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Developmental exposure to lead (Pb) alters the expression of the human tau gene and its products in a transgenic animal model

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Abstract

Tauopathies are a class of neurodegenerative diseases associated with the pathological aggregationof the tau protein in the human brain. The best known of these illnesses is Alzheimer's disease (AD); a disease where the microtubule associated protein tau (MAPT) becomes hyperphosphorylated (lowering its binding affinity to microtubules) and aggregates within neurons in the form of neurofibrillary tangles (NFTs). In this paper we examine whether environmental factors play a significant role in tau pathogenesis. Our studies were conducted in a double mutant mouse model that expressed the human tau gene and lacked the gene for murine tau. The human tau mouse model was tested for the transgene's ability to respond to an environmental toxicant. Pups were developmentally exposed to lead (Pb) from postnatal day (PND) 1-20 with 0.2% Pb acetate. Mice were then sacrificed at PND 20, 30, 40 and 60. Protein and mRNA levels for tau and CDK5 as well as tau phosphorylation at Ser396 were determined. In addition, the potential role of miRNA in tau expression was investigated by measuring levels of miR-34c, a miRNA that targets the mRNA for human tau, at PND20 and 50. The expression of the human tau transgene was altered by developmental exposure to Pb. This exposure also altered the expression of miR-34c. Our findings are the first of their kind to test the responsiveness of the human tau gene to an environmental toxicant and to examine an epigenetic mechanism that may be involved in the regulation of this gene's expression.

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.06.001.

Keywords

Alzheimer's disease; Neurofibrillary tangles; Lead; Tauopathy; microRNA

1. Introduction

Tauopathies are a class of neurodegenerative diseases associated with the pathological aggregation of the tau protein in the human brain. There are approximately thirty known tauopathies, with different clinical, pathological and biological characteristics (Caillet-Boudin et al., 2015). Some tauopathies are considered familial and are related to precise mutations of the tau gene, while others are sporadic in nature with no direct genetic links. Perhaps the best-known tauopathy is Alzheimer's disease (AD), the most common form of dementia affecting elderly people over the age of 65 and the sixth leading cause of death in the United States (Alzheimer's, 2015). A key pathological feature of AD is the aggregation of the tau protein and its deposition within neurons in the form of neurofibrillary tangles (NFTs). In the healthy brain, tau stabilizes microtubules and supports intracellular transportation. If tau is hyperphosphorylated, its binding affinity for tubulin is lowered, increasing the probability that tau will aggregate and form tangles. The aberrant phosphorylation of tau compromises microtubule stability, axonal transport and, therefore, cognitive function (Gendron and Petrucelli, 2009).

Patients that develop AD prior to age 65 are considered to have Early Onset AD and may exhibit hereditable mutations in the *Amyloid Precursor Protein* (APP), *Presenilin1* (PSEN1) or *Presenilin2* (PSEN2) genes (Liddell et al., 2001). Most individuals that develop AD (>95%) have sporadic or Late Onset AD (LOAD) (Bekris et al., 2010). Genome analyses have failed to identify a clear genetic etiology for this form of AD, suggesting the possibility that environmental exposures and related epigenetic modifications play an important role in the pathogenesis of sporadic forms of AD and other tauopathies (Karch and Goate, 2014; Lunnon and Mill, 2013; Rosenthal and Kamboh, 2014).

The environment contains a number of toxicants that are risk factors for the development of neurodegenerative diseases. These include, but are not limited to, pesticides (such as DDT), metals, and air pollution (Dosunmu et al., 2007). The heavy metal lead (Pb) still poses a danger as an environmental toxicant. While extant research does not provide clear evidence that Pb exposure is the cause of AD or other tauopathies, the identification of relationships between Pb poisoning and cognitive decline began to surface after several longitudinal and cross-sectional studies were performed on the elderly. The Normative Aging Study, a prospective continuing study started by the Veterans Administration in 1963, has shown that participants with higher levels of Pb in the blood/bone exhibited diminished performance in cognitive measures such as the Wechsler Adult Intelligence Scale-Revised and Mini-Mental state examination (Payton et al.,1998; Weisskopf et al., 2007, 2004; Wright et al., 2003).

