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Genome-wide expression and methylation profiling in the aged rodent brain due to early-life Pb exposure and its relevance to aging

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Abstract

In this study, we assessed global gene expression patterns in adolescent mice exposed to lead (Pb) as infants and their aged siblings to identify reprogrammed genes. Global expression on postnatal day 20 and 700 was analyzed and genes that were down- and up-regulated (≥2 fold) were identified, clustered and analyzed for their relationship to DNA methylation. About 150 genes were differentially expressed in old age. In normal aging, we observed an up-regulation of genes related to the immune response, metal-binding, metabolism and transcription/transduction coupling. Prior exposure to Pb revealed a repression in these genes suggesting that disturbances in developmental stages of the brain compromise the ability to defend against age-related stressors, thus promoting the neurodegenerative process. Overexpression and repression of genes corresponded with their DNA methylation profile.

Keywords

gene expression; Pb (lead); aging; methylation arrays

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder whose clinical manifestations appear in old age. Cognitive decline and many of the hallmark pathological features of AD, are present in normal aging individuals. This poses a challenge of distinguishing AD from normal aging. The majority of AD cases occur in the elderly; however, it is still unresolved whether AD is a disease of old age or whether it has earlier origins. Late-onset AD (LOAD), a common sporadic form of the illness, may be influenced by epigenetic factors acquired during early developmental stages. Accumulating evidence suggests that LOAD may result from the combined effects of variations in a number of genes and environmental factors.

The Normative Aging Study (NAS) investigated a link between past non-occupational Pb exposure and cognitive decline. It was reported that higher levels of Pb in blood and/or bone were accompanied by poor cognitive performance in different cognitive tests including

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Wechsler Adult Intelligence Scale-Revised (WAIS-R), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) and Mini-Mental state examination (MMSE) (Payton et al., 1998; Weisskopf et al., 2004; Weisskopf et al., 2007; Wright et al., 2003). In a sub-group of the Nurses' Health Study (NHS), Weuve et al., (2009) reported that higher levels of Pb in tibia bone was associated with poor test scores of MMSE. Although it is not clear cut that Pb exposure is a risk for AD, a large body of clinical evidence supports a link between occupational lead exposure and cognitive decline. Cognitive decline is an intermediate stage towards the development of AD, as evident from an annual conversion rate of 28% from MCI to AD (Schmidtke and Hermeneit, 2008).

In animal models, it is strongly suggested that exposure to Pb during development could result in promoting the pathogenesis of AD (Basha et al., 2005a). In our lab's previous findings, rats that were developmentally exposed to Pb were monitored for their lifetime expression of the APP gene. We found that APP mRNA expression was induced transiently in the neonates; however, these rats exhibited a delayed overexpression 20 months later after cessation of the initial Pb exposure. This increase of APP mRNA expression was proportionally accompanied by a rise in the levels of the transcription factor specificity protein 1 (SP1) which is one of the regulators of the APP gene. As expected with the up-regulation of APP gene expression, elevation of APP and its amyloidogenic Aβ products were also observed in old age. Interestingly, these were not observed when the rats were exposed to Pb during old age. These findings were also confirmed on primates, which express amyloid plaques and other pathological features that are absent in normal non-transgenic rodents. Our lab obtained the brains of 23-year-old monkeys that were exposed to Pb during infancy and we found that APP protein and mRNA, and Aβ levels were elevated. Also, immunohistochemistry revealed the presence of AD-pathology, including Aβ plaques in the frontal association cortex (Wu et al., 2008). Thus, these data suggest that developmental Pb exposure could result in promoting AD-related amyloidogenesis.

The mechanisms by which Pb exerts its acute and chronic AD-related effects are still under extensive research. The observed delayed increase in SP1 and APP are suggested to be as a result of a decrease in the activity of the DNA methyltransferase 1 (DNMT1), a methylating enzyme specific for the cytosines in CpG dinucleotides (Wu et al., 2008). Pb also induces oxidative stress and damage; it was found that developmental exposure to Pb resulted in elevation of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG), a reactive oxygen species (ROS), that was not accompanied with the expected increase in the DNA repair enzyme 8-oxoguanine DNA glycosylase (Ogg1) activity (Bolin et al., 2006, Wu et al., 2008, Zawia et al., 2009). Clearance of Aβ is also impaired by Pb exposure. Behl and colleagues have found that Pb accumulates in the choroid plexus (CP) where it reduces the activity of the insulin degrading enzyme (IDE) leading to disturbances in the metabolic clearance of Aβ and thus a rise in its levels (Behl et al., 2009). In addition, neprilysin (NEP), a protease that is involved in monomeric and oligomeric degradation of Aβ, was found to be specifically affected by Pb, which decreased its mRNA expression and protein (Huang et al., 2011). Pb also has been linked to induce hyperphosphorylation of tau and data suggest that maternal Pb exposure resulted in increased hyperphosphorylated tau in offspring mice (Li et al., 2010) that can be explained by the decrease in the expression of protein phosphatase 2A (PP2A) and PP5 (Rahman et al., 2011).

