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Infantile Postnatal Exposure to Lead (Pb) Enhances Tau Expression in the Cerebral Cortex of Aged Mice: Relevance to AD

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Abstract

The sporadic nature in over 90% of Alzheimer's disease (AD) cases, the differential susceptibility and course of illness, and latent onset of the disease suggest involvement of an environmental component in the etiology of late onset AD (LOAD). Recent reports from our lab have demonstrated that molecular alterations favor abundant tau phosphorylation and immunoreactivity in the frontal cortex of aged primates with infantile lead (Pb) exposure (Bihaqi and Zawia, 2013). Here we report that developmental Pb exposure results in elevation of protein and mRNA levels of tau in aged mice. Western blot analysis revealed aberrant site-specific tau hyperphosphorylation accompanied by elevated cyclin dependent kinase 5 (CDK5) levels in aged mice with prior Pb exposure. Mice with developmental Pb exposure also displayed altered protein ratio of p35/p25 with more Serine/Threonine phosphatase activity at old age. These changes favored increase in tau phosphorylation, thus providing evidence that neurodegenerative diseases may be in part due to environmental influences that occur during development.

Keywords

aging; Alzheimer's disease; hyperphosphorylation; CDK5; lead; tau

1. Introduction

The neurodegenerative disorder Alzheimer's disease (AD) is the most prominent form of dementia among elderly in Western countries (Hebert et al., 2003). At the pathological level,

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AD is characterized by excessive accumulation of two major proteinaceous aggregates, senile plaques composed of aggregated amyloid beta (Aβ), which is derived from the β-amyloid precursor protein (APP), and neurofibrillary tangles enriched with hyperphosphorylated tau. These pathological hallmarks are responsible for synaptic loss in the brain resulting in dementia (Selkoe, 1991, Tanzi and Bertram, 2008).

Although the familial early onset (<65years) form of AD (EOAD) has been linked to mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN 1), presenilin 2 (PSEN2), late onset AD (LOAD) that represents the majority of AD cases (~95%) is sporadic in nature and has no genetic association (Liddell et al., 2001). Moreover, the pathological manifestation of AD which occur in old age, may have been set in motion prior to such a stage, and possibly during early stages of brain development (Zawia and Basha, 2005). The developing brain is specifically vulnerable to the neurotoxic effects of lead (Pb), a widely recognized neurotoxin (Mushak et al., 1989). While there are no conclusive evidence available which can pin point a precise correlation between Pb exposure and AD, however studies in the ageing individuals have shown a possible association between Pb exposure and cognitive decline in human subjects (Nordberg et al., 2000, Weisskopf et al., 2007). This was corroborated by recent findings from a 2-year lifespan study in rodents which demonstrated that rodents with developmental exposure to Pb showed cognitive deficit as aged adults, which is consistent with changes in amyloid biomarkers of AD (Bihaqi et al., 2013).

Evidence of the association between exposure to Pb and cognitive decline in humans is present from several longitudinal and cross-sectional studies in the elderly. Cognitive decline is an intermediate stage towards the development of AD. The Normative Aging Study (NAS) is a prospective longitudinal study that the Veterans Administration started in 1963 to monitor the effect of aging on different health conditions (Peters et al., 2010) with sub-groups of population investigated for a link between past non-occupational Pb exposure and cognitive decline. Participants in NAS were examined by several investigators, with different cognitive tests, sample size and time periods, who reported that higher levels of Pb in blood and/or bone were accompanied by poor cognitive performance in different cognitive tests including 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, Weisskopf et al., 2007). In a sub-group of the Nurses' Health Study (NHS), a longitudinal study established in 1976 to monitor women health, (Weuve et al., 2009) reported that in 587 women exposed to Pb, higher levels of Pb in tibia bone were associated with poor test scores on MMSE. Moreover, in a cross-sectional study that involved 530 women of age between 65–87 years, blood Pb concentrations of ≥8μg/dL significantly resulted in a poor performance in several cognitive tests including MMSE, incidental memory test and digit symbol test (Muldoon et al., 1996). Despite the negative effects of Pb exposure on cognitive functions in elderly population as suggested by those longitudinal and cross-sectional studies, the need for studies that link the exposure to Pb, especially in infantile or childhood stages, and incidence of AD arises.

For over a decade our work had focused on Pb exposure and amyloidosis. Recently we have turned our attention to the co-existent tau pathology. Furthermore, neurofibrillary

degeneration devoid of amyloidosis has been visualized in several tauopathies like Guam parkinsonism dementia complex, dementia pugilistica, corticobasal degeneration, Pick's disease and FTDP-17 tau (fronto-temporal dementia with parkinsonism linked with chromosome 17 and tau mutations) and progressive supra nuclear palsy (Iqbal et al., 2010). Furthermore, dementia in humans is best correlated with the presence of neurofibrillary pathology than β-amyloidosis.

