lead exposure paradigms during different developmental periods, the data are fairly consistent in pointing to structural and functional alterations in NMDAR complexes in the rat hippocampus that have significant effects on signaling, synaptic plasticity and a variety of cognitive functions (Toscano and Guilarte 2005). In addition, many of the findings regarding lead effects on the NMDARs resemble changes in NMDAR subunit expression described in the brain of schizophrenic patients (Tsai and Coyle 2002; Konradi and Heckers 2003).

In this regard, developmental lead exposure has been suggested as a significant risk factor for the expression of schizophrenia later in life (Opler *et al.* 2004; Opler *et al.* 2008). The potential link between developmental lead exposure and schizophrenia may be the disruption of glutamatergic synaptic activity—specifically, hypoactivity of NMDARs (Guilarte 2004; Opler *et al.* 2004). NMDAR function is modulated in complex ways and in part by substances that modulate the endogenous NMDAR glycine site. One such substance is d-serine, which together with its catabolic enzyme (which is augmented in the brain of schizophrenics (Madeira *et al.* 2008) and activator has been associated with an increased risk of schizophrenia (Labrie *et al.* 2009). D-serine is synthesized from L-serine by the enzyme serine racemase (Srr). In support of a Srr dysfunction in schizophrenia patients is the observation of reduced levels of D-serine in serum and CSF (Hashimoto *et al.* 2003; Bendikov *et al.* 2007), reflecting an abnormality in available D-serine in the pathogenesis of schizophrenia (Labrie *et al.* 2009). The differential expression of genes relevant to schizophrenia and cognitive function have been described in SrrY269 mutant mice in which there is a lack of Srr activity and associated cognitive disturbances (Labrie *et al.* 2009). In particular, SrrY269 mice displayed an elevated level of hippocampal Ttr, Enpp2, Kl, Igf2, Fcrl1, Prlr, Otx2 and Cldn2 gene expression and these elevations were reversed by D-serine or clozapine administration. Six of these 9 genes (Enpp2, Kl, Igf2, Fcrl1, Prlr, Otx2) were significantly affected by lead exposure and all but Igf2 (which was decreased in expression in both males and females) was increased in males and decreased in females exposed to lead. These findings provide new molecular insight into the possible relationship between developmental lead exposure and the risk of schizophrenia and may suggest possible gender-based vulnerability, which will need to be assessed clinically.

Sex differences have been identified in the brain and although the brains of men and women are highly similar, differences have been described that have important implications for each sex (Ngun *et al.* 2010). Sex-related differences in the brain uniquely affect biochemical processes, may contribute to the susceptibility to specific diseases, and may influence specific behaviors (Ngun *et al.* 2010). However, there is little information in the literature comparing gene expression profiles in the brain, including the hippocampus, in control male and female rats. In the current study, we found a relatively small number of genes differentially expressed in control males compare to control females and in the majority of cases, there was greater expression of the differentially regulated genes in females compared to males. In one of the few studies that provides information on this topic, Biala *et al.* (Biala *et al.* 2010), examined gene expression profiles from hippocampal extracts prepared from Wistar rats and reported that 1,680 genes were differentially expressed in control males and females. The gender difference in gene expression was decreased to 11% (191 genes) by prenatal stress (Biala *et al.* 2010). In the present study, we observed far fewer differentially expressed genes in control male vs. female animals. Although the reasons for the discrepancy between our data and the findings reported by Biala *et al.* (2010) are not clear, they may be related to differences in criteria for defining differentially expressed genes as well as potential strain differences (Wistar vs. Long Evans). In another study, Torbati *et al.*

(Torbati *et al.* 2008) reported that only 61 genes (36 functionally recognized genes) showed statistically significant differential gene expression (both up- / down-regulation) at 10 days of age between male and female Sprague Dawley rat pups.