Recently we monitored the lifespan profiles of AD-related biomarkers in both mice and primates (Dosunmu et al., 2009); however, that study plus our previous work focused on the patterns of expression and levels of a small set of AD associated genes. The purpose of this study is to perform a global assessment of gene expression and DNA methylation patterns to determine the scope of changes that occur in old age as a result of developmental exposure to Pb and their relationship to epigenetic pathways. Our goal is to shed light on

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developmental mechanisms that may explain the onset and/progression of a disease in old age. The findings from this study are intended to clarify the background of global gene expression and DNA methylation in our developmental animal exposure model of AD, so as to obtain a total picture of what was occurring at time of exposure and define changes that may impact AD-related outcomes as well as those that are involved in normal aging.

2. Materials and Methods

2.1 Animal exposure

C57Bl6 mice were bred in-house at the University of Rhode Island. The experiment was designed as in previous studies (Basha et al., 2003; Basha et al., 2005a). Twenty-four hours after the birth of a new mouse dam is Post-natal Day One (PND1). Male pups from the different dams were randomized, pooled and divided into the two following groups: 1) Control-no exposure to Pb and 2) Pb/E - *in utero* Exposure to Pb beginning on gestational day 13 until PND20. In the control conditions, the mice had freely accessible deionized drinking water. In the Pb/E exposure, 0.2% Pb-acetate (Sigma Chemical Company, St. Louis, MO) was added to the deionized drinking water of the pregnant female and the Pb/E dam group was exposed to the Pb drinking water through the mother's milk. Food was freely accessible throughout the study. The animals were housed at constant temperature (21±2°C) and relative humidity (50±10%) with a 12-hour light/dark cycle (light: 07:00-19:00 hours). The selected time points of PND 20 and 700 represented early and late time points of the animal for microarray analysis. Normal aging corresponds to C20 vs. C700 and Pb reprogrammed aging is C20 vs. E700. The animals were sacrificed following CO_2 exposure at day twenty and at two years of age and the total frontal cortical brain regions were isolated and stored at -80°C until future use. All animal procedures were conducted in accordance to the protocol approved by the Institutional Animal Care and Use Committee of the University of Rhode Island.

2.2 RNA extraction

RNA from the neocortex of three animals per age and condition was isolated using the TRIzol method (Invitrogen, Carlsbad, CA). Briefly, 100mg of tissue was homogenized in one ml of TRIzol reagent and, 200μl of chloroform was added to each sample. After the five minute incubation, samples were centrifuged for 12,000 × g for 15 minutes. The RNA recovered from the aqueous phase was mixed with equal volumes of 100% isopropanol and incubated for one hour at -80°C. After incubation, RNA was precipitated by centrifugation at 12,000 × g for 15 minutes, the supernatant was discarded and the pellet was washed twice with 75% and 100% ethanol, each. The RNA was further cleaned using standard methods from the Qiagen RNeasy Mini Kit (Valencia, CA).

2.3 NimbleGen cDNA microarray

RNA from the mouse neocortex, (three biological replicates/age/condition; 12 arrays total) was hybridized to the NimbleGen 12 × 135K array chip (Madison, WI). The samples were sent for array hybridization to the Keck Biotechnology Resource Laboratory at Yale University in New Haven, and the NimbleGen protocol was followed. Briefly, 10μg of total RNA was reverse transcribed to synthesize first strand cDNA, which was followed by second strand cDNA synthesis. The cDNA products were cleaned up using RNase A, and after re-precipitation, the QC of the cDNA was checked on an OD spectrophotometer and the Agilent Bioanalyzer. The cDNA samples were then labeled with Cy3 and after cleanup and quantification was incubated at -20°C until array hybridization.

The samples were resuspended in Sample Tracking Control and hybridization solution master mix was added to each sample. After aligning of the H × 12 mixer to the array, 6 μl

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of each sample was loaded to the fill port of each array. The samples were hybridized to the arrays at 42°C for ≥ 16 hours, and then washed with a series of three different wash buffers. After the last wash, the arrays were dried in the Nimblegen Microarray Dryer and scanned using the GenePix 4000B Scanner and software. The fluorescent images were imported to the NimbleScan software and images were extracted. The experimental metrics report was generated to assess the quality of the microarray experiment, then the pair reports generated for each image. The NimbleScan software normalized the raw expression data using quantile normalization methods and normalized gene calls were generated using the Robust Multichip Average (RMA) algorithm (Bolstad et al., 2003; Irizarry et al., 2003). Data analysis was performed in-house at the University of Rhode Island.

2.4 NimbleGen cDNA methylation array

DNA from the neocortex of three animals per age and condition was extracted from brain samples using standard procedures per the QIAamp kit. These data from these two groups was utilized for this analysis: C20 and E700. For preparation for the microarray, DNA was isolated and methylated according to the methlyated DNA immunopreciptation (MeDIP) protocol from the Weber group (Weber et al., 2005). Briefly, tissue from the frontal cortex was homogenized in 300μl TE buffer. 300μl of Lysis buffer (20mM Tris, pH8; 3mM EDTA; 20mM NaCl; 1% SDS; and 20μl proteinase K) was added and the mixture incubated for 5 hours at 55°C. The DNA was extracted with 1 volume of phenol and then one volume of chloroform. The DNA was then precipitated and resuspended in TE. The DNA was then fragmented using restriction enzyme *AluI* (NE BioLabs, Ipswich, MA) and then split into two groups: one set of samples served as the input and frozen until later use and the other set was methylated. The DNA set aside for methylation was diluted to 4μg, denatured for 10 minutes and allowed to cool for 10 minutes. 51μl of 10X IP buffer (100nm Na-Phosphate, pH 7; 1.4M NaCl, 0.5%Triton X-100) and 10μl of 5-methyl cytidine (Calbiochem-EMD Biosciences, Darmstadt, Germany) was added to the IP mixture and incubated for 2 hours at 4°C. Dynabeads Dynabeads M-280 Sheep anti-Mouse IgG (Invitrogen, Carlsbad, CA) were then added to the sample to collect the methylated DNA. The IP linked beads were then washed in 1X IP buffer and then collected using a magnet. The beads were resuspended in 250μl Proteinase K digestion buffer (50mM Tris pH 8; 10mM EDTA, 0.5% SDS) with 7μl Proteinase K added. The suspension was incubated for 3 hours at 50C. The DNA was then extracted with 1 volume of phenol and then one volume of chloroform. The DNA was then precipitated and resuspended in TE.

