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HUMAN TAU GENE EXPRESSION IN A TRANSGENIC ANIMAL MODEL ALTERED BY DEVELOPEMENTAL Pb EXPOSURE

BY

MIRIAM ERLINE DASH

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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2015

ABSTRACT

Alzheimer's Disease (AD), the sixth leading cause of death in the United States, is a neurodegenerative disease characterized by a decline in memory and cognitive function. Demographics show that more than 90% of patients are first diagnosed with AD after the age of 65, classified as Late Onset AD (LOAD). The remaining 10% are said to have Early Onset AD. Research has shown that no mutation is linked to LOAD. Thus we hypothesize that environmental and/or epigenetic factors may be playing a major role in LOAD. AD traits coincide with the presence of two pathological hallmarks found in the brain: amyloid-beta plaques and neurofibrillary tau tangles. The microtubule associated protein tau (MAPT) is primarily found in neuronal axons. A feature of AD is the hyperphosphorylation of tau, lowering its binding affinity to the microtubules and increasing the chance of toxic aggregates to entangle. This *in vivo* study uses a MAPT transgenic mouse model knocked out for murine tau and knocked in for the human tau gene. Lifespan protein profiles of total tau, phosphorylated tau (Ser396) and related kinases (CDK5) were compared to wild-type profiles to validate the genomic insertion of the transgene. The mouse model was then tested for the transgene's ability to be altered by an environmental toxin. Pups were exposed to lead (Pb) from postnatal day (PND) 0-20 with 0.2% Pb acetate through the drinking water of the dam. Mice were sacrificed at PND 20, 30, 40, 50 and 60. Protein and mRNA levels of tau and CDK5 were investigated. Protein levels of Ser396 were also measured. DNMT1 protein levels were measured to evaluate the ability of Pb to affect epigenetic regulators in this mouse model. We

can conclude that this human tau transgene has been genomically inserted in a similar location to the endogenous murine tau gene. The human tau transgene can also be manipulated by environmental exposures, such as the heavy metal Pb, making this mouse line a good model to use for future AD studies. Our findings are the first of their kind to test the responsiveness of the human tau gene to an environmental toxin, specifically lead (Pb).

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INTRODUCTION

Alzheimer's Disease (AD) is the most common form of dementia affecting elderly people over the age of 65, and is currently the sixth leading cause of death in the United States. The disease affects 5 million Americans and 35 million people worldwide (Alzheimer's Association, 2015; Querfurth, 2010). Due to our population now able to live longer as well as numerous baby boomers reaching the targeted age of 65 and older, numbers are estimated to triple by 2050 (Herbert, 2013). At the clinical level, AD can be classified as a neurodegenerative disease most commonly characterized by a decline in memory and cognitive function (APA, 2007). These traits coincide with the presence of two pathological hallmarks found in the brain: amyloid-beta plaques and neurofibrillary tau tangles. Hallmarks are often correlated with synaptic loss in the brain resulting in the characteristic dementia (Selkoe, 1991). Although in AD we frequently see plaques and tangles present together, it is possible that each can exist independently of the other. Diseases such as Guam parkinsonism dementia complex, dementia pugilistica, corticobasal degeneration, Pick's disease, FTDP-17 tau (frontotemporal dementia with parkinsonism linked with chromosome 17 and tau mutations), and progressive supranuclear palsy have neurofibrillary degeneration absent of amyloid-beta plaques (Iqbal et al., 2010). Furthermore, research has shown that neurofibrillary tangles correlate best with the occurrence of dementia in humans versus the presence of plaques (Burns et al., 1997; Serrano, 2011).

Patients that develop AD prior to age 65 are considered to have Early Onset AD (EOAD) and exhibit hereditable mutations in *Amyloid Precursor Protein* (APP),

Presenilin1 (PSEN1) or *Presenilin2* (PSEN2) genes (Liddell, 2001). Most individuals that develop AD (>95%) have sporadic or Late Onset AD (LOAD) (Bekris, 2010). No mutation, other than a risk associated with carrying certain susceptibility alleles, has been linked to LOAD. The failure to identify a clear genetic etiology for LOAD suggests the likelihood that environmental exposures and/or epigenetic modifications play an important role in initiating and influencing the cascade of events that leads to this form of AD.

Epidemiological studies have revealed that developmental exposure to environmental toxicants can lead to epigenetic changes to our DNA. This is made more apparent when monozygotic twins have dichotomous outcomes for AD, indicating the effect of obvious environmental influences (Mastroeni et al., 2010; Raiha et al., 1997). More recently, a 2014 study investigated the impact of early life exposure to a high fat diet on the cerebral vasculature and perivascular clearance of amyloid-beta. Clearance pathways involving the Apolipoprotein ε-4 (APOε4) allele were altered in a wild type C57BL/6 mouse model further supporting the concept that the epigenome is highly vulnerable and susceptible to changes from the environment during development (Hawkes, 2014). Earlier findings had previously demonstrated that the APOε4 allele is the main genetic risk factor in increasing one's risk for developing LOAD (Querfurth, 2010).

The environment possesses many contaminants that are risk factors for developing neurodegenerative diseases, which includes, but is not limited to, pesticides (such as DDT), metals, and air pollution (Stein, 2008). The heavy metal lead (Pb) still poses a danger as an environmental toxicant. Exposure occurs via

commonly used materials from construction materials to batteries. 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 (NAS) is a prospective longitudinal study started by the Veterans Administration in 1963 to monitor the effect of aging on different health conditions with sub-groups of populations investigated for a link between past non-occupational Pb exposure and cognitive decline (Peters et al., 2010). Participants with recorded higher levels of Pb in the blood/bone corresponded with lower cognitive performance recorded by the Wechsler Adult Intelligence Scale-Revised (WAIS-R), Consortium to Establish a Registry for AD (CERAD) and Mini-Mental state examination (MMSE) (Payton et al., 1998, Wright et al., 2003, Weisskopf et al., 2004 and Weisskopf et al., 2007).

Cognitive decline is an intermediate phase in the progressive steps of AD. Data from longitudinal studies provided by NAS show that Pb is a key factor to investigate. By intertwining developmental exposure research with Pb investigations, studies have shown that perinatal and childhood exposure to Pb can disrupt neuropsychiatric function, impair cognition, and produce behaviors associated with attention deficit function (Senut et al, 2012). Some recent work (Bihaqi & Zawia, 2011, 2014; Wu, 2008) focused on the Amyloid Cascade Hypothesis. This theory asserts that the deposition of the amyloid-β peptide in the brain parenchyma initiates a sequence of events that ultimately leads to AD dementia. The Amyloid Cascade Hypothesis brings together histopathological and genetic information and has been influential in research conducted by academia and

the pharmaceutical industry (Karran, 2011). Previous work on this theory has shown that when wild-type C57BL/6 mice were exposed to 0.2% Pb acetate in early life (PND 0-20), a transient increase was parallel with the exposure in the expression of AD genes followed by delayed overexpression of the AD related genes and enzymes. This includes mRNA levels of APP, Sp1 and BACE as well as proteins levels of Aβ, APP, Sp1 and BACE activity (Bihaqi & Zawia, 2014). These findings support the theory that the presence of Aβ plaques correlates with AD dementia symptoms.

In addition to examining AD genes, the epigenome and influential changes caused by Pb is a central focus in current research studies (Kim, 2014; Goodrich, 2015). Epigenetic changes occur to our DNA without altering the DNA sequence. These changes are modifications that can enhance or repress the transcription of certain genes. DNA methylation occurs mainly at CpG rich sites on the DNA. Bihaqi and Zawia (2011) investigated epigenetic modifications primarily related to DNA methylation in primates exposed to Pb during early life. Results showed that there was a decrease in the protein levels of the maintenance DNA methyltransferase (DNMT1) and a decrease in the protein levels of the *de novo* DNA methyltransferase (DNMT3a) in the primate frontal cortex. The decrease in both DNMT1 and DNMT3a is correlated with an upregulation of AD related genes (APP, BACE1, SP1) are normally repressed later in life in aging brains (Wu, 2008). Post-translational histone marks such as acetylation or methylation can lead to chromatin being more or less accessible to transcriptional machinery therefore respectively increasing/decreasing gene activity. H3K9ac, H4K8ac, and H4K12ac and H3K4me2

are all activating marks that were investigated. In healthy aging brains, these marks increase. However, upon developmental Pb exposure a decrease in these marks occurred, a finding possibly related to certain genes' repression (Bihaqi &Zawia, 2011). Briefly summarized, alteration in DNA-methylation appears to be consistent with the latent up-regulation of AD-related genes, while histone modifications correlate better with global gene repression observed later in life caused by early-life exposure to Pb (Dosunumu et al, 2009). Thus, it appears that global gene repression may be mediated by histones, while upregulation of genes late in life may involve DNA hypomethylation.