In conjunction with this data collected in humans, recent research has demonstrated that mice exposed to Pb postnatally performed worse during cognitive behavior tests as aged adults (Bihaqi and Zawia, 2013). These behavioral deficits correlated with the changes in the protein and gene expression levels of tau, phosphorylated tau, related enzymes and

transcription factors (Bihaqi et al., 2014; Bihaqi and Zawia, 2013). More recently we discovered that epigenetic mediators such as microRNA may paly a role in mediating these changes in gene expression (Masoud et al., 2016). Developmental Pb studies completed in the wild-type mice were replicated in non-human primates. Results from the primate study were consistent with those found in the mouse model. Furthermore, the exposed primates showed a development of NFTs at the end of life, a classic pathological marker of AD (Bihaqi and Zawia, 2013).

To translate the results seen in our rodent and primate studies into a human application, we have implemented the use of a transgenic mouse model that possesses the gene for human tau. Prior studies have provided strong evidence for the ability of an environmental toxin to affect the murine tau gene, but there is a critical need to examine the relationship between toxins and the human tau gene because the mouse and human genes produce different protein products. The mouse gene does not produce all of the isoforms produced by the human gene. This whole line of investigation would have to come to a dead end if it was discovered that the human gene was not sensitive to the effects of Pb exposure.

The present study utilizes a transgenic mouse that has a homozygous knock-out of the murine tau gene and is hemizygous for the human tau gene. On a molecular level, this transgene includes the promoter, intronic regions, and regulatory regions of the human MAPT gene. The transgenic mouse model develops all six isoforms of the human protein tau, including both 3R and 4R isoforms. Since the adult mouse brain contains exclusively the 4R tau isoform, while the adult human brain contains roughly equal amounts of 3R and 4R, this model has a representative human tau ratio (Andorfer et al., 2003).

The primary purpose of the current research was to explore the utility of this transgenic mouse model in the study of interactions between environmental toxicants and the human tau gene. Our investigation examined whether the human tau transgene is responsive to exposure to a developmental toxicant by determining the effect of this exposure on tau protein and gene expression. In addition, we surveyed the impact of the toxicant on a population of miRNA that target the mRNA for MAPT.

2. Methodology

2.1. Animals and exposure

This study employed the transgenic mouse model B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J [Jackson Laboratory Stock 005491, Bar Harbor, ME]). No endogenous mouse MAPT is detected in these mice, but all six isoforms (including both 3R and 4R forms) of human MAPT are expressed. Mice were bred in the Animal Care Facility at the University of Rhode Island (Kingston, RI). All experiments were performed in accordance with the standard guidelines and protocol approved by the University of Rhode Island Institutional Animal Care and Use Committee (IACUC) with supervision of the university's veterinarian.

The transgenic mice were divided into two groups. One group of mice was exposed to Pb during the first 20 days of postnatal life according to the protocol we have published

previously (Bihaqi et al., 2014). Mice in the second cohort were not exposed to Pb and served as controls. The first group was exposed to 0.2% Pb Acetate via the drinking water of their respective dams. Lead acetate (500 mg) was dissolved in 250 mL of deionized water. This exposure protocol causes the concentration of Pb in the cerebellum of PND 20 rodents (0.25+/- 0.07 μg/g) to be approximately three times the level seen in control animals (Zawia and Harry, 1996). Blood levels have been shown to be 46.43 (μg/dl during Pb exposure but are reduced to basal levels in adults (Basha et al., 2005). Exposure began 24 h after birth (PND 1) and continued until weaning (PND 20). Mice were euthanized with CO_2 at PND 20, PND 30, PND 40 and PND 60 for protein and mRNA measures and at PND 20 and PN D50 for miRNA assays. For each time point and exposure group there was an n = 3. The brains were removed and placed on ice followed by immediate storage at −80°C.