Nimblegen (Madison, WI) tilling arrays were used to scan 16,000 CpG islands in promoter regions. The samples were sent for array hybridization to the Keck Biotechnology Resource Laboratory at Yale University in New Haven, and the NimbleGen protocol was followed. Before labeling, the samples were run for a QC check to ensure methylated DNA on the Agilent 2100 Bioanylzer (Santa Clara, CA). The samples were labeled, pooled and hybridized to the array at 42°C for ≥ 16 hours. After hybridization the arrays were washed and after the last wash, the arrays were dried in the Nimblegen Microarray Dryer and scanned using the NimbleGen MS200 Data collection software. The fluorescent images were imported to the NimbleScan software and images were extracted. Data analysis was performed in-house at the University of Rhode Island.

2.5 Bioinformatics

The raw data from the microarray slide was managed and analyzed in-house using open-source software such as the R programming language and Bioconductor Library. Custom written code using Perl was used for data preparation. We averaged the normalized data of the three samples for each age group. Then, we produced the scatter plots using the “plot” function that is available in R, as shown in Figure 1. The data of age group C20 was used as

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the base values to identify the two fold changes in other age groups. For the heatmaps, we employed the hierarchical clustering method using the "heatmap.2" function that is available in the "gplots" package in R. The differentially expressed genes from each experiment were listed and a comparative analysis was carried out to discover the unique and common genes across samples. For this purpose, we used the "vennDiagram" function that is available in the "LIMMA" package in the Bioconductor Library.

We performed functional analysis using the web-based software tool Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) as described by Huang et al. (2009). The parameters used for analysis in this study were Gene Ontology (GO) Molecular Function term, Interpro name in Protein Domains, KEGG_Pathway and SP_PIR_Keywords in the Functional Categories section. Gene transcripts with unknown function and hypothetical gene transcripts were removed from the list of analyzed genes. Although gene transcripts may overlap in functional analyses, redundant terms and non-informative terms were excluded, for one gene-one category pie-chart analysis.

Statistically significant differential expression (down- or up-regulation) between PND 20 and PND 700 from control and Pb-exposed animals was determined by student t-test ($p \leq 0.01$) which was corrected for multiple comparisons using false discovery rate FDR (Q) with a threshold of $Q<0.5$. We identified the overlap of all significant gene transcripts across the experimental groups and generated heatmaps of uniquely down- and up-regulated genes at ages 20 and 700 of control and exposed animals.

DNA methylation data of control PND 20 and exposed PND 700 were normalized and $\log_2$-transformed to be correlated with gene expression data. The correlation coefficient of the differential gene expression and methylation with the associated *p* value were calculated and shown in the results. We mapped the averaged normalized data of DNA methylated locations to genes using perl scripts. The genes with 2 fold differential methylation based on C20 values were identified and plotted as shown in Figure 5.

2.6 Real-time PCR validation

A select number of genes on the array that were significantly expressed between normal aging and Pb-reprogrammed aging were validated using a two-step real-time polymerase chain reaction protocol. Genes that were most significant in the groups of down- and up-regulated genes and genes of interest to the study were examined. The genes validated were olfactory receptor 1140, kelch-like 1, cytidine 5′-triphosphate synthase, protein kinase C theta, aldehyde oxidase 1, transient receptor potential cation channel subfamily C member 6, methyltransferase-like 4, and choline dehydrogenase.

The first step was cDNA synthesis. The RNA was reverse transcribed to create cDNA, using the SA Biosciences RT^2 first strand kit (Qiagen Company, Frederick, MD). Briefly, 500ng of RNA was mixed with 2μl of 5X gDNA Elimination Buffer and 3μl of nuclease free H_2O. The reaction was incubated at 42°C for 5 minutes and chilled on ice for 1 minute. Then 10μl of RT cocktail (4μl of 5X RT Buffer 3; 1μl of primer and external control mix; 2μl of RT Enzyme Mix 3 and 3μl of H_2O) was added to the RNA mixture and then incubated at 42°C for 15 minutes and the reaction stopped at 95°C for 5 minutes. 91μl of H_2O was added to the cDNA mixture and was stored at -20°C until use in Real-Time PCR.