As previously mentioned, two pathological hallmarks of AD exist: Aβ plaques and neurofibrillary tau tangles. The tau gene is involved in AD pathogenesis and its regulation is established during development via epigenetic programming. The tau protein is primarily found in neuronal axons. Tau is crucial for stabilization of neuronal microtubules, thereby supporting intracellular transportation (Madelkow & Madelkow, 1994). A feature of AD includes hyperphosphorylation of tau at serine and threonine sites; a state that lowers its binding affinity to microtubules and increasing the chance of toxic aggregates to entangle (Lee et al, 2004). This aberrant phosphorylation compromises microtubule stability, axonal transport and, therefore, cognitive function (Alonso et al, 1996; Salehi et al, 2003). Given these pathological tau abnormalities, it is important to conduct experimental research investigating links between the tau gene and AD. Studies have shown that the biomarker of amyloid-beta plaques tends to plateau before any signs of cognitive decline become apparent making other neurological biomarkers such as tau and

synaptic loss more useful in tracking dementia. Findings such as these suggests that amyloid-beta by itself may not be sufficient to produce the clinical AD syndrome; only when paired with neuronal loss and tau filaments will cognitive decline begin to occur (Sperling, 2010). Research investigating these connections is best done within animal studies that may shed light on a comparable link with humans. Animal model (*in vivo)* studies, in comparison to *in vitro* studies, are ideal for complex diseases such as AD. *In vivo* experiments can provide an opportunity to observe whole system interactions regarding the drug and/or toxin being administered and its affect on the gene of interest (Autoimmunity Research Foundation, 2012). These connections result in more applicable data in relation to the entire biological system.

In addition to work done on amyloid-beta in primates and wild-type mice, recent research by Bihaqi and Zawia (2013, 2014) has begun to investigate lead's affect on the tau protein. The frontal cortex of primates exposed to 0.2% Pb postnatally showed elevated levels of total tau, phosphorylated tau, and cyclin-dependent kinase 5 (CDK5 is a serine-threonine kinase found primarily in neurons). Ser/Thr phosphatase activity was also investigated and found to be elevated; suggested to be a result from the higher amount of phosphorylated tau found in AD brains and to counteract the higher activity of CDK5 (Bihaqi & Zawia, 2013). Developmental Pb studies completed in primates were replicated in wild type C57BL/6 mice. Results from the mouse model were consistent with those found in the primate study (Bihaqi & Zawia, 2014).

Applying the results seen in the wild-type mouse model to a relevant human

application requires a transgenic mouse model. This research utilizes a transgenic mouse model that has a homozygous knock-out for the murine tau gene and a hemizygous knock-in for the human tau gene. On a molecular level, this transgene includes the promoter, intronic regions, and regulatory regions of the human MAPT gene. It should be noted that the adult profile of tau isoforms differs between humans and mice. Alternative splicing of a single tau gene generates six isoforms. Tau isoforms are described as either 3 repeat (3R) or 4 repeat (4R), depending on whether exon 10 was included in the final splicing. The adult mouse brain contains exclusively the 4R tau isoform. Comparatively, the adult human brain contains roughly equal amounts of 3R and 4R (Andorfer, 2003). The transgenic mouse model used in this study develops all six isoforms including both 3R and 4R isoforms.

Transgenic mice thus far have been the best models to study LOAD. They provide insight on the pathogenesis and physiology of the disease including molecular mechanisms, pathways towards neuronal damage, and risk factors of neuroinflammation and neurodegeneration (Medina,2014). Unfortunately, problems still arise with transgenic insertions. Transgenes can be incorporated into host DNA at random locations through illegitimate recombination and cellular DNA repair activity. This can lead to injected transgenes not necessarily behaving in every way that their corresponding endogenous genes do (Yan, 2013). Thus before beginning a full study with a new transgenic mouse model, the behavior of the transgene in its placed environment must be verified with a proof-of-concept study. Once the model has proven to behave similarly to the wild-type mice, studies can be performed to test the transgene's responsiveness to drugs, toxins, or other

environmental influences.

The purpose of the current research is to examine this transgenic mouse model as a proof-of-concept study for the human tau gene and its responsiveness to the environmental toxicant lead. Two hypotheses were examined: 1) The B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J mouse line expresses the human transgenic tau gene temporally and spatially similar to the murine tau gene; 2) The human tau gene within the B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J mouse line can be altered by developmental Pb exposure. The B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J mouse line was used to determine the transgenic tau animal model's temporal expression of Alzheimer's like tauopathies across the lifespan. Through a lifespan analysis, the transgenic mouse model was compared to the protein expression profile of the wild-type studies previously published (Bihaqi, Zawia, 2014).

The mouse line was then developmentally exposed to Pb to monitor the ability of the transgenic human tau gene's ability to be affected by an environmental toxin. Levels of tau and its posttranslational modifications in Pb-exposed versus non-Pb-exposed transgenic mice were compared. Epigenetic regulators were evaluated for Pb-induced manipulations. Establishing these fundamental features of the mouse model determined the ability of this transgene to be used for future Pb studies.

To date, only data examining the murine tau promoter and the impact of the environment has only been published (Lahiani-Cohen, 2011). Data examining the human tau promoter in this aspect has not been published. Epigenetic studies on the

human tau gene have only been done in post-mortem Alzheimer's patients (Iwata, 2014 Millan, 2014), which leads to potential confounding variables. The present study has opened up avenues for future research to look specifically at modifications occurring within the human tau promoter of mice possessing this transgenic gene.

METHODOLOGY

Animals and Exposure: A transgenic mouse model (B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J [Jackson Laboratory Stock 005491, Bar Harbor, ME]) that is a homozygous knockout for the murine tau gene and a hemizygous knock in for the human tau gene was used. Although no endogenous mouse MAPT is detected, 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. Brains for the lifespan study (n=3 for each time point) were dissected at postnatal day (PND) PND 20, PND 30, PND 60, PND 120, PND 150, PND 180, PND 210, PND 240, and PND 450. Time points were chosen to provide a profile of AD-related proteins across the lifespan of the transgenic model. Mice were euthanized with CO_2 and brains were dissected on ice followed by immediate storage at -80°C.

A second cohort of mice was exposed to Pb during the first 20 days of postnatal life according to the protocol we have described previously (Bihaqi, 2014). Mice in the first group were not exposed to Pb and served as controls. The second 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, 1996). Blood levels have been shown to be 46.43 μg/dl during Pb exposure but are reduced to basal levels in adults (Basha, 2005). The blood levels of Pb during exposure are higher than the current CDC level of concern (5 μg/dL), but are consistent with the levels seen in children and adults exposed to

environmental Pb (Ettinger, 2010; Mazumdar, 2012) and well below the 70 µg/dL considered to represent a medical emergency (Ettinger, 2010). Exposure began 24 hours after birth (PND 0) and continued until weaning (PND 20). Mice were euthanized with CO_2 at PND 20, PND 30, PND 40, PND 50 and PND 60. 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.

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'-CGA AGT GAT GGA AGA TCA CG-3' and transgene antisense: 5'-GTC TTG GTG CAT GGT GTA GC-3'. Amplification was conducted under the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The amplified product was electrophoresed on a 2% agarose gel at 100 volts for 90 min and imaged on the Amersham Typhoon Imager Scanner FLA 9000 (GE Life Sciences, Piscataway, NJ).

Protein Levels: Western blots were utilized to measure and compare protein levels of total tau, phosphorylated tau Ser396, cyclin dependent kinase 5 (CDK5), and DNMT1. 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 RIPA buffer, 2 µL protease inhibitor, and 10µL phosphatase inhibitor. The homogenates were placed on ice for 10 min before being centrifuged at 8000x RPM for 10 min at

4°C. The supernatants were collected and aliquoted. Lysate protein concentrations were determined by a Bicinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL). Standard samples ranged from 1-30 µg in 4 µL. Lysate samples were run in triplicate on a 96 well plate and absorbency differences were read at 560 nm using the Spectra-Max Multimode plate reader (Molecular Devices, Sunnyvale, CA). Following protein standardization, samples were prepared for western blotting according the concentrations listed in *Table 1*.

A nuclear extraction was necessary to increase the sensitivity for DNMT1. Nuclear extractions from cerebral cortical brain tissue were carried out using NE-PER Nuclear and Cytoplasmic Extraction reagents according to the manufacturer's instructions (Thermo

Scientific Pierce, Rockford, IL). Nuclear lysate protein concentrations were determined by a Bicinchonic acid assay and prepared for western blotting by loading 100 µg of protein (according *Table 1*).

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

to the housekeeper protein GAPDH or β-Actin. Total tau and phospho-tau blots were normalized to GAPDH. CDK5, Sp1, and DNMT1 were normalized to β-Actin.