2.2. Genotyping

Pups born in-house were weaned, separated by sex, and genotyped at PND 20. Tail snips of 2 mm were taken from each mouse and a DNA extraction was performed on each sample. The resulting DNA was amplified with the following primers— transgene sense: 5′-CGAAGTGATGGAAGATCACG-3′ and transgene antisense: 5′-GTCTTGGTGCATGGTGTAGC-3′. Amplification was conducted under the following conditions: 94 °C for 2min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The amplified product was electrophoresed on a 2% agarose gel at 100 V for 90 min and imaged on the Amersham Typhoon Imager Scanner FLA 9000 (GE Life Sciences, Piscataway, NJ).

2.3. Protein levels

Western blots were utilized to examine protein levels of total tau, phosphorylated tau Ser396, and cyclin dependent kinase 5 (CDK5). The following protocol was used to prepare samples for total tau, Ser396, and CDK5: 50 mg of cerebral cortical brain tissue was homogenized in 1 mL of radioimmunoprecipitation assay (RIPA) lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid and 2 μL protease inhibitor, and 10 μL phosphatase inhibitor. The homogenates were placed on ice for 10min before being centrifuged at 8000× RPM for 10 min at 4°C. Lysate protein concentrations were determined by a bicinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL), at 560 nm using the Spectra-Max Multimode plate reader (Molecular Devices, Sunnyvale, CA). Following protein standardization, samples were prepared for western blotting according to the concentrations listed in Table 1.

Samples were separated on polyacrylamide gels at 85 V for 90min and then transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked at room temperature and incubated overnight with blocking buffer and primary antibody (Table 1) with gentle shaking at 4°C. The membranes were then washed with TBST three times and incubated with the appropriate secondary antibody. The membranes were washed 3 times with TBST and 3 times with TBS. Membranes were imaged on the Odyssey® Infrared Imaging System (Li-Cor, Lincoln, NE). All blots were normalized to housekeeping proteins

GAPDH or β-Actin. Total tau and phospho-tau blots were normalized to GAPDH. CDK5 was normalized to β-Actin.

2.4. Immunohistochemistry

Immunohistochemistry was used to visually confirm the presence of hyperphosphorylated tau in 13-month-old mice. While our study involved developmental profiles, we used older mice to confirm the presence of tau immunoreactvity and to observe for pathological deposits associated with the presence of the human tau gene. These normally do not fully manifest until an older age. According to a protocol previously published (Subaiea et al., 2015), mice were deeply anesthetized with an intraperitoneal injection of 0.1 ml/10 g of xylazine-ketamine mixture (100-10 mg/ml) and were perfused transcardially with 100 ml of perfusion wash containing 0.8% sodium chloride, 0.8% sucrose, 0.4% dextrose, 0.034% anhydrous sodium cacodylate, and 0.023% calcium chloride. Mice were then perfused with 100 mL of perfusion fix containing 4% paraformaldehyde, 4% sucrose, and 1.07% anhydrous sodium cacodylate, and their brains were removed. The extracted brains were post-fixed in the perfusion fix solution overnight and then cryopreserved in 30% sucrose solution at −20 °C. Fixed brains were sent to NeuroScience Associates (Knoxville, TN) and stained with 1:2500 phospho-tau Thr181 (Thermo Scientific Pierce, Rockford, IL).

2.5. RNA and micro RNA (miRNA) isolation and real-time PCR

Total RNA was isolated from Pb-exposed and non-exposed cerebral cortex tissue according to the TRIzol method (Invitrogen, Carlsbad, CA). Purity and concentration were confirmed using a Nanodrop UV/vis Spectrophotometer (Thermo Scientific, Wilmington, DE). First strand complementary DNA (cDNA) was synthesized from 1 (μg total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) for mRNA and NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen, CA) for miRNA. cDNA was amplified using real-time polymerase chain reaction (PCR). The SYBR Green quantitative real-time PCR assay was performed in 12.5 μl (20 μl for miRNA) reactions using 1 μl cDNA template, 1 X SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.5 μM forward and reverse primers, and deionized H_2O. The primers utilized can be found in Table 2. Amplification was performed on ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA) following the standard protocol: 50°C for 2min followed by 95 °C for 10min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Results were analyzed with with ViiA 7, and expression was reported relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA with the $2^{-\Delta\Delta C_T}$ method for mRNA and $2^{-\Delta C_T}$ for miRNA which was normalized to small nucleolar RNA 202 (sno202).