The cDNA underwent RT-PCR using the ABI 7500 real-time instrument (Applied Biosystems, Carlsbad, CA) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin as endogenous controls. The primers used were designed, synthesized and plated by SA Biosciences as a custom PCR array. To reproduce RT-PCR experiment, the accession

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number, catalog number and reference position for each primer is listed in Table 1. Briefly, each RT-PCR reaction mix contained the following: One μl of cDNA, one μl each of 200nM sense and antisense primers, 10.5μl of nuclease free H_2O and 12.5μl of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each sample was performed in triplicate and amplified using the following standard protocol: Initial step was 50°C for two minutes followed by 95°C for ten minutes, then 50 cycles of 95°C for fifteen seconds and 60°C for one minute. RT-PCR products were checked with agarose gel to confirm no non-specific products were formed. Results were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method (Livak and Schmittgen, 2001).

3. Results

The data reported below is for the four groups utilized for this study, with three animals averaged for each group: PND C20, C700, E20 and E700. Normal aging corresponds to C20 vs. C700 and Pb reprogrammed aging is C20 vs. E700. The Pb levels in the two age groups, PND 20 and 700 has been monitored and published in previous studies (Basha et al., 2005b). We found that at post-exposure 20 days, the Pb blood levels were 46.13 ± 1.95 μg/dl and brain levels were 0.41 ± 0.04 μg/g wet weight. Background levels (≤ 2 and ≤ 0.2 blood and brain, respectively) were seen in C20, C700 and E700.

3.1 Aging profile in developmentally Pb-exposed mice

We determined the relationship between groups by creating scatter plots of the array's gene transcripts, as shown in Figure 1. The number of genes that were altered between C20 and E20 were minor, indicating that in our study, early life showed few alterations in global gene expression (Figure 1A). In the normal aging profile, there were more dispersed points, indicative of gene expression alterations (Figure 1B). It should be noted that the animals that were Pb-reprogrammed had many more dispersed points *as well as* a drift towards global repression of genes (Figure 1C).

3.2 Functional analysis of down- and up-regulated genes

We restricted analysis to 2 fold differences in gene expression alterations between samples, so as to investigate the gene transcripts that showed the greatest magnitude of change. We identified 501 down-regulated and 647 up-regulated 2 fold gene changes out of 44,170 gene transcripts. To investigate these genes that were altered in normal aging and by Pb reprogrammed aging, we functionally analyzed and grouped the genes by relevant biological functions using the DAVID software, in addition to literature searches (Figure 2). Since little global difference was seen between the C20 and E20 samples, we used only C20 as a baseline for the early profile. The Venn diagram in Figure 2A shows that 142 gene transcripts were down-regulated as a result of normal aging, and the expression of 359 unique gene transcripts were repressed in old age due to Pb-reprogrammed aging.

For functional analysis, we based our categories on the most common ontological classifications that appeared in the output and created pie charts based on function (Figure 2B and 2C). Gene transcripts whose functions are unknown or hypothetical were not included in the count. Gene categories down-regulated (repressed) during normal aging in the rodent were primarily olfactory receptors followed by genes associated with signal transduction, transcription factors, and metal binding. While Pb does not alter the types of genes affected, there is an increase in the size of repressed genes by almost five fold in most categories.

In terms of up-regulation (Figure 2D), we found that 224 gene transcripts were highly expressed in the normal aging rodent brain; however, 423 unique genes were up-regulated in

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animals in Pb-reprogrammed aging. While global analysis of gene expression showed a preponderance of a downward shift in gene expression, there were many more uniquely up-regulated genes than those that are repressed in the aged brain. Up-regulated genes were similar to those in the down-regulated profile, however, there were more down-regulated genes related to transcription factor and DNA-binding proteins as well as genes involved with the immune response (Figure 2E).. More genes related to transcription factor and DNA-binding proteins as well as genes involved with the immune response were up-regulated in the aging brain. It is interesting to note that olfactory receptors, which are vital for the life of the rodent, are highly repressed in old age while pro-inflammatory genes are greatly up-regulated in old age. Reprogrammed aging by Pb does not uniformly increase the numbers of up-regulated genes in each category of genes; however, more genes associated with the immune response are increased (Figure 2F). On the other hand, the numbers of genes involved in DNA or metal binding are not increased by the early Pb exposure.

3.3 Significant genes

A hierarchical clustering method grouped all significantly differentially expressed genes on a single heatmap (Figure 3). The down-regulated genes (Figure 3A) showed a higher degree of repression in the younger animals than in the older animals, and the inverse is true of the up-regulated genes (Figure 3B). Our determination of the most significant changes found 150 genes with p values ranging from 7.62 E^{-05} to 0.01. The top 40 significant genes are seen in Table 2 (The complete list of significant genes is in the supplementary data). For many genes, where there is lack of annotation, or the function is of a hypothetical nature, those genes were eliminated from the final list.

3.4 Gene analysis validation through Real-time PCR

We validated the results obtained from our microarray analysis through RT-PCR. We chose a select subset of genes found to be the most significantly altered between normal aged and Pb-reprogrammed aged animals (Figure 4). The olfactory receptor 1140 did not show any amplification in the PCR experiment (data not shown). The rest of the results indicate that RTPCR gene amplification closely resembled array gene expression. In some cases, the expression in the PCR data was much greater, indicative of the greater sensitivity of the method.