Gene	**Protein**	**Gel**	**Transfer**	**Blocking**	**Primary Antibody**	**Secondary Antibody**
Tau	10 µg	10%	40 min	1 hour in 5% BSA	1:5000	1:10,000 30 min
CDK5	40 µg	10%	30 min	20min in 5% BSA	1:1000	1:10,000 60 min
Ser396	40 µg	10%	30 min	20min in 5% BSA	1:500	1:5,000 60 min
DNMT1	100 µg	7%	60 min	No blocking	1:500	1:5,000 60 min
GAPDH					1:1000 1hr RT	1:10,000 60 min
β-Actin					1:1000 1hr RT	1:10,000 60 min

Table 1. Antibodies for Western Blotting

Behavioral Testing: Spatial learning and memory were tested using the Morris water maze (MWM) according to the method of Morris and colleagues (Vorhees, 2006). The apparatus consisted of a white 48" diameter water tank that is 30" in height and filled with water to a depth of 14". A non-toxic washable white paint was added to the water to increase opacity. Four distinct visual cues surrounded the pool, distinguishing four equal quadrants. A clear Plexiglas platform square of 10cm was kept submerged and hidden 0.5 cm below the surface of the water.

Habituation trials preceded the start of the MWM experiment. Each animal was allowed to swim freely for 60 sec without the hidden platform in order to acclimate to the procedure. On the following day, each mouse received three trials per day for 8 days with a 10 min inter-trial interval. Each of the three trials started

from a different quadrant not containing the hidden platform. Mice were allowed to swim until they found the immersed hidden platform or for a maximum time of 60 sec. A mouse that failed to locate the platform would be placed upon the platform for a maximum of 10 sec to encourage awareness of the location. A retention test (Probe Trial) was performed on the ninth day, and the percentage of time and distance traveled in the target quadrant for 60 sec was recorded. Ten days following Probe Trial 1, a second Probe Trial was conducted. Swim distance and latencies to locate the platform were videotaped and analyzed with a computerized video tracking system (ObjectScan, Clever Systems Inc, Reston, VA).

Immunohistochemistry: To visually confirm the ability of the transgenic mouse model to achieve hyperphosphorylated tau, 13-month old mice were prepped for immunohistochemistry. According to a protocol previously published (Subaiea, 2015), mice were deeply anesthetized with an intraperitoneal injection of 0.1 ml/10 g of xylazine-ketamine mixture (100 mg/ml-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. Thereafter, mice were 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 Thr231 (Thermo Scientific Pierce, Rockford, IL).

RNA Isolation and Real-Time PCR: Total RNA was isolated from Pb-exposed and non-exposed (control) frontal 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). cDNA was amplified using real-time polymerase chain reaction (PCR). The SYBR Green quantitative real-time PCR assay was performed in 12.5 μL 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 2 min followed by 95°C for 10 min, then 40 cycles of 95°C for 15 sec 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.

GENE	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'
DNMT1	5'-AGC TAC CTG CTC TGG CTC TG-3'	5'-GAG TCT TCG ACG TCA CAC CA-3'
GAPDH	5'-AGG TCG GTG TGA ACG GAT TTG-3'	5'-TGT AGA CCA TGT AGT TGA GGT CA-3'

Table 2. Primer Sequences for qPCR

Statistical Analysis: Western blot bands were quantified used the Li-Cor Odyssey infrared image system. Real time PCR curves were quantified using Viia 7 Real-Time PCR System software. All measurements were made in triplicate and all values are presented as mean ± standard error of the mean. To statistically test the second hypothesis examining protein and mRNA levels between treatment and control groups, one-way ANOVA for each gene was performed with the Tukey-Kramer multiple-comparison post hoc text. Effect sizes were calculated to assess the magnitude of the findings for time points that were found to be significantly different between treatment and control. The significance of interaction between treatment groups of each protein expression was determined by two-way analysis of variance (ANOVA) and the Tukey-Kramer multiple-comparison post hoc test. All analyses were done using SigmaStat 3.5 computer software. The level of significance for each ANOVA was set at $\alpha=0.05$.

RESULTS

The human tau transgene is temporally and spatially expressed in a mouse model comparable to wild-type profiles.

Genotyping of the mice population revealed clear results of mice carrying the transgene versus non-carrier controls. Bands for the transgenic human tau gene were either present or absent after imaging *(Figure 1)*. Mice aged 13 months showed visual signs of hyperphosphorylation in multiple cerebral cortical regions such as in the inner capsule *(Figure 2)* as well as the external capsule *(Figure 3)*.

Protein expressions of total tau *(Figure 4A)*, phospho-tau Ser396 *(Figure 5A),* and CDK5 *(Figure 6A)* were examined by Western blot analysis at different time points within the lifespan of the mice unexposed to lead. Results revealed normalized tau levels were first elevated during early life (PND 20). Following this elevation, a peak at mid-life (PND 180), a dip at PND 270, and rise at PND 450 in the tau protein expression was observed. The tau protein expression pattern quantified in the wild-type mouse is comparable *(Figure 4B)*.

Results also revealed normalized p-tau Ser396 to GAPDH in the transgenic mouse model followed a similar pattern to the wild-type protein expression *(Figure 5A)*. A mid-life peak in phosphorylated tau occurs in both models. In the transgenic model a peak occurs at PND 180, while in the wild-type mouse a peak occurs at PND 270. After a decrease in protein levels around PND 270 the phosphorylated Ser396 levels starts to elevate again around PND 450. The pattern is matched in the wild-type mouse model *(Figure 5B)*.

Results for the lifespan protein profile of CDK5 in this transgenic mouse model also reflect a similar pattern to the wild-type mouse model *(Figure 6)*. Protein levels are around the same for PND 20 and PND 180 in the wild-type mice. The transgenic mice reflect the same pattern. Variation occurs at the mid-life peak. The transgenic mouse model peaks at PND 210 while the wild-type model peaks at 270. Both models start to elevate again towards the end of life.

The human tau transgene exacerbates cognitive deficits.

In the 8 days, an overall trend of transgenic mice performing worse in the behavior test than the controls was observed. Repeated-measure ANOVA indicated no significant difference in the performance, assessed by measuring escape latency, between the non-transgenic control group and the transgenic mice group in this task *(Figure 7)*. Probe trials 1 and 2 assessed the percent of time spent in the correct quadrant that once contained the hidden platform in previous trials. Results revealed that in both trials, transgenic mice spent less time in the correct quadrant but no significant difference in retention between the transgenic mice and the non-transgenic controls was noted *(Figure 8)*.

Postnatal lead exposure alters AD biomarkers in the transgenic mouse model.

Protein levels of cerebral cortical total tau normalized to GAPDH were analyzed by Western blot after postnatal dosing with 0.2% Pb acetate for 20 days *(Figure 9A).* One-way ANOVA revealed a statistical decrease in Pb-exposed mice at PND 50 compared to controls ($p=0.002$). η^2 for this effect is 0.253 revealing that

25% of the variance was shared between the treatment and outcome. A two-way ANOVA revealed a statistically significant interaction between the time points and exposure (p= <0.001). The effect of Pb exposure is dependent on the time point. η^2 for this interaction is 0.512 revealing that 51% of the variance was shared by the interaction effect *(Figure 9B).* Total tau mRNA was evaluated for Pb's ability to affect expression. Analysis of total tau mRNA levels shows that postnatal Pb exposure has a similar up-and-down regulation effect on the expression of tau at the transcriptional level. Similar to protein expression, a significant decrease occurred in tau gene expression in Pb-exposed mice compared to controls at PND 50 (p=0.021) η^2 for this effect is 0.108 revealing that 11% of the variance was shared between the treatment and outcome *(Figure 9C)*.

The cerebral cortical levels of phosphorylated tau Ser396 normalized to GAPDH were analyzed by Western blot after postnatal lead exposure *(Figure 10A).* Results from a one-way ANOVA revealed a significant increase of phosphorylated protein expression in Pb-exposed mice at PND 20 and PND 30 compared to controls (p=0.021 and p=0.018, respectively). η^2 for these effects are 0.156 and 0.158, respectively. This reveals that 16% of the variance at PND 20 and 16% of the variance at PND 30 was shared between the treatment and outcome. Subsequently, a statistically significant decrease in Pb-exposed phosphorylated protein expression at PND 50 was found compared to controls (p=0.001). η^2 for this effect is 0.207 revealing 21% of the variance shared between Pb exposure and the outcome (*Figure 10B*). Two-way ANOVA findings revealed a statistically significant interaction

between the time points and exposure ($p < 0.001$). η^2 for this interaction is 0.510 revealing that 51% of the variance was shared by the interaction effect.

Cerebral cortical CDK5 normalized to β-actin was analyzed by Western blot to measure Pb's ability to affect the kinase's protein expression levels (*Figure 11A*). CDK5 levels were elevated at every time point in Pb-exposed mice compared to controls (*Figure 11B*). At PND 40 and PND 60, a significant difference was revealed by a one-way ANOVA ($p=0.008$ and $p=0.004$, respectively). η^2 for these effects are 0.228 and 0.243, respectively. This reveals that 23% of the variance at PND 40 and 24% of the variance at PND 60 was shared between the treatment and outcome. A two-way ANOVA revealed that the effect of Pb exposure does not depend on the time point. There is not a statistically significant interaction between the time points and exposure ($p=0.067$). η^2 for this interaction is 0.127 revealing that only 13% of the variance was shared by the interaction effect. CDK5 mRNA levels followed a similar pattern as the protein expression (*Figure 11C*). One-way ANOVA results revealed no statistical significance between the treatment groups for CDK5 gene expression.