2.6. Statistical analysis

Western blot bands were quantified using the Li-Cor Odyssey infrared image system. Real time PCR curves were quantified using Viia7 Real-Time PCR System software. All measurements were made in triplicate and all values are presented as mean istandard error of the mean. Analyzing the protein and mRNA levels of the Pb-exposed group versus the control groups tested the responsiveness of the transgene to Pb exposure. One-way analysis of variance (ANOVA) for each gene was performed with the Newman-Keuls multiple-comparison post hoc test. Newman-Keuls was chosen as the preferred method for a stepwise

approach to pairwise comparisons of the mean responses due to increased power among smaller number of treatment groups. To determine the significance of interaction between Pb-exposure and the time points investigated, a two-way ANOVA and a Newman-Keuls multiple-comparison post hoc test was performed for each gene's protein expression. Effect sizes were calculated to assess the magnitude of the interaction between Pb-exposure and time. The level of significance for each ANOVA was set at $\alpha = 0.05$. All analyses were performed using Sigma Stat 3.5 computer software.

3. Results

3.1. Genotyping and immunohistochemical characterization of the human tau phenotype

Genotyping and immunohistochemistry were conducted on aged sibilings of the experimental cohorts as part of the model validation. Analysis of DNA obtained from the tails of PND20 mice revealed clear differences between mice carrying the transgene versus non-carrier controls. Immunohistochemical staining for phosphorylated tau (Thr181) in older (13 month) transgene carriers demonstrated clear evidence of hyperphosphorylation in multiple cerebral cortical regions, including the internal capsule (Supplementary Fig. 1A) and the external capsule (Supplementary Fig. 1B).

3.2. Effect of Pb exposure on biomarkers of neurodegeneration

Protein levels of cerebral cortical total tau normalized to GAPDH were analyzed by Western blot after postnatal dosing with 0.2% Pb acetate for 20days (Fig. 1A). One-way ANOVA revealed a dramatic increase in total tau protein levels in the brains of Pb-exposed mice at PND 20 and 30 which then returned to basal levels by PND 40-60 ($p=0.008$, $p=0.006$ respectively) (Fig. 1B). A two-way ANOVA revealed a statistically significant interaction between the time points and Pb-exposure ($p=<0.001$). The interaction effect, η^2, for this relationship is 0.512 revealing that 51 % of the variance was due to an interaction between time and exposure. Tau mRNA was evaluated for Pb's ability to affect expression. Analysis of tau mRNA levels shows that at PND 20, postnatal Pb exposure has a similar up-regulation effect on the expression of tau at the transcriptional level (Fig. 1C).

The cerebral cortical levels of phosphorylated tau Ser396 normalized to GAPDH were analyzed by Western blot after postnatal lead exposure (Fig. 2A). Results from a one-way ANOVA revealed a significant increase of phosphorylated protein expression in Pb-exposed mice at PND 20 and PND30, which also normalized at PND40-60 ($p=0.001$ and $p=0.001$, respectively) (Fig. 2B). Two-way ANOVA findings revealed a statistically significant interaction between the time points and exposure ($p < 0.001$). The h^2 for this interaction is 0.510 revealing that 51% of the variance is attributed to the interaction between exposure and time.