3.5 Gene expression and methylation profiles

The most apparent mechanism that may mediate latent effects in gene expression is an epigenetic memory that is imprinted in tissue that will entail long-term consequences. DNA methylation arrays of C20 and E700 were used to explore and study gene expression and methylation correlation. We plotted the $\log_2$-transformed differential gene expression values against methylation intensity signals (Figure 5). The results shows a correlation coefficient r = -0.0459 with p value = 0.000769. Hypermethylated genes that were down-regulated clustered in the top left quadrant indicating a role of DNA methylation in gene silencing. Other quadrants, which consisted of much smaller sets of genes, did not exhibit a clear correlation between gene expression and methylation. Plotting gene expression and methylation profile of genes with 2-fold change alongside each other in a line plot as shown in Figure 6, revealed a consistent inverse-correlation between gene expression and methylation signals. We also found that the methylation pattern early in life is maintained in old age with minor changes related to reprogrammed developmental exposure. Consistent with gene expression profiling, the methylation profiles show that only a small percent of gene are affected by the developmental exposure to Pb.

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4. Discussion

One of the first indications that epigenetics was involved in LOAD came from studies from our lab (Basha et al., 2005b; Wu et al., 2008; Zawia et al., 2009) which showed that Pb exposure occurring during brain development pre-determined the expression and regulation of AD-related genes later in life, influencing the course of amyloidogenesis and oxidative DNA damage via a process that involved DNA methylation. These studies focused on AD-related genes and their regulatory regions; however a global assessment of gene expression and DNA methylation were not undertaken. Here we present findings on the global picture vis-a-vis gene expression and DNA methylation in the aging brain developmentally exposed to Pb.

The overall aging patterns in the adolescent and old mice indicated specific signatures. Global analysis of gene expression shows a preponderance of a downward shift in gene expression, however, there were many more uniquely up-regulated genes than those that are repressed in the aged brain. In Pb-reprogrammed aging, there was a global down shift of total gene expression. Although normal aging was associated with the down-regulation of genes, prior exposure to Pb repressed these genes even further. It is important to note that these results reflect activity in many cell types and not just neurons. Published work has shown that normal aging in mice may have altered differential gene expression with age, and a global repression is seen on a genome wide scale in humans and primates (Loerch et al., 2008).

Early life exposure to Pb does not alter the type of gene transcripts affected; it only increases the size of repressed gene categories represented, by almost 5 fold. The same was true for up-regulated genes, although the functional categories were different in representation. Up-regulated categories indicated that there were more genes related to transcription factor and DNA-binding proteins as well as genes involved with the immune response. The aging brain experiences neurodegenerative and inflammatory effects consistent with what is seen in this microarray.

Stringent statistical analysis to identify the most significant changes in gene expression (p values between 0.001 to 0.005) that were altered due to normal aging or following developmental exposure to Pb led to the emergence of a striking pattern. We found that the majority of the significant genes fall under four categories: metabolic enzymes, transduction/ transcription, immune response, and metal binding. The overall pattern and theme observed following such statistical analysis, is that these genes are uniquely up regulated during normal aging. These genes are suspected to play a role in combating or reacting to triggers and stressors in old age. Developmental exposure to Pb suppresses the expression pattern of these genes unique to aging and may thus disable the brain's capacity to defend against neurodegenerative damage. This has been seen in multiple studies as reviewed by Bouton and Pevsner (2000), in that Pb induces cellular defense mechanisms against toxic insults such as metallothioneins, stress response proteins, NF-Kappa B and heat shock proteins. Transcription factor binding altered by Pb has also been seen in our previous studies of Pb and transcriptional regulation of AD related genes by SP1 and Oct-2 (Bakheet et al., 2007; Basha et al., 2005a).

Epigenetics is a major mechanism that accommodates gene-expression changes in response to gene-environment interactions (Holliday, 2006; Morange, 2002). Epigenetics refers to modifications in gene expression that are primarily influenced by DNA methylation. DNA methylation is largely seen as a gene silencer, and in our study we do see a global repression of genes in Pb-perturbed animals. The methylation data analysis reveals a consistent inverse relationship between gene expression levels and methylation intensity in particular, the role

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of hypermethylation in gene silencing. It is interesting to note that two years after exposure to Pb, the cerebral methylation profile of adolescent (C20) control animals matches that of aged animals exposed to Pb as infants (E700), with few variations. The methylation profile shows alterations in only a small percent of genes due to developmental exposure to Pb. Future work will focus on specific genes and their methylation signal at different sites of the same gene. It is also important to note that, there is more overall methylation (hypermethylation) at E700 than at C20, consistent with the global repression of gene expression observed.

5. Conclusion

The data generated from the microarray analysis screened over 40,000 gene transcripts that covered the mouse genome, and identified 150 genes for a transcriptional profile of normal aging and environmentally perturbed aging. The genes that appear to be involved in immune responses, metal binding, metabolism, transcription, and transduction feature very prominently during old age. In normal aging, those genes are up-regulated, perhaps as a compensatory response to stressors acting on the aging brain.. Prior developmental Pb exposure caused an overwhelming repression of these genes. DNA methylation appears to play an important role in the downregulation of these genes suggesting that early life exposure to Pb interferes with the methylation pattern of genes, which is then sustained throughout life, and has an impact on an animal's ability to respond in old age.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Early-life exposure to a pollutant reprograms global gene expression in old age through epigenetic mechanisms.
2. Genes associated with the immune response, metal binding, metabolism and transcription are up-regulated in the aging brain.
3. Developmental exposure to a pollutant (Pb) results in the repression of these genes in old age.
4. Reprogrammed gene expression appears to correlate with alterations in DNA methylation.