Postnatal lead exposure alters epigenetic regulators.

The protein levels of cerebral cortical DNMT1 normalized to β-actin were analyzed by Western blot after postnatal dosing with 0.2% Pb acetate for 20 days (*Figure 12A*). One-way ANOVA analysis results a statistically significant decrease in Pb exposed expression at PND 60 compared to controls ($p=0.002$). η^2 for this effect is 0.219. Two-way ANOVA findings revealed a statistically significant interaction

between the time points and exposure (p= <0.001)(*Figure 12B*). η^2 for this interaction is 0.336 revealing that 34% of the variance was shared by the interaction effect.

Figure 1. Genotyping
Lane 1 is the positive control. Lane 2 is the negative control. Lanes 3-8 are DNA samples from mice being genotyped for carriers of the transgene.

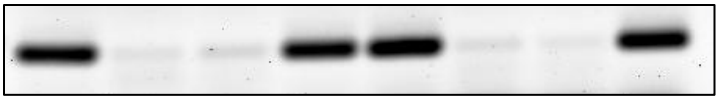


Figure 2. Immunohistochemistry
Axon from the internal capsule stained with Phospho-tau Thr 181

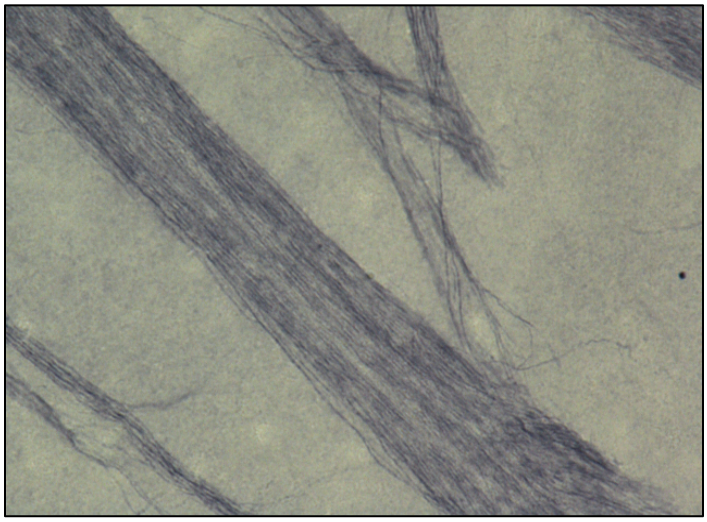


Figure 3. Immunohistochemistry
External capsule stained with Phospho-tau Thr 181

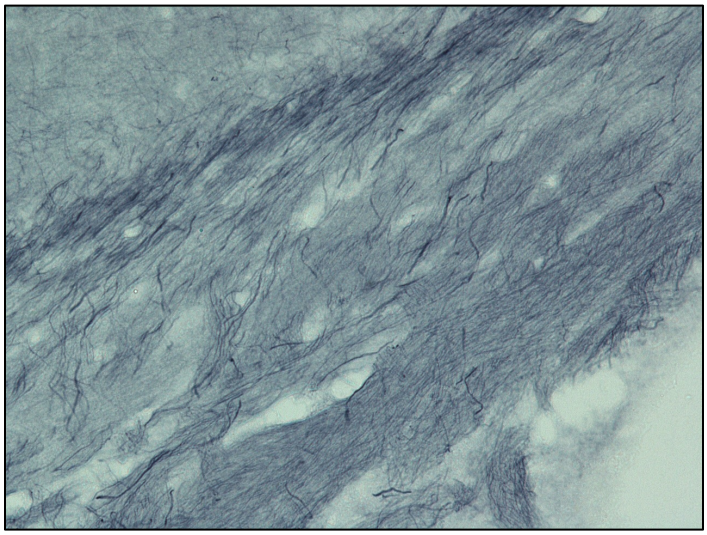
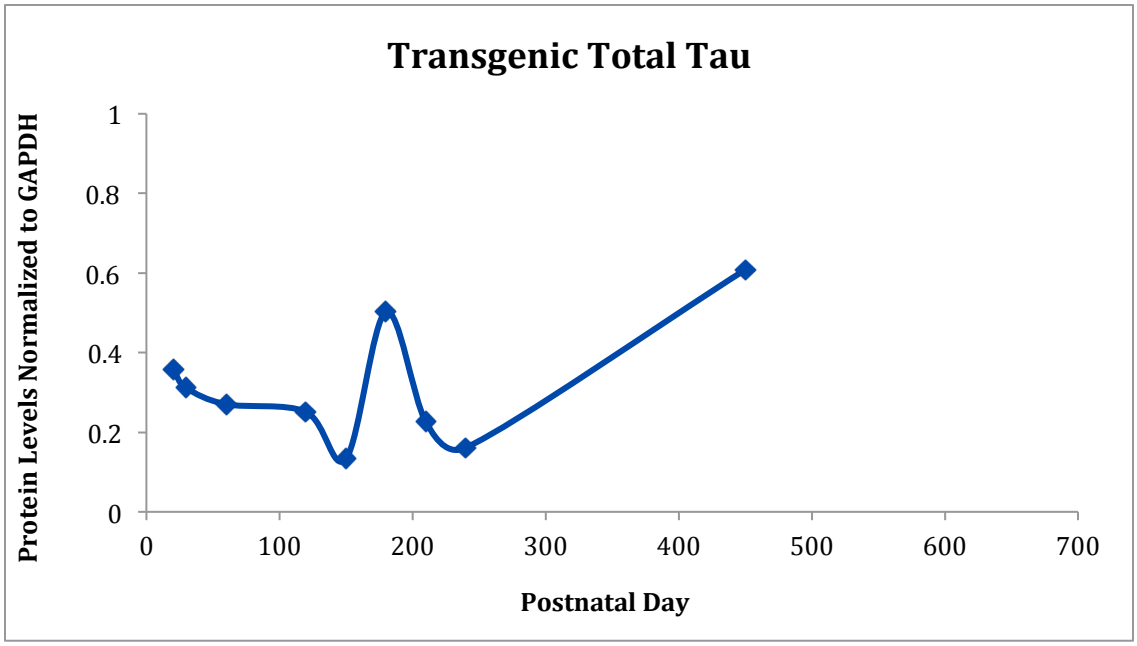


Figure 4. Total tau lifespan

A. Transgenic mouse model's lifespan protein expression of total tau



B. Wild-type mouse model's lifespan protein expression of total tau

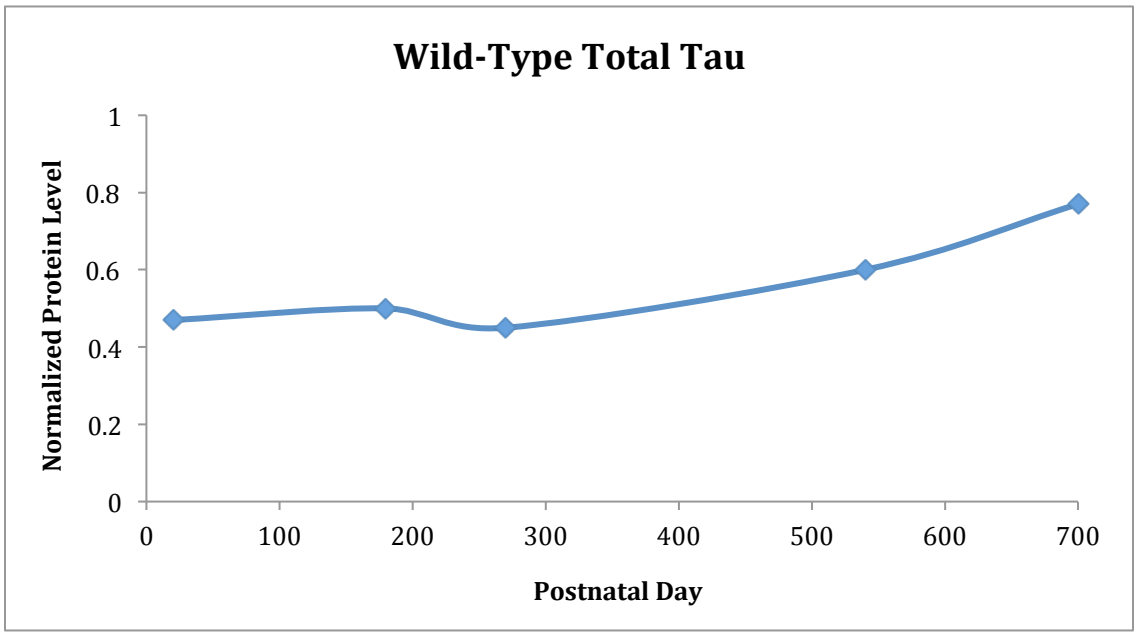
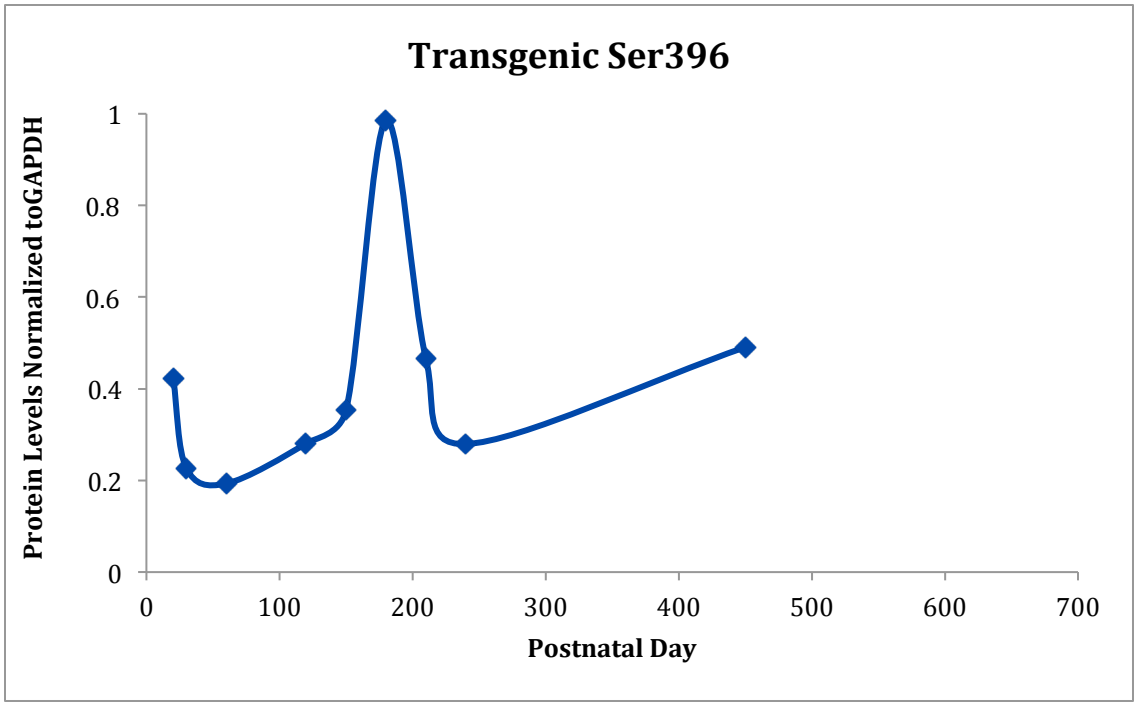