Cerebral cortical CDK5 normalized to β-actin was analyzed by Western blot to measure the effect of Pb on the kinase's protein expression levels (Fig. 3A). CDK5 levels in Pb-exposed mice showed an up-regulation trend at every time point as compared to controls (Fig. 3B). At PND 40 and PND 60, a one-way ANOVA test reported a significant difference ($p = 0.001$ and $p< 0.001$, respectively). A two-way ANOVA revealed that the effect of Pb exposure does

not depend on the time point. Stated differently, there is no statistically significant interaction between the time points and exposure ($p = 0.067$). η^2 for this interaction is 0.127; a finding that reveals just under 13% of the variance can be attributed to the interaction effect between Pb exposure and time. CDK5 mRNA levels also followed a pattern of consistent upregulation in the Pb-exposed mice compared to unexposed controls (Fig. 3C). One-way ANOVA results revealed no statistical significance between the treatment groups for CDK5 gene expression.

Recent studies by us have suggested that microRNAs may play a role in mediating Pb-induced changes in gene expression. We have shown previously that exposing wild-type mice to Pb produces a transient increase in the expression of miRNA that target *APP* mRNA and the mRNA for proteins involved in promoter methylation (Masoud et al., 2016). This increase in the expression of select miRNA coincided with a period of decreased protein expression that followed the Pb exposure, suggesting that the miRNA might be inhibiting the translation of these proteins. In the present study, we found that Pb exposure produced an analogous and significant ($p=0.037$) increase in the expression of miR-34c between PND20 and PND50 (Fig. 4). This miRNA has been shown to target the mRNA for human MAPT (Wu et al., 2013).

4. Discussion

Extant findings for wild-type mice have provided evidence that developmental Pb exposure has an effect on tau, its kinase's and phospho-tau (Bihaqi et al., 2014). The expressed hypothesis of this study was focused on identifying the responsiveness of the human tau transgene to an environmental exposure. Initial genotyping confirmed that transgenic mice adequately express human tau and that murine endogenous tau was expectedly absent due to its deletion. Immunohistochemical staining in aged mice with an antibody for phospho-tau site Thr181 validates that the mouse model has high levels of phosphorylated tau in various brain structures as they age. This is in accordance with previously published findings (Andorfer et al., 2003).

Western blotting and quantitative real time PCR demonstrated that Pb has an effect on the human tau gene transcription and translation. Previous studies have shown postnatal exposure in wild-type mice increases levels of tau, phosphorylated tau, and its related enzymes (Bihaqi et al., 2014). We tested to see the same pattern within the transgenic mouse model between PND 20-60. The findings from this current study illustrated that Pb indeed has an effect on the human tau gene which occurs within a stage-specific developmental window and then normalized by adulthood. These observations are consistent with numerous studies reported by our group on the impact of developmental Pb exposure and gene expression and more recently shown to occur for tau (Bihaqi et al., 2014). The latent effects of elevated tau levels in late life seen in the wild-type mice are possibly being established and further aging of these mice would be expected to reveal a delayed upregulation in this biomarker in old age.

The phosphorylated tau serine 396 protein levels follow the same upregulation pattern of the total tau protein levels (Figs.1–2). This infers that as more total tau is being translated,

phosphorylation of the protein is also increasing; suggesting that the hyperphosphorylation of tau is substrate driven. The total tau mRNA levels illustrate that the upregulation of transcription at PND 20 results in excessive protein translation and subsequent phosphorylation at PND 20 and 30. At PND 40 and PND 60 the transcriptional levels are similar in pattern to the protein levels.

Additional western blotting showed consistent elevated levels of CDK5 in Pb-exposed mice compared to controls. This was analogous to the two-year Pb exposure study done in the wild-type mouse model (Bihaqi et al., 2014); however, it is important to note that both CDK5 levels and gene expression are belatedly elevated on PND 40 and such elevation is sustained throughout their adulthood. This suggests that while CDK5 can contribute to the posttranslational modification of tau, it is not responsive to substrate levels and may be activated by alternate pathways. It is also important to note that Ser396 is a major target of glycogen synthase kinase 3 (GSK3), a second kinase of tau (Kimura et al., 2014). CDK5 was the primary kinase evaluated in this study because of its importance in abnormal tau phosphorylation as well as many GSK3 sites, including Ser396, needing to be primed by CDK5 initially (Kimura et al., 2014).