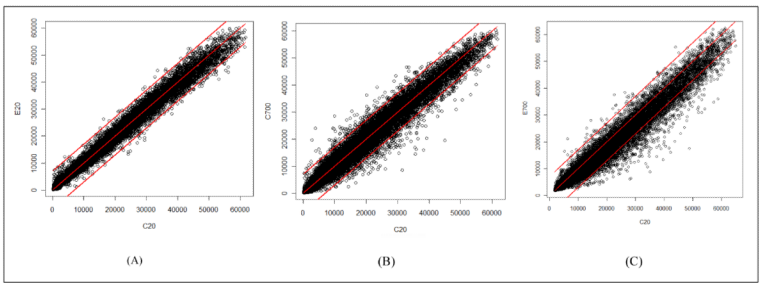

Figure 1. Scatter plots depicting overall gene expression patterns in young and old animals and those developemntally-exposed to Pb

(A) early-life perturbation: PND20 control vs developmentally Pb-exposed PND20 animals; (B) normal aging: control PND20 vs. control PND700; and (c) Pb-reprogrammed aging: control PND20 vs devleopmental exposed PND700. Each group represents data pooled from three animals. Both young and old animals are offspring from the same cohort. The axis numbers represent fluorescent intensity.

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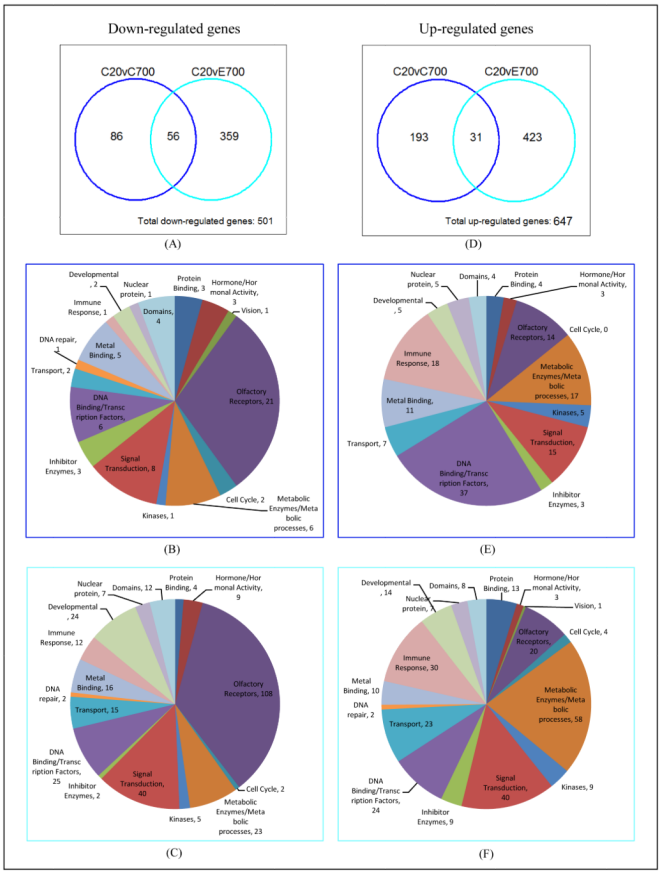

Figure 2. Functional annotations of genes from control aging rodents and those with early-life exposure to Pb

The Venn diagram in (A) represents down-regulated genes in the normal aging mouse brain in control animals as well as those exposed to Pb early in life [PND 700 is compared to PND 20 as the baseline (C20)]; (B) shows the functional groups that are down-regulated in normal aging, and (C) displays down-regulated genes due to early-life exposure to Pb. The Venn diagram in (D) represents up-regulated genes in the aging mouse brain in control animals as well as those exposed to Pb early in life [PND 700 is compared to PND 20 as the baseline (C20)]; (E) shows the functional groups that are up-regulated in normal aging, and (F) displays up-regulated genes due to early-life exposure to Pb. Normal aging is shaded in purple to distinguish genes that change solely due to aging. The frontal cortex from three analysis was arrayed separately (n=3) and the data was grouped from the three animals for C20, C700 and E700 groups.

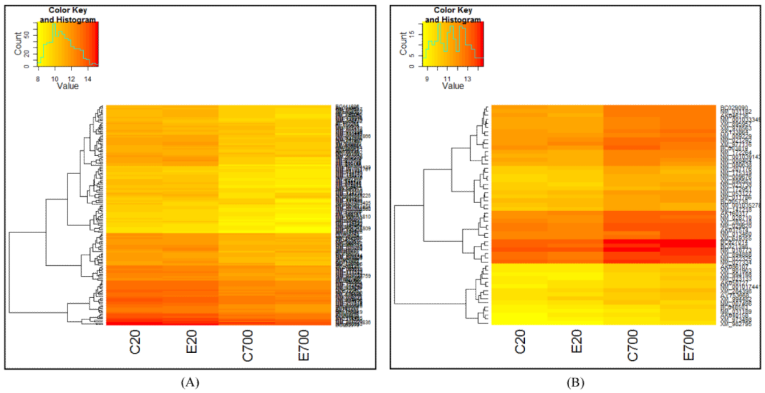

Figure 3. Hierarchical clustering of the most significantly altered genes

Shown through heat maps is the expression patterns of significant two fold differential gene expression ($p \leq 0.01$) in the normal aging brain and in aged brains developmentally exposed to Pb. The yellow rows indicate the genes exhibiting the least degree of change; the red rows indicate the highest degree of change as well as the genes relationships to each other. The rows designate the relationship of the genes to each other and the columns indicate the age of the animals. Down-regulated genes (A) and up-regulated genes (B) are shown; different genes are represented horizontally and their relationships to each other in the dendogram are shown vertically.