Figure 5. Ser396 lifespan

A. Transgenic mouse model's lifespan protein expression of phospho-tau Ser396



B. Wild-type mouse model's lifespan protein expression of phospho-tau Ser396

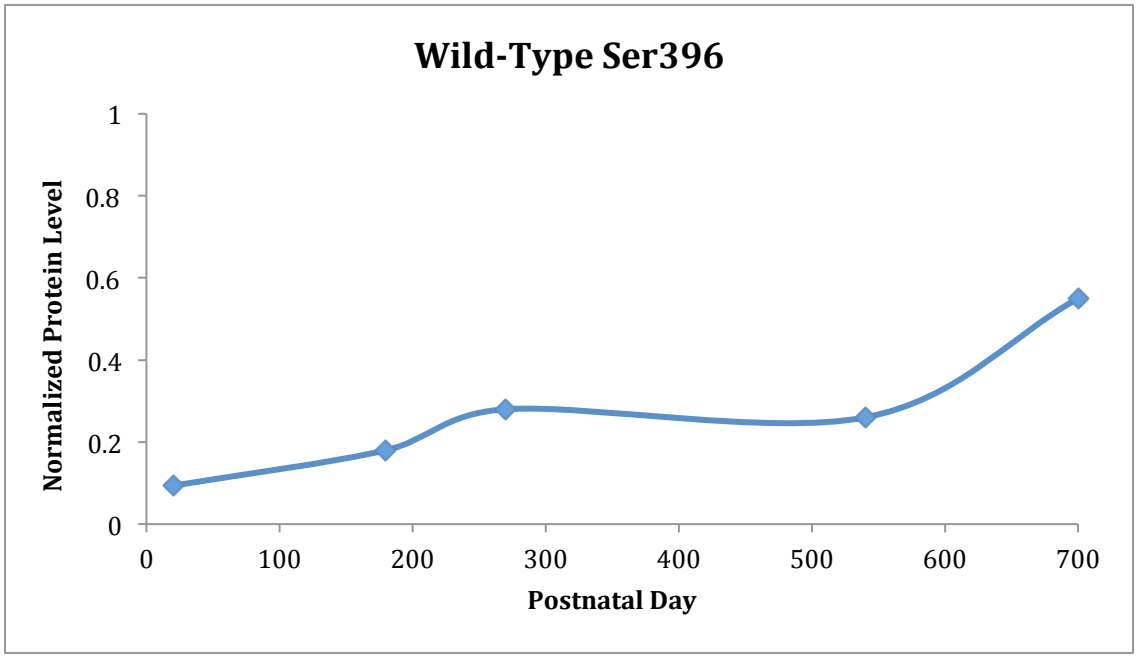
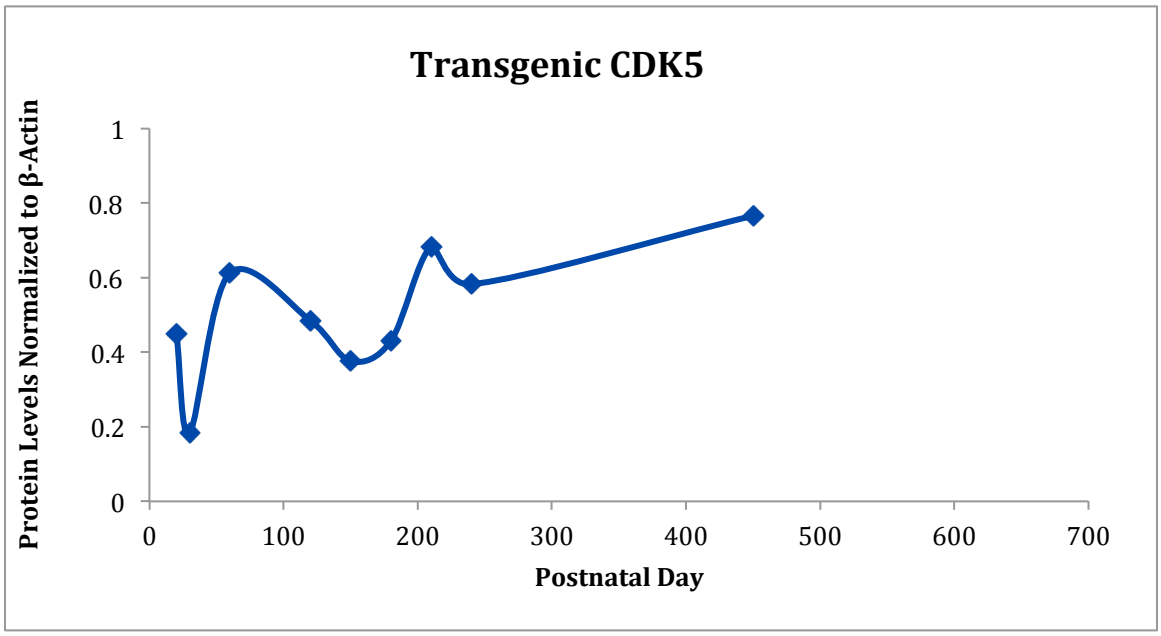