Although changes in gene expression provide valuable information they are not necessarily predictive of cellular function. Gene expression is vital for allowing a cell to produce its gene products allowing flexibility to adapt to a variety of external signals. Thus any process (or exposure) that alters gene expression patterns is potentially disruptive to the cell and its ability to adapt to changing demands. However, information concerning gene expression changes is only one piece of a larger, complex puzzle. The current findings will need to be followed up with protein expression studies as well as studies of possible post-translational modifications of proteins to better understand how lead exposure could influence hippocampal functionality. It is also possible that the current data actually under-estimate potential effects of lead on hippocampal gene expression profiles. The current pool of RNA was extracted from the whole excised hippocampus. Recent data suggest that there may be region-specific changes in hippocampal gene expression in response to lead exposure (ex., (Guilarte and McGlothan 1998; Zhang *et al.* 2002) that may need to be studied in greater detail in future studies.

In summary, post-weaning exposure to environmentally relevant levels of lead produced significant changes in the expression a variety of genes in the hippocampus and the present results show that the response of the brain to a given lead exposure may vary depending on sex. Based on the current data from animals with this particular type of lead exposure (i.e., post-weaning exposure), it is not possible to ascribe any particular gene expression changes observed to any particular cognitive function or dysfunction that might be associated with this type of lead exposure. The functional analyses performed revealed a complex array of genes differentially affected in males and females by lead exposure but did not reveal particular definitive overarching biological themes or annotations containing significant numbers of differentially expressed genes across treatment groups. Further work is necessary to further investigate the functional significance of sex-based differences in gene expression in brain as a consequence of lead exposures that occur during different developmental periods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Highlights

- Postnatal lead exposure has a significant effect on hippocampal gene expression patterns
- At least one set of genes was affected in opposite directions in males and females
- Differentially expressed genes were associated with diverse biological pathways

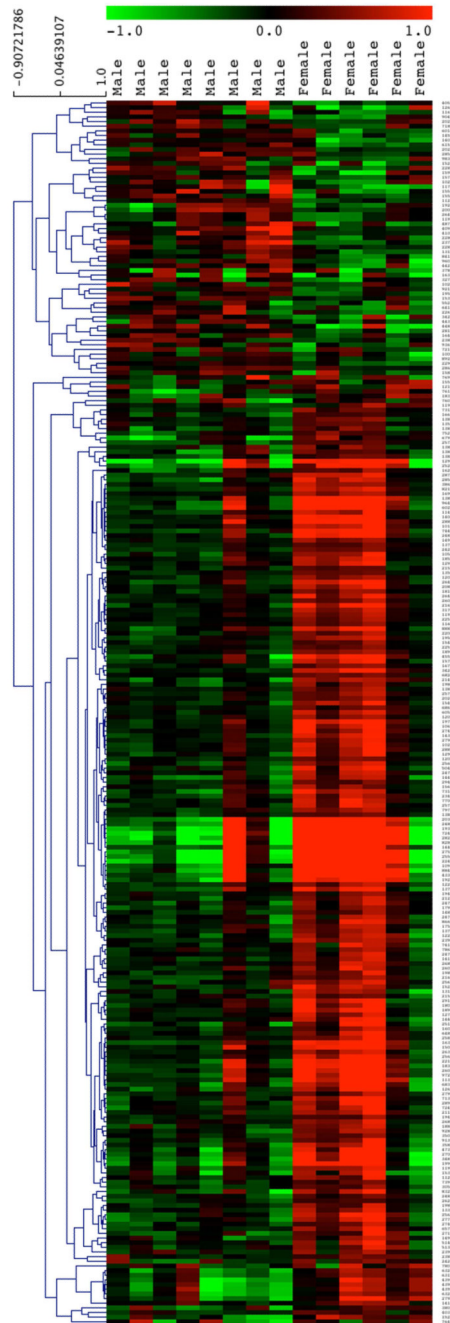


Figure 1.