MicroRNAs (miRNA) are small non-coding RNA of ~22 nucleotides that act as important post-transcriptional regulators of gene expression by binding to the 3′ un-translated regions of their target mRNAs, resulting in mRNA degradation or the inhibition of protein translation (Goodall et al., 2013). It has been shown that miR-34c binds to human MAPT mRNA and thus inhibits the expression of MAPT protein (Wu et al., 2013). In the present study we demonstrate that early postnatal Pb exposure causes an increase in the expression of this miRNA that coincides with the post-exposure normalization of tau expression that we describe above. This data suggests that miRNA, including miR-34c, may play a critical role in “correcting” the transient Pb-induced over-expression of tau, similar to the role suggested for miRNA in the normalization of protein expression in wild-type mice exposed to Pb (Masoud et al., 2016).

In summary, these studies demonstrate for the first time that exposure to an environmental toxin can modulate a human gene and biomarkers associated with AD and tauopathies. When compared with our previous work using wild-type animals, the present results indicate that the human tau gene may in fact be more sensitive than the murine tau gene to developmental Pb exposure. Future studies will explore the combined effects of the environment and susceptible genes across the lifespan through old age. Furthermore, epigenetic pathways that maybe associated with this genetic reprogramming will be examined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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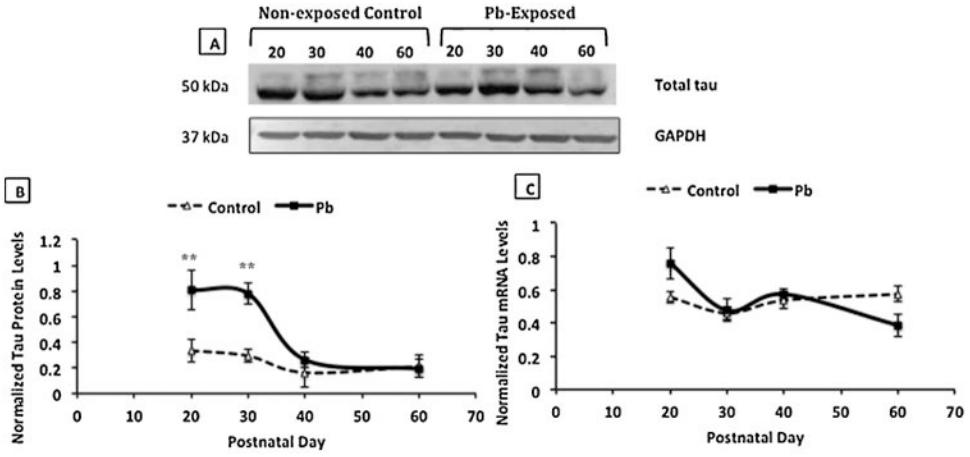

Fig. 1.

Total tau expression in mice with postnatal exposure to Pb. (A) Changes in the developmental profiles of tau protein expression in the cerebral cortex of control and developmental exposure group (Pb). (B) Quantification of protein expression by normalization to GAPDH. (C) Quantification of mRNA levels of total tau normalized to GAPDH. Each data point in the bar diagram represents the mean ± SEMs (n = 3) for protein and gene expression. **p < 0.010 as compared to unexposed controls. *p < 0.05 as compared to unexposed controls and independently verified. Total tau protein and gene expression was altered by postnatal Pb exposure.