Figure 6. CDK5 lifespan

A. Transgenic mouse model's lifespan protein expression of CDK5



B. Wild-type mouse model's lifespan protein expression of CDK5

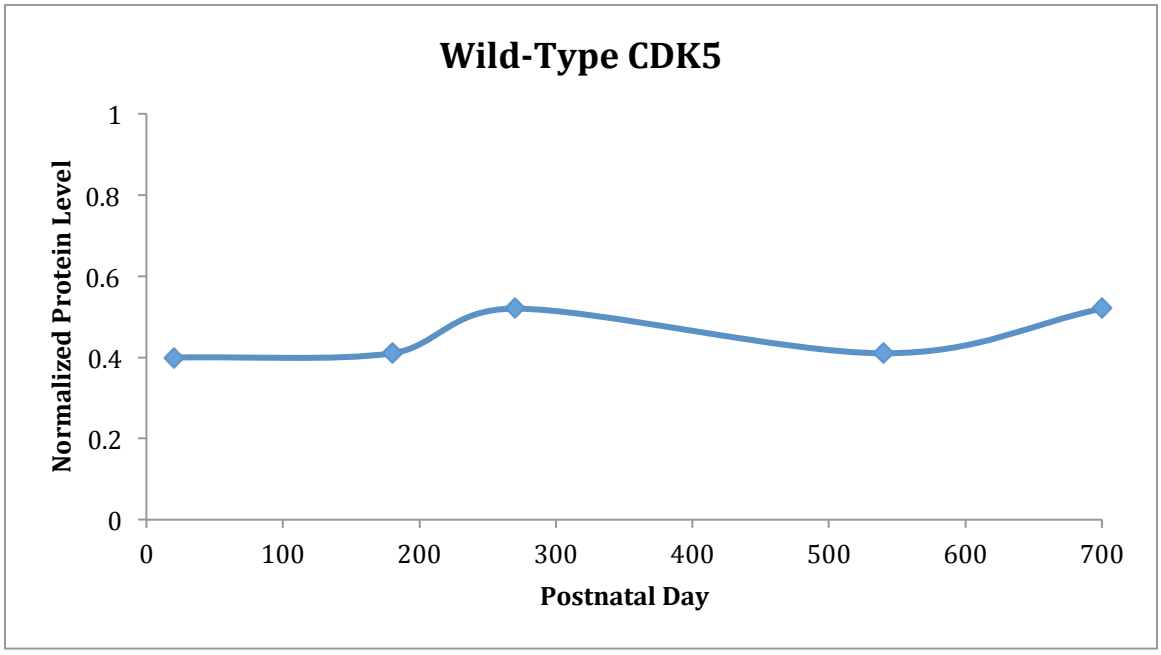


Figure 7. Morris Water Maze Results

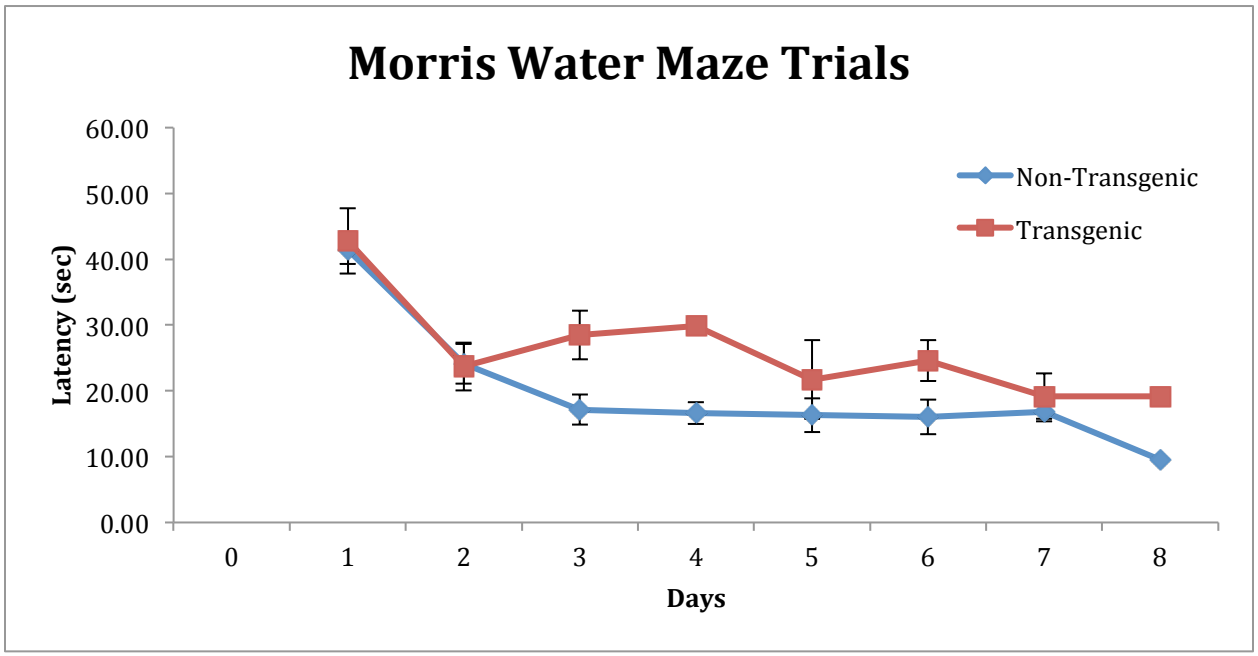


Figure 8. Probe Trial Results

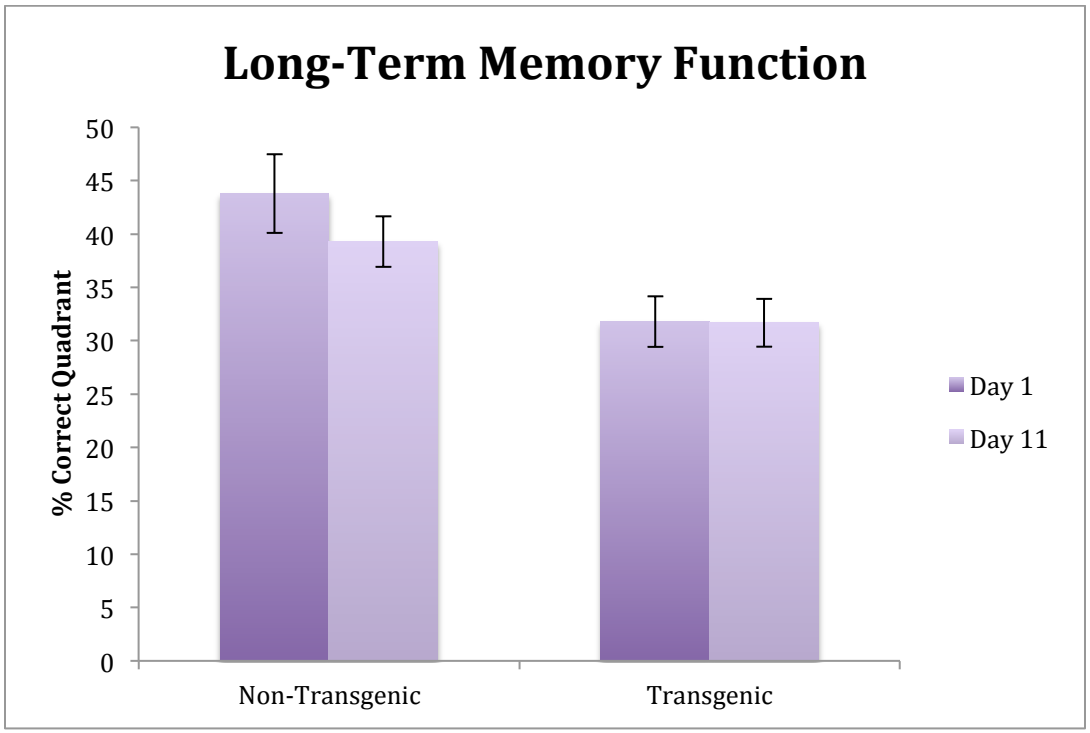
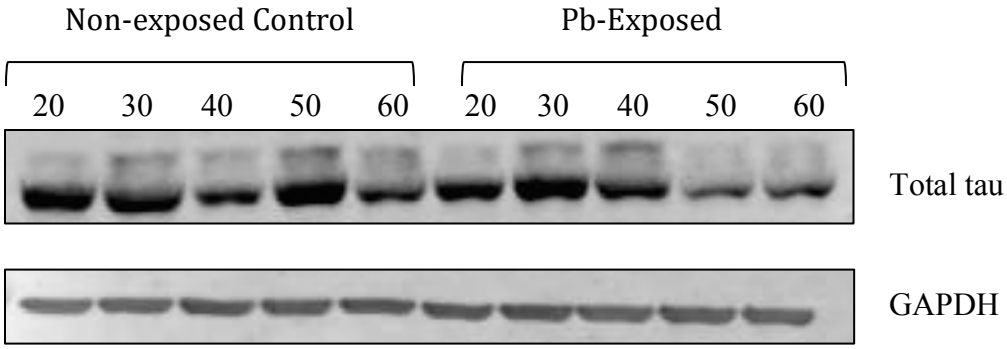
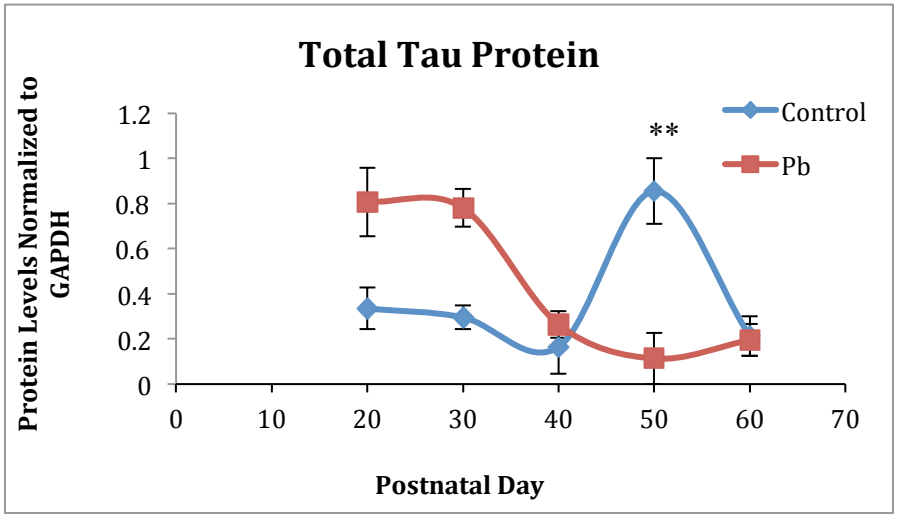


Figure 9.