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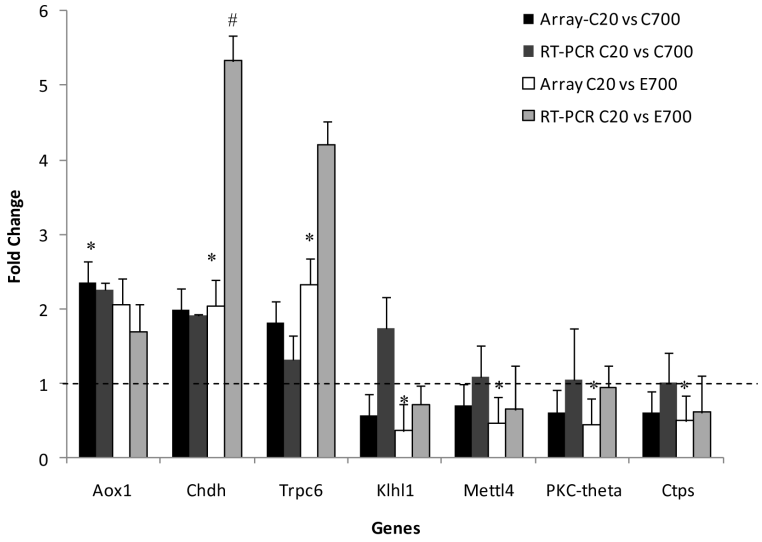

Figure 4. Gene validation through RT-PCR

Comparison of array and RT-PCR fold change data for selected genes changed from normal aging (C20 vs C700) and Pb-reprogrammed aging (C20 vs E700). Values over 1 (dashed line) are up-regulated and the values under 1 indicate genes that are down-regulated. The values ≥ 2 and values ≤ 0.5 are significantly up-regulated and down-regulated, respectively. "*" denotes significance has been determined by student's t-test ($p \leq 0.01$). "#" denotes significance has determined by student's t-test ($p \leq 0.05$). The genes that were up-regulated in the array were aldehyde oxidase 1 (Aox1), choline dehydrogenase (Chdh) and transient receptor potential cation channel subfamily C member 6 (TRCP6). The genes that were down-regulated in the array were kelch-like 1 (KLHL1), methyltransferase like 4 (METTL4), protein kinase C-theta (PKC-theta) and 5′ cytidinetriphosphate synthase (CTPS).

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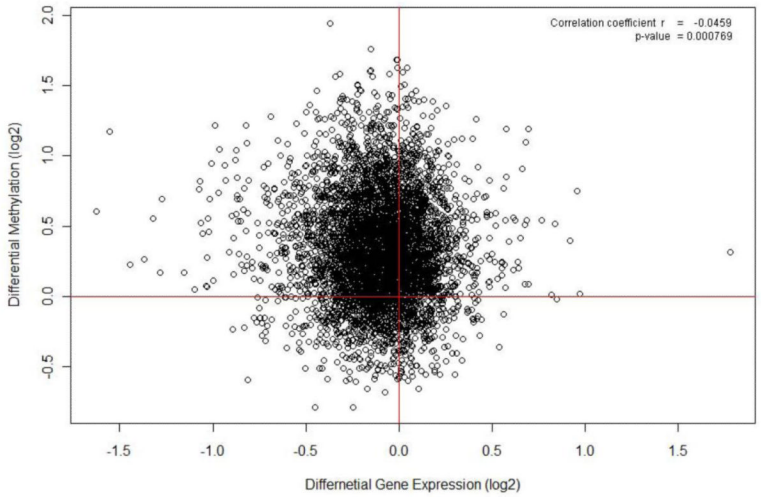

Figure 5. Differential Gene Expression and Methylation Correlation

A scatter plot of differential gene expression of control PND20 and developmentally-exposed PND700 (E700 - C20) vs the differential methylation of control PND20 and developmentally-exposed PND700 (E700 - C20). Methylation data were linearly transferred and $\log_2$ was applied to gene expression and methylation signal intensity values.

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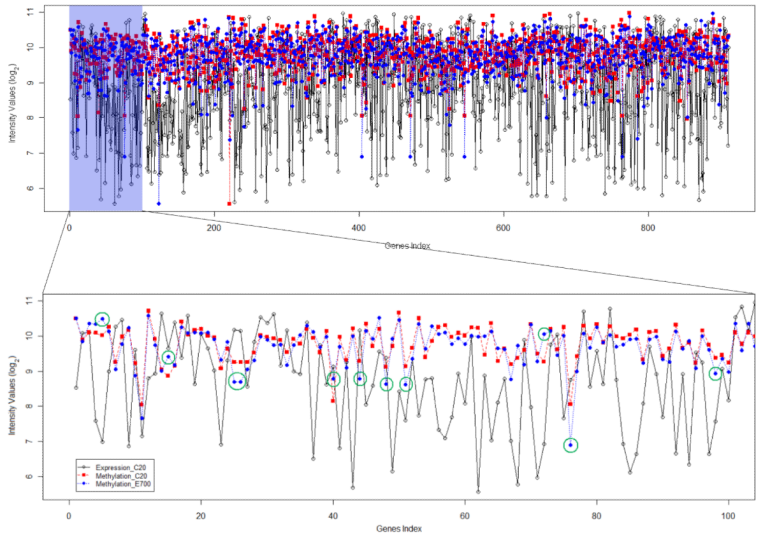