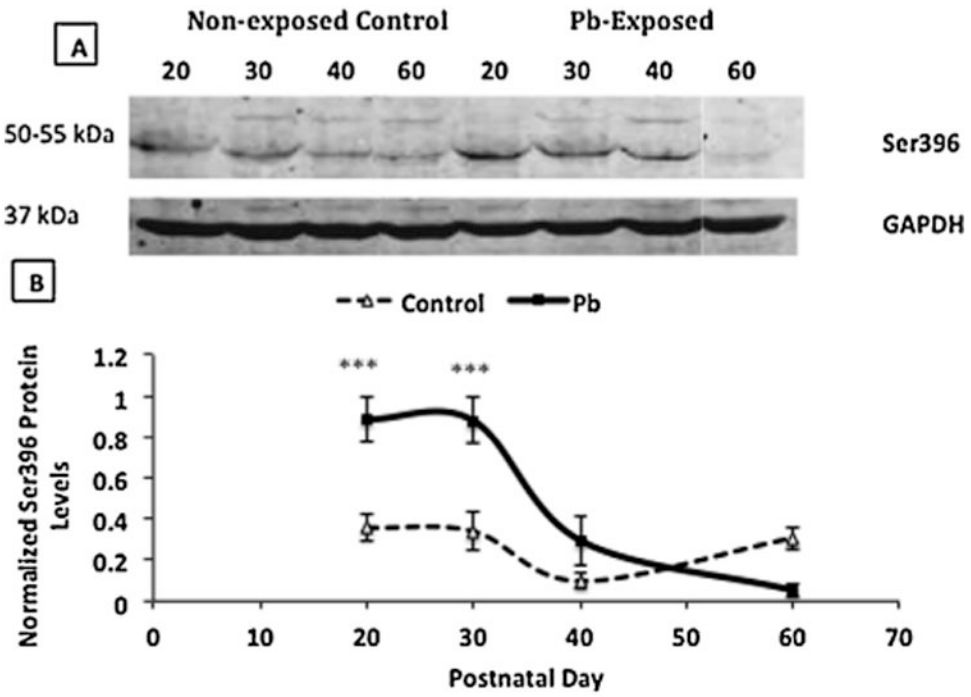

Fig. 2.

Phospho-tau Ser396 expression in mice with postnatal exposure to Pb. (A) Changes in the developmental profiles of phosphor-tau Ser396 protein expression in the cerebral cortex of control and developmental exposure group (Pb). (B) Quantification of protein expression by normalization to GAPDH. Each data point in the bar diagram represents the mean±SEMs (n = 3) for protein expression. ***$p < 0.001$ as compared to unexposed controls. Phopho-tau Ser936 protein expression was altered by postnatal Pb exposure.

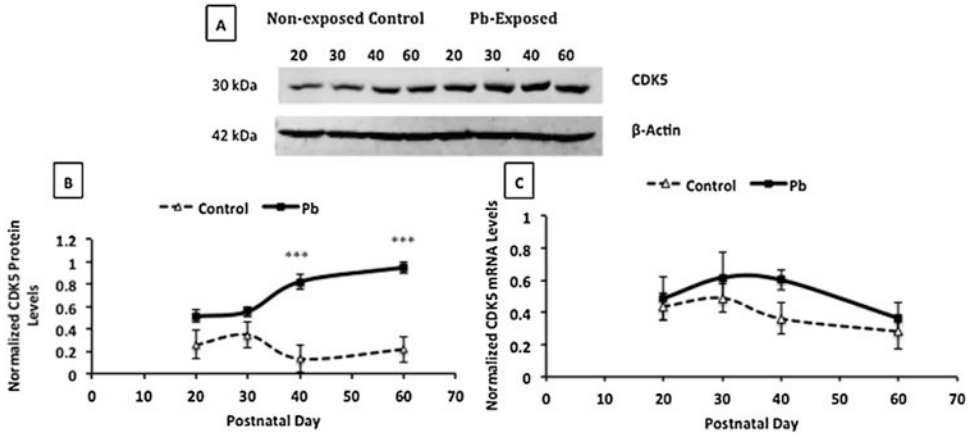

Fig. 3.

CDK5 expression in mice with postnatal exposure to Pb. (A) Changes in the developmental profiles of CDK5 protein expression in the cerebral cortex of control and developmental exposure group (Pb). (B) Quantification of protein expression by normalization to β-actin. (C) Quantification of mRNA levels of CDK5 normalized to β-actin. Each data point in the bar diagram represents the mean ± SEMs (n = 3) for protein and gene expression. ***$p < 0.001$ as compared to unexposed controls. CDK5 protein and gene expression was altered by postnatal Pb exposure.