Hierarchical cluster analysis of differentially expressed genes in hippocampus from male or female non-lead exposed control animals. The majority of differentially regulated genes between control male and female rats showed a greater expression in females than in males. Green indicates down-regulation of a gene; red indicates up-regulation relative to the median. Dendrogram on the left side of the gene list indicates clustering.

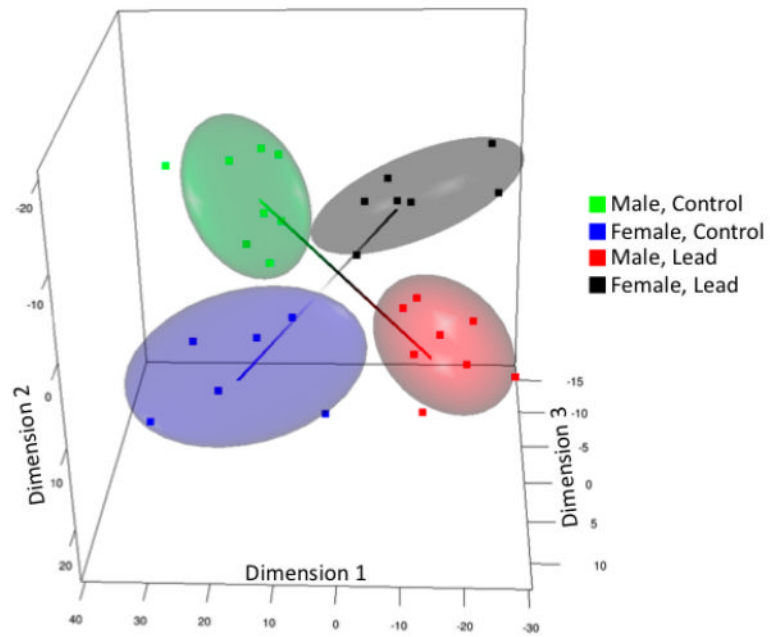


Figure 2. Multi-dimensional scaling analysis of the gene expression response to lead. Male and female groups occupy separate, diagonally opposite segments, indicating a significant gender-specific response that is in opposite directions in male vs. female animals.

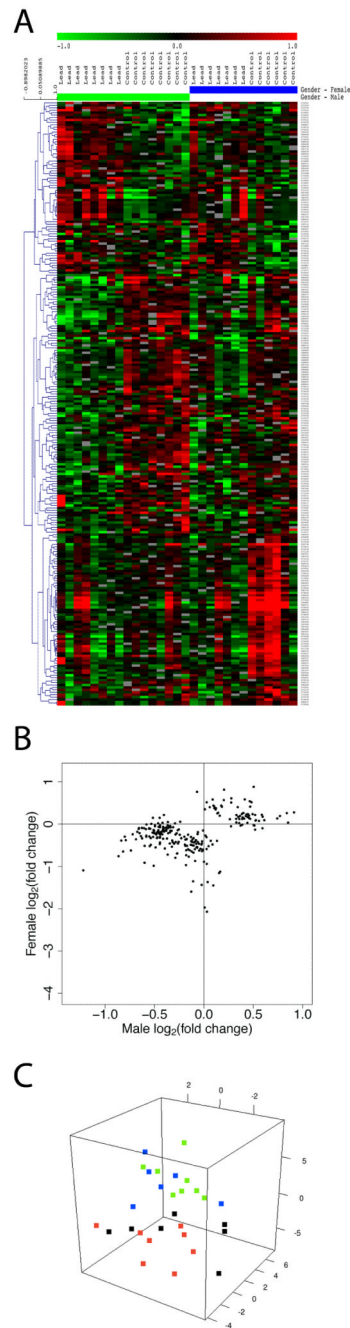


Figure 3.

Significant overall effect of lead exposure without a significant difference between male and female gene responses. **A.** Hierarchical cluster analysis of differentially expressed genes in hippocampus from control and lead exposed female or male rats. Green indicates down-regulation of a gene; red indicates up-regulation relative to the median. **B.** Scatter plot comparing male and female average gene expression responses. This set of genes did not show a significant sex-based differential response to lead exposure. **C.** Multi-dimensional scaling analysis of the gene expression shown in panel A. Color legend is the same as in Figure 2.