A. Western blot of total tau normalized to GAPDH: Pb vs Controls



B. Total Tau protein expression: Pb vs Controls



C. Total tau mRNA expression: Pb vs Controls

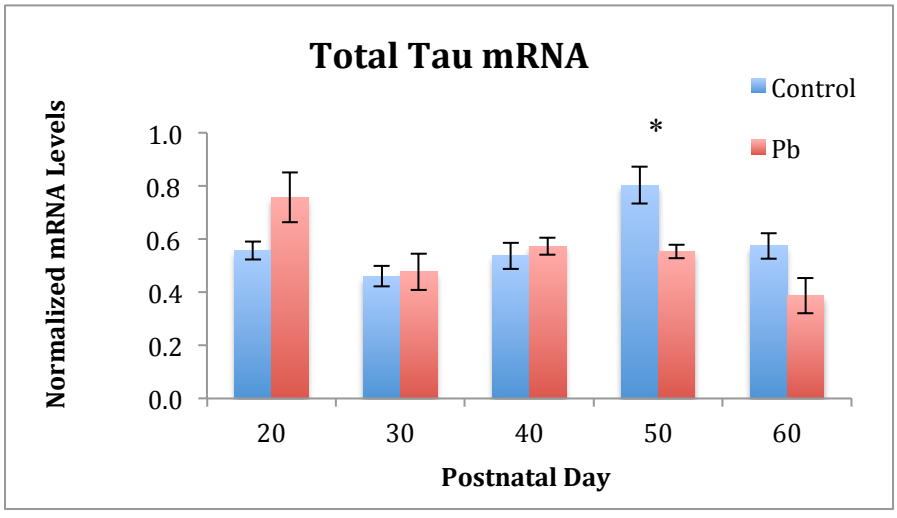
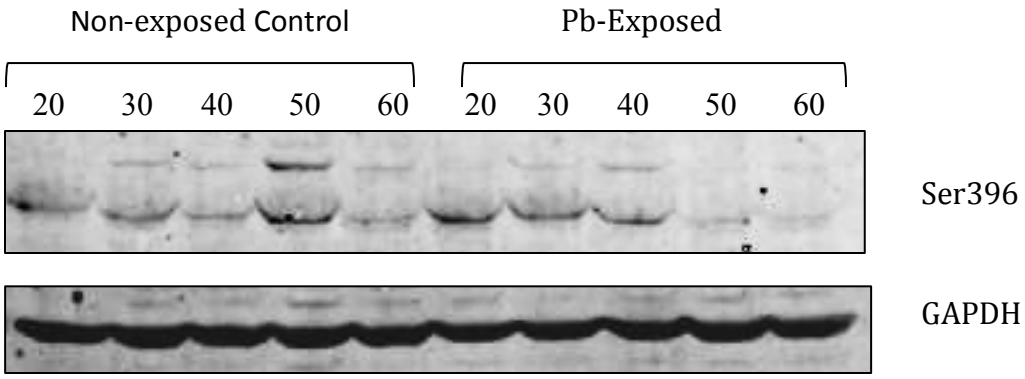


Figure 10.
A. Western blot of phospho-tau Ser396 normalized to GAPDH: Pb vs Controls



B. Phospho-tau Ser396 protein expression: Pb vs Controls

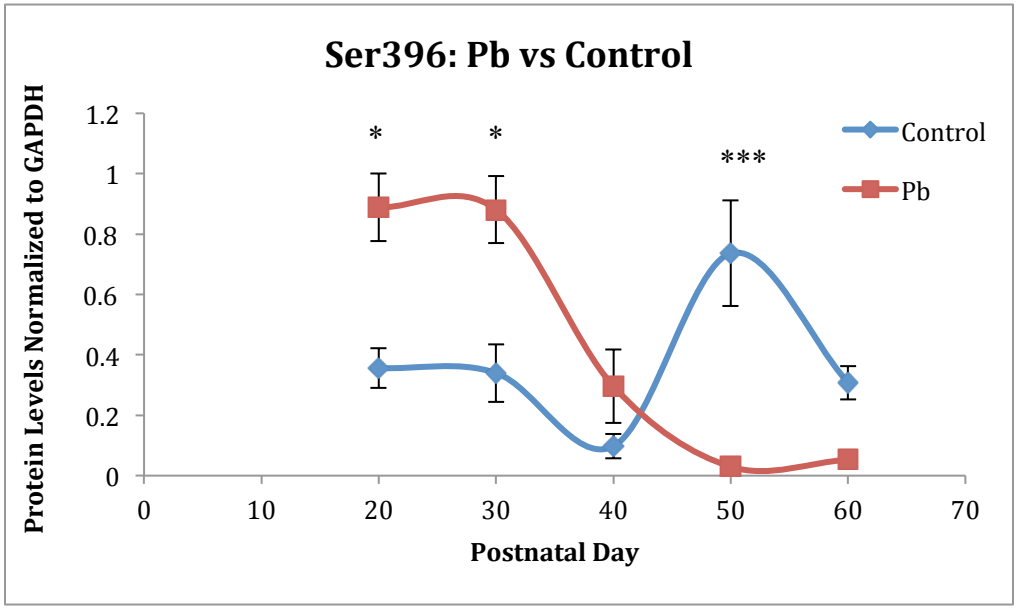
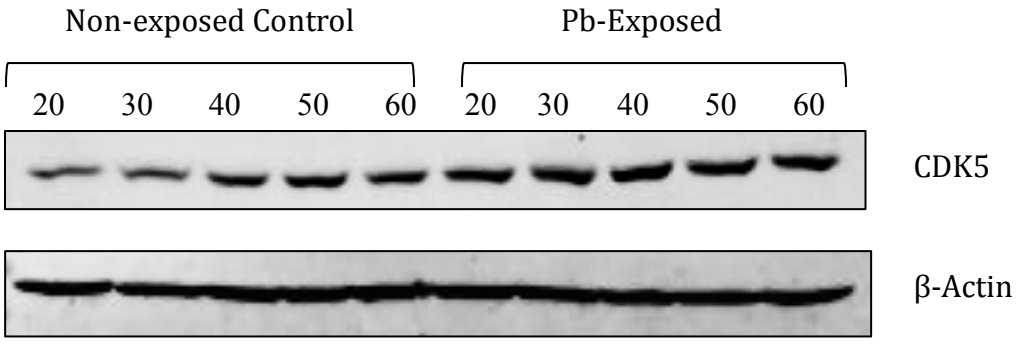
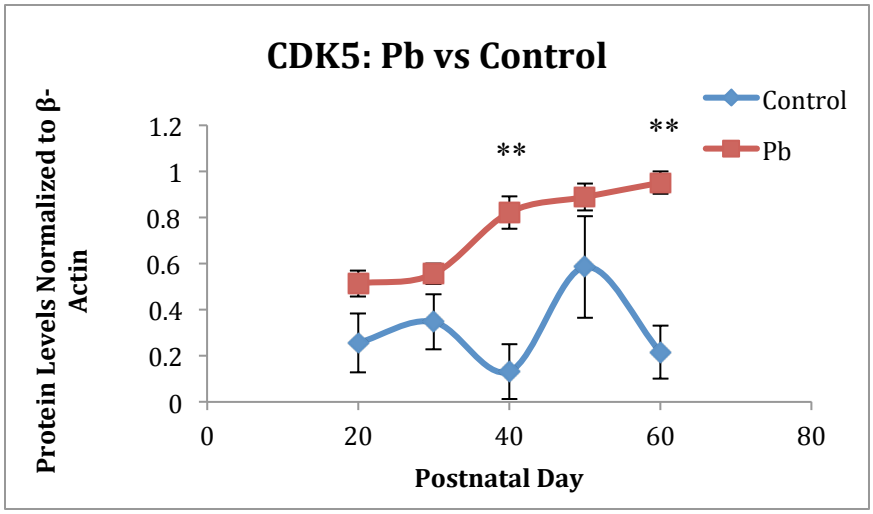