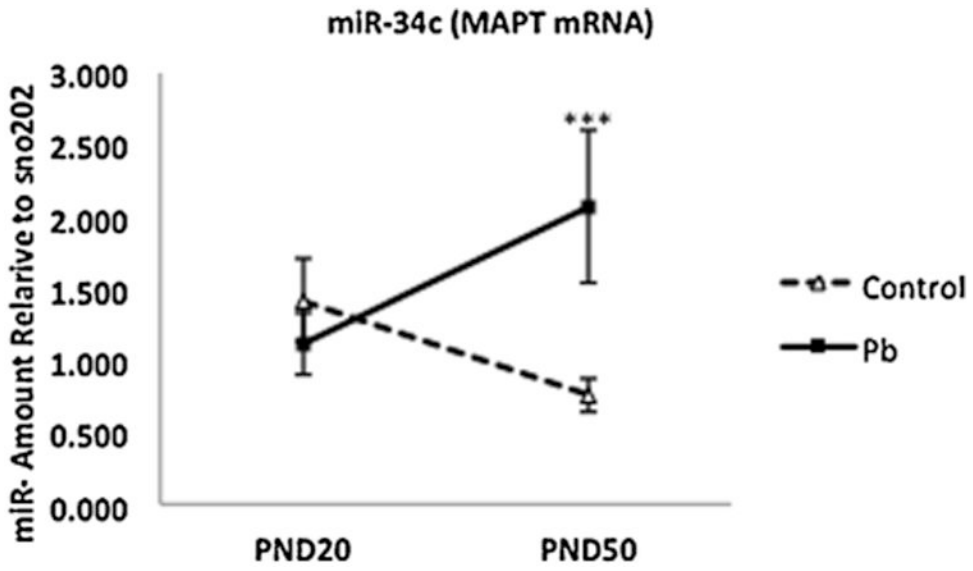

Fig. 4.

Expression of miR-34c relative to sno202 in control animals (dotted line) and Pbexposedanimals (PbE, solid line) at PND 20 and PND 50. Results expressed as mean ± SEMs (n = 3 with measurements in triplicates). *p<0.05 as compared to unexposed controls.

Table 1

Western blot protein concentrations and antibody ratios.

Gene	Protein	Gel	Transfer	Blocking	Primary Antibody	Secondary Antibody
Tau	10 μg	10%	40min	1h in 5% BSA	1:5000	1:10,000 30min
CDK5	40 μg	10%	30min	20min in 5% BSA	1:1000	1:10,000 60min
Ser396	40 μg	10%	30min	20min in 5% BSA	1:500	1:5,000 60min
GAPDH					1:1000 1h RT	1:10,000 60min
β-Actin					1:1000 1h RT	1:10,000 60min

Table 2

Primer Sequences for qPCR.

Target	FORWARD PRIMER	REVERSE PRIMER
Tau	5′ -TGA ACC AGG ATG GCT GAG C-3′	5′-TTG TCA TCG CTT CCA GTC C-3′
CDK5	5′ -GGC TAA AAA CCG GGA AAC TC-3′	5′-CCA TTG CAG CTG TCG AAA TA-3′
SP1	5′ -TCA TAC CAG GTG CAA ACC AA-3′	5′-AGG TGA TGT TCC CAT TCA GG-3′
GAPDH	5′ -AGG TCG GTG TGA ACG GAT TTG-3′	5′-TGT AGA CCA TGT AGT TGA GGT CA-3′
miR-34c	[miRBase ID: MI0000403]: 5′-AGGCAGTGTAGTTAGCTGATTGC-3′	Universal primer provided with the kit
Sno202	5′ -GCTGTACTGACTTGATGAAG-3′	5′-CATCAGATGGAAAAGGGTTC-3′