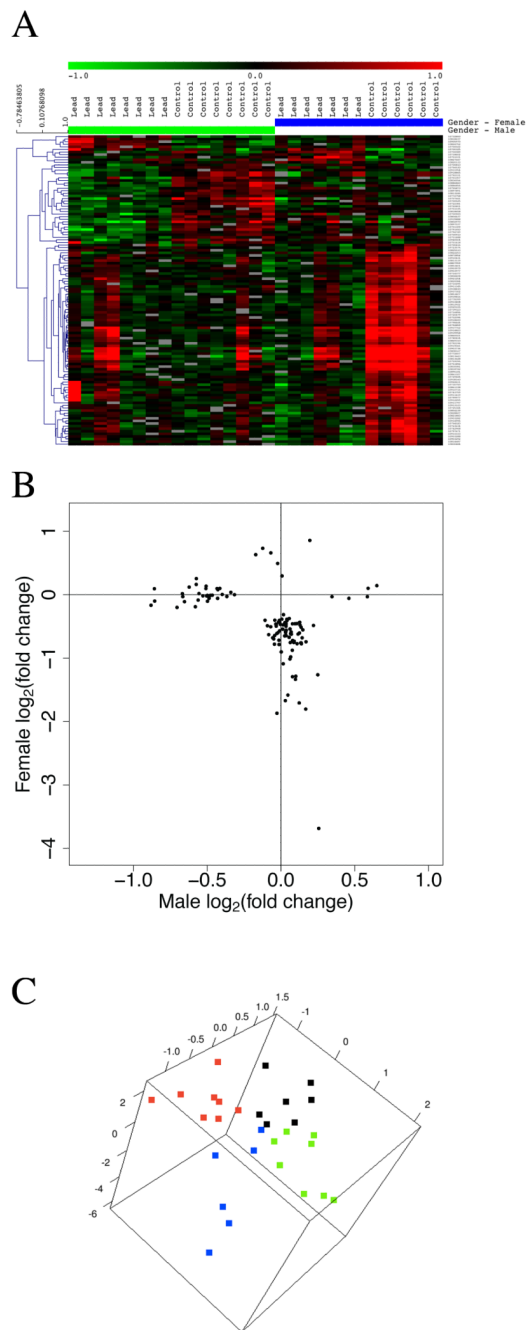


Figure 4. Significant overall effect of lead exposure with differences in response in males and females. A majority of the genes in this set were responsive to lead exposure either in males or females, but not in both. **A.** Hierarchical cluster analysis of differentially expressed genes in hippocampus from control and lead exposed female or male rats. Green indicates down-regulation of a gene; red indicates up-regulation relative to the mean. Dendrogram on the side of gene list indicates clustering. **B.** Scatter plot comparing male and female average gene expression response. **C.** Multi-dimensional scaling analysis of the gene expression shown in panel A. Color legend is the same as in Figure 2.