Figure 11.
A. Western blot of CDK5 normalized to β-Actin: Pb vs Controls



B. CDK5 protein expression: Pb vs Controls



C. Total tau mRNA expression: Pb vs Controls

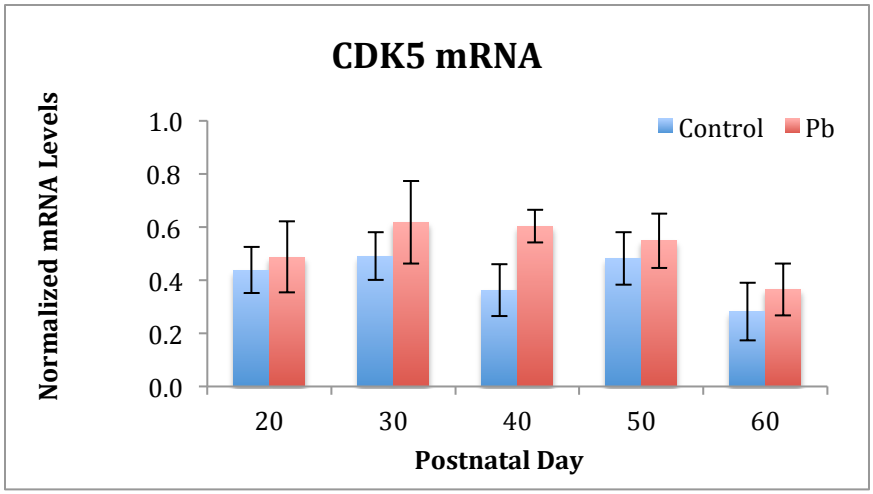
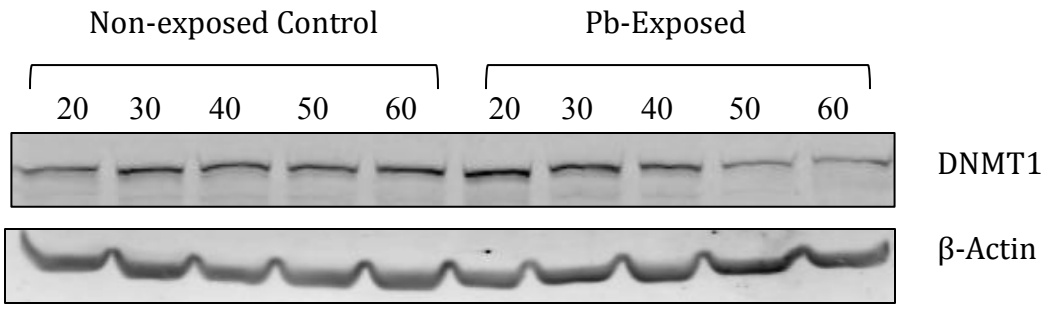
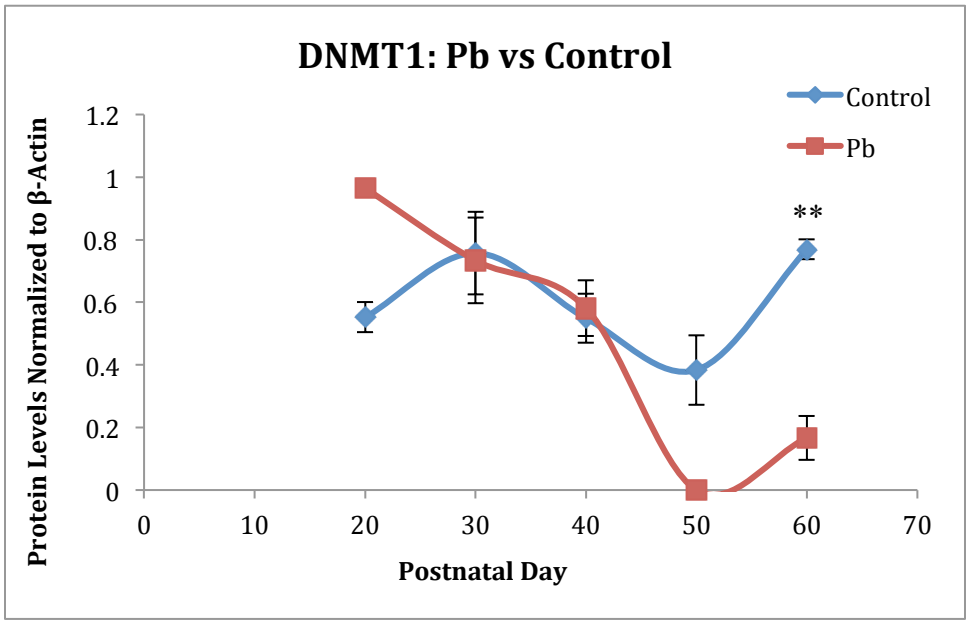


Figure 12.
A. Western blot of DNMT1 normalized to β-Actin: Pb vs Controls



B. DNMT1 protein expression: Pb vs Controls



DISCUSSION

The present study has two hypotheses. Testing the first illustrates that the human tau transgene is temporally and spatially located similarly to the endogenous murine tau gene, which validates that the mouse model being examined is adequate to undergo further studies. The results recorded because of manipulations from drugs and/or toxins will be a result from the transgene being altered molecularly rather than being inserted in the wrong genomic location. Second, the study investigates the effect of postnatal lead exposure on the developmental time frame of Alzheimer's related genes. Although extant findings have shown that Pb has an effect on tau, its kinase's and phospho-tau, this study is the first to explore the direct effects of an environmental toxin on the human tau gene.

Initial genotyping confirms that transgenic mice adequately express human tau and that both the transgene and endogenous tau are absent in the non-transgenic mice. Immunohistochemistry staining in aged mice with an antibody for phospho-tau site Thr231 validates that the mouse model has high levels of phosphorylated tau in various brain structures as they age. This is in accord with the literature (Andorfer, 2003). The Morris Water Maze (MWM) behavior study evaluates mice on hippocampal-dependent spatial learning and memory. The results demonstrate that the presence of the human tau gene correlates with lower performance in the MWM. Transgenic mice compared to controls also spent less time in the correct quadrant in both Probe Trial 1 and 2. This finding indicates forgetfulness of the platform location, or loss of long-term memory function.

The similar lifespan protein patterns seen in the transgenic mice for total tau, phospho-tau site Ser396 and CDK5 in comparison to the wild-type mouse model (Bihaqi, 2014) qualitatively verify that the genomic location of the transgene is the same as the endogenous murine tau gene. The enzymes related to, as well as any post-translation modifications occurring to the human tau gene, are consistent with the murine gene. The higher intensities of the peaks seen within the transgenic mouse model are occurring because transgenes are commonly overexpressed in comparison to their comparative endogenous genes (Elder, 2010). At PND 20 in the transgenic mouse model, there is a high peak of tau and phopho-tau. In combination with overexpression of the transgene protein, this can be explained by fetal tau maintaining high levels in the postnatal brain. Fetal tau is the expression of only the shortest form of tau (3R) (Jovanov-Milošević, 2012) As mice age, the amount of 3R tau expressed is roughly equal to the amount of 4R tau (Chen, 2010).

Western blotting and quantitative real time PCR demonstrated that Pb has an effect on the human tau gene's transcription and translation. Previous studies have shown postnatal exposure in wild-type mice increases levels of tau, phosphorylated tau, and its related enzymes (Bihaqi, 2014). We expected to see the same pattern within the transgenic mouse model between PND 20-60. In fact, we find that Pb-exposed total tau and phosphorylated tau protein levels are elevated at PND 20 and PND 30. At PND 40, elevated levels are not maintained and decrease towards control levels. At PND 50 control levels are greater than Pb-exposed levels. At PND 60 levels start to converge, effectively restoring control protein levels. Combined, the findings illustrate that Pb has an effect on the human tau gene but it is not

maintained within the adolescent stage of life. The latent effects of elevated tau levels in late life seen in the wild-type mouse model are not being established and maintained within the time points investigated.

Additional western blotting showed consistent elevated levels of CDK5 in Pb-exposed mice compared to controls. This is analogous to the two-year Pb exposure study done in the wild-type mouse model (Bihaqi, 2014). The elevated kinase level with lower levels of phosphorylated protein is understood by knowing Ser396 is a major target of GSK3. GSK3 is a second kinase of tau. 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, 2014).

Furthering our understanding of these gene alterations requires a look into epigenetics. Epigenetic modifications occur at a transcriptional level to alter gene silencing or activation. mRNA levels of tau and CDK5 were altered by developmental Pb exposure in this transgenic mouse model. Therefore, epigenetic modifications, such as DNA methylation, are targets of exploration because of the changes seen. Western blotting showed initial increased protein expression at PND 20 of DNMT1 in Pb exposed mice compared to controls. DNMT1 returned to control levels for PND 30 and 40. Protein levels then proceeded to fall below controls at PND 50 and 60. Decreased global DNMT1 levels correlate to less gene silencing. The results from this present study confirm that the transgenic mouse model is useful in studying further epigenetic changes such as DNA methylation at additional time points and possibly histone modifications.

Future studies should explore the peak of tau, phospho-tau and CDK5 occurring in young to mid adulthood of this transgenic mouse line. We believe that unknown recovery pathways may be reversing the postnatal lead exposure effects of elevated tau and phospho-tau levels. This exposure effect may not be able to be recovered after the adulthood peak of tau, phosphorylated tau and CDK5. A meticulous time point study to determine the pattern of proteins and expression around each protein peak after postnatal Pb exposure should be investigated. Ultimately, it would be interesting to determine postnatal Pb exposure's precise timing role on the Alzheimer's related human tau gene. This knowledge could lead to more direct diagnosis and therapeutic efforts by pinpointing when AD related genes start to become abnormally elevated.

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