Figure 6. Gene expression and methylation profiles

A line plot of gene expression intensity values of control PND20 (C20) alongside the methylation intensity values of control PND20 and developmental exposed PND700 (E700) for the selected genes that have ≥2 fold differences in gene expression alterations between samples. The top part of the figure shows all selected genes while the bottom part shows to the first 100 genes. The circled E700 methylation values indicated the genes with noticeable difference between C20 and E700 in methylation values. Gene expression and methylation intensity values were log2-transformed.

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Table 1

Primer list for SA Biosciences custom RT-PCR array.

Gene Name	Accession	Catalog Number	Reference Position
Olfactory receptor 1140	NM_146642	PPM36374	689
Kelch-like 1	NM_053105	PPM39954	2856
Cytidine 5′- triphosphate synthase	NM_016748	PPM24712	1788
Protein kinase C theta	NM_008859	PPM04035	3025
Aldehyde oxidase 1	NM_009676	PPM26932	3072
Transient receptor potential cation channel subfamily C member 6	NM_013838	PPM04056	3054
Methyltransferase-like 4	NM_176917	PPM41961	1861
Choline dehydrogenase	NM_172264	PPM35704	609
Actin, beta	NM_007393	PPM02945	182
Glyceraldehyde-3-phosphate dehydrogenase	NM_008084	PPM02946	309

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Table 2

Most significantly altered genes

Gene Name	Accession	*P* value	*Q* value
Down Regulated Genes: Normal Aging (C20 vs. C700)			
Olfactory receptor 1140	NM_146642	0.00032	0.0397201
Olfactory receptor 1196	NM_146464	0.000623	0.0653306
Armadillo repeat containing 9	AK019512	0.001083	0.097071
Tigger transposable element derived 4	NM_207278	0.001453	0.118282
Tumor-associated calcium signal transducer 1	NM_008532	0.00164	0.1293019
Makorin, ring finger protein, 1	BC003329	0.003429	0.2036118
Serine/threonine kinase 17b (apoptosis-inducing)	BC006579	0.003481	0.2052681
Olfactory receptor 821	NM_146776	0.004796	0.2519902
Paired box gene 5	NM_008782	0.008716	0.3530649
Deafness, autosomal dominant 5 homolog (human)	AK155135	0.008724	0.3530649
Down Regulated Genes: Pb Reprogrammed Aging (C20 vs. E700)			
Kelch-like 1 (Drosophila)	NM_053105	7.62E-05	0.0138233
Zinc finger, DHHC domain containing 9	NM_172465	0.0001041	0.017518
SRY-box containing gene 4	NM_009238	0.0002195	0.0309102
Semaphorin 6A	BC062979	0.0002583	0.0349115
Olfactory receptor 728	NM_001011809	0.0003507	0.0421781
Ankyrin repeat domain 1	NM_181404	0.0003685	0.0439465
Olfactory receptor 58	NM_011001	0.0004234	0.0491179
Adenylate cyclase activating polypeptide 1	NM_009625	0.0004416	0.0504052
Trans-acting transcription factor 7	BC113150	0.0005488	0.0597574
Olfactory receptor 485	NM_001011810	0.0005703	0.06116
Up Regulated Genes: Normal Aging (C20 vs. C700)			
Histocompatibility 2, T region locus 22	BC003819	5.61E-05	0.0107106
Fer-1-like 3, myoferlin (C.elegans)	AK086107	0.0003392	0.0413795
Arylsulfatase G	NM_028710	0.0006639	0.0684958
Integrin beta 2	NM_008404	0.0007695	0.0760127
Transcription factor AP4	NM_031182	0.0012829	0.108067
Major vault protein	NM_080638	0.0026613	0.173249
Aldehyde oxidase	NM_009676	0.0029233	0.1836249
Src family associated phosphoprotein 2	NM_018773	0.0051528	0.2605469
Methyltransferase like 7A	NM_027334	0.006102	0.2899472
Procollagen C-endopeptidase enhancer 2	NM_029620	0.006225	0.2927196
Up Regulated Genes: Pb Reprogrammed Aging (C20 vs. E700)			
Transient receptor potential cation channel, subfamily C, member 6	AK133864	0.00010855	0.0179826
Eukaryotic translation initiation factor 4E member 3	BC027014	0.00020028	0.0289048
Cathepsin Z	NM_022325	0.00025915	0.0349155
Ribosomal protein S19	NM_023133	0.0007459	0.0745462
Coiled-coil domain containing 18	AK040158	0.00081893	0.0797693

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Gene Name	Accession	*P* value	*Q* value
Choline dehydrogenase	NM_172264	0.00128442	0.108067
Kinesin family member 13B	XM_283218	0.00132082	0.1096178
Histocompatibility 2, Q region locus 1	AK037574	0.00183872	0.1394476
Myogenin	NM_031189	0.00188253	0.1413305
Corticotrophin releasing hormone receptor 2	AY753668	0.00230183	0.1608218

* For the complete list of altered genes, please see the Supplementary data.