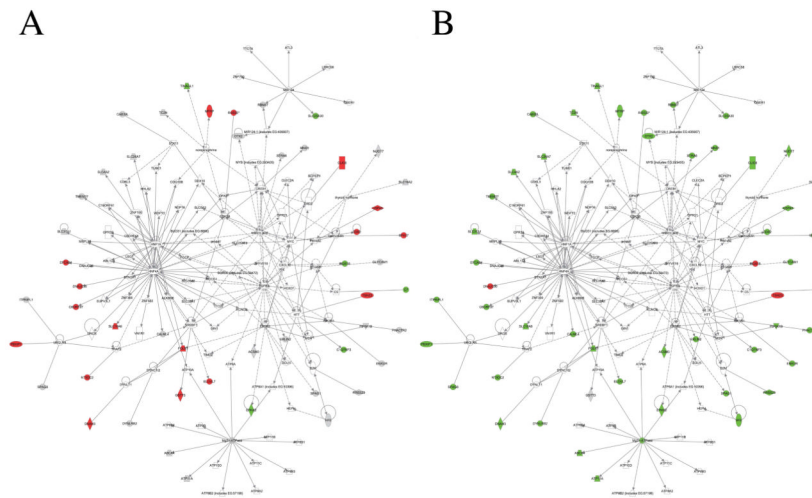


Figure 6. Network analysis. This network was obtained through Ingenuity Pathway Analysis of gene sets differentially expressed in male or female lead-exposed rats. The network is displayed as nodes (genes/gene products) and biological relationships between nodes. Solid lines denote direct interactions; dotted lines represent indirect interactions between genes. The average gene expression data from male (A) and female (B) rats was mapped to the corresponding genes in the IPA-derived network. Green indicates 1.3 fold or larger decreases; red indicates 1.3 fold or larger increases. A majority of differentially expressed genes in this network showed opposite responses in lead-exposed female rats (B, green nodes) compared to male rats (A, red nodes). A subset of genes showed the opposite or no response (grey) in lead-exposed male rats (A). A limited set of genes with common up- or down-regulation is also seen (nodes red or green in both A and B).

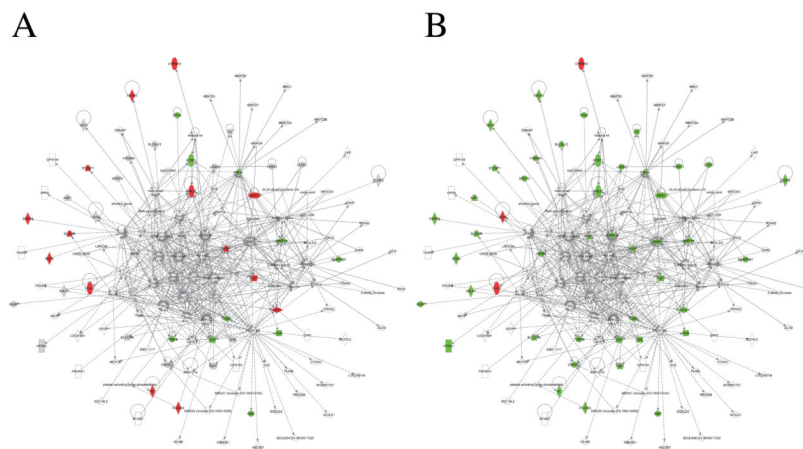


Figure 7. Network analysis. This network was obtained through Ingenuity Pathway Analysis of gene sets differentially expressed in male or female lead-exposed rats. The network is displayed as described in Figure 6. Green indicates 1.3 fold or larger decreases; red indicates 1.3 fold or larger increases. A majority of differentially expressed genes in this network showed down-regulation in lead-exposed female rats (B, green nodes) with a subset of these genes showing the opposite (red) or no response (grey) in lead-exposed male rats (A). A limited set of genes with common up- or down-regulation is also seen (nodes red or green in both A and B).

Table 1

Differential Expression of Hippocampal Genes in Control Males and Females

Process (Functional Annotation Clustering)	No. of genes	P value
Genes showing higher expression in females than in males (203 out of 244 genes)		
Extracellular region	31	7.4E-7
Glycoprotein	42	8.1E-6
Signal (Signal peptides)	29	3.5E-3
Plasma membrane associated	43	7.0E-6
Ion binding	43	6.5E-5
(Metal ion binding)	39	7.8E-4
Ion transport	18	5.8E-5
(Metal ion transport)	10	8.0E-3
Response to hormone stimulus	13	1.9E-3

Table 2

Genes Differentially Expressed in Opposite Directions in the Hippocampus of Male and Female Lead-Exposed Animals.

Affymetric Probe ID	Gene Symbol	Description	Fold Change Male	Fold Change Female
10768357	Rgs18	regulator of G-protein signaling 18	-0.331	0.381
10707370	LOC298314	similar to solute carrier family 25, member 5	-0.524	0.041
10724150	LOC365319	hypothetical LOC365319	-0.531	0.122
10791818	LOC364597	similar to 60S ribosomal protein L21	-0.283	0.254
10832563	Gst3	glutathione S-transferase, theta 3	0.403	-0.034
10916232	Spa17	sperm autoantigenic protein 17	0.228	-0.301
10804127	Fgf1	fibroblast growth factor 1	0.154	-0.461
10860867	Pon1	paraoxonase 1	-0.007	-0.458
10922615	LOC501126	similar to hypothetical protein MGC26733	0.011	-0.306
10903290	RGD1565493	similar to DKFZP434I092 protein	0.050	-0.569
10903298	RGD1565493	similar to DKFZP434I092 protein	0.176	-0.440
10816807	Pbxip1	pre-B-cell leukemia homeobox interacting protein 1	0.248	-0.351
10912977	Zmynd10	zinc finger, MYND-type containing 10	0.219	-0.379
10765503	RGD1562658	similar to RIKEN cDNA 1700009P17	0.230	-1.011
10725387	Cdr2	cerebellar degeneration-related 2	0.120	-0.863
10825100	Car14	carbonic anhydrase 14	-0.002	-0.799
10777108	Sod3	superoxide dismutase 3, extracellular	0.257	-0.627
10853396	Steap1	six transmembrane epithelial antigen of the prostate 1	0.191	-0.708
10876551	Igfbp1	insulin-like growth factor binding protein-like 1	0.057	-0.772
10787401	Slc5a5	solute carrier family 5 (sodium iodide symporter), member 5	0.116	-0.691
10782986	Otx2	orthodenticle homolog 2 (Drosophila)	0.095	-1.656
10724116	Folr1	folate receptor 1 (adult)	0.447	-1.782
10856673	Slc4a5	solute carrier family 4, sodium bicarbonate cotransporter, member 5	0.366	-2.509
10880872	Pla2g5	phospholipase A2, group V	0.163	-0.985
10866606	Slco1a5	solute carrier organic anion transporter family, member 5	0.030	-0.796
10739035	Ace	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	0.196	-1.735
10750296	Clic6	chloride intracellular channel 6	0.601	-2.742
10759762	K1	Klotho	0.269	-2.988
10909428	Mfrp	membrane frizzled-related protein	0.414	-2.784
10871561	Cldn19	claudin 19	0.228	-0.516
10931558	Efcab1	EF hand calcium binding domain 1	0.173	-0.610

Table 3

Confirmation by qPCR of Relative Changes in Gene Expression Identified by Array Analysis.

Gene	RefSeq	Array Data (Fold Change from Control)		qPCR Data (Fold Change from Control)	
		Male	Female	Male	Female
<i>A2m</i>	NM_012488	1.18	-2.10	1.26 ± 0.33	-2.18 ± 0.27
<i>Clic6</i>	NM_176078	1.82	-15.52	1.56 ± 0.74	-14.52 ± 6.35
<i>Cldn1</i>	NM_031699	1.11	-3.62	1.39 ± 0.34	-2.27 ± 0.23
<i>Enpp2</i>	NM_057104	1.13	-5.52	1.21 ± 0.36	-2.60 ± 0.56
<i>Ephx2</i>	NM_022936	-1.66	-2.68	-0.60 ± 0.11	-5.60 ± 1.52
<i>Gsta4</i>	NM_001106840	1.25	-1.63	-1.13 ± 0.05	-1.16 ± 0.15
<i>Mfrp</i>	NM_001108137	1.51	-16.12	1.30 ± 0.27	-11.07 ± 8.62
<i>Dnajc30</i>	NM_001109024	1.53	1.27	1.14 ± 0.17	-1.08 ± 0.19