Current published studies by us on primate brains exposed to Pb as infants have shown that developmental exposure to Pb induces a latent overexpression of tau accompanied by site specific hyperphosphorylation of tau as well as increased tau immunoreactivity in the frontal cortex (Bihaqi and Zawia, 2013). In this manuscript we examine the cerebral cortices from a lifespan study carried out in a cohort of mice with developmental and adult Pb exposure scenarios in order to profile stage- and exposure specific changes in the tau mRNA and protein, its phosphorylated forms, as well as other critical intermediates involved in its regulation.

2. Materials and methods

2.1. Animal exposure

Mice of C57BL/6 strain were bred in-house at the University of Rhode Island. Postnatal day 1 (PND 1) was designated as 24 hours after birth. Male pups from different dams were pooled and placed randomly to different dams (10 mice/dam). The mice were divided into four groups. The control group received regular tap water. The second group (PbE, the early Pb exposure group) was exposed to 0.2% Pb acetate from PND 1 to PND 20 through drinking water of the dam. The third group (PbA, the adult lead exposure group) was exposed to Pb daily for 3 months starting at 7 months of age. The fourth group (PbEA, the early and adult Pb exposure group [re-exposure as adults]) was exposed to Pb developmentally (PND 1 to PND 20) and during adulthood (7–9 months of age). The mice were sacrificed and their brains dissected out at PNDs: 20, 180, 270, 540, and 700 and their frontal cortices were stored at −80°C until use (Fig 1). (Figure 1)

2.2. Sample preparation and Western blotting

Brain cortical tissues were lysed or homogenized in RIPA lysis buffer (lysis buffer (150 mM NaCl, 25 mM Tris-HCl at pH 8.0, 1% NP-40, 10 mM NaF, 1mM Na3VO4) containing 1% protease inhibitors cocktail (Sigma-Aldrich, MO), The samples were sonicated and vortexed for 5min prior to centrifugation at 10,000 x g for 20 min at 4°C to pellet the cell debris, the supernatant obtained was used for Western blot analysis. Protein concentration was determined by using a BCA kit (Pierce Biotechnology Inc. Rockford, IL). Immunoblotting for total tau, phosphorylated tau and associated proteins was determined following over night exposure at 4°C to the following antibodies diluted at 1:1000: mouse anti-Tau 46, anti-phospho-Tau [rabbit anti pThr-212 (Sigma Aldrich, MO), rabbit anti p-Thr-181, mouse anti pSer-396 (Cell Signaling technology, MA), rabbit anti p-Ser 235 (Abcam, MA)], rabbit anti-CDK5, and rabbit anti p35/25 (C64B10) (Cell Signaling technology, MA). On the following day, membranes were washed and exposed for 1 h to goat anti mouse/goat anti rabbit IRDye® 680LT infrared dye (LI-COR Biotechnology, NE), diluted at 1:10,000. The images

were developed using Odyssey infrared imaging system (Model- 9120, LI-COR Biotechnology, NE). As a control for equal protein loading, membranes were stripped and reprobed for 2 h with mouse glyceraldehydes 3 phosphate dehydrogenase (GAPDH) antibody diluted at1:5000 (Sigma-Aldrich, MO) at room temperature followed by washing and re-exposure to goat anti mouse IRDye® 680LT infrared dye. After transferring to a polyvinylidene fluoride (PVDF) membrane, the gel was stained with Bio-safe Coomassie blue stain (Bio-Rad, Hercules, CA) to assess the loading of the samples. The protein expressions of the various protein analyzed in the present study where normalized against GAPDH, which is normally used as reference gene product.

2.3. Total RNA isolation, synthesis of complementary DNA, and real-time polymerase chain reaction

Total RNA from control and exposed brain tissue was extracted according to TRIzol method (Invitrogen, CA). First strand complementary DNA (cDNA) was synthesized from 1.5 μg of total RNA using the iScript cDNA kit (Bio-Rad, CA). cDNA was then amplified using real-time PCR. The SYBR Green qRT-PCR assays were performed in 25 μl reactions in replicates using 1.5 μl of cDNA template, 1×SYBR Green master mix, 0.4 μM forward and reverse primers, and deionized water. The following primer pairs were used: tau forward primer 5′-GTG GCC AGG TGG AAG TAA AA-3’, tau reverse primer 5′-TGG AAG ACA CAT TGC TGA GG-3′; CDK5 forward primer: 5′-TGG TGA AGC AGG CAT CTG AG-3′, CDK5 reverser primer 5′-CCA TTG CAG CTG TCG AAA TA-3′; GAPDH forward primer 5′-TGG TGA AGC AGG CAT CTG AG-3′, GAPDH reverse primer 5′-TGC TGT TGA AGT CGC AGG AG-3′. Amplification was undertaken on an ABI PRISM 7500 machine (Applied Biosystems, Foster City, CA, USA) with sequence detection software version 1.3, and expression was reported relative to GAPDH mRNA with the $2^{-\Delta\,\Delta Ct}$ method.

2.4. Ser/Thr phosphatase assay

Evaluation of serine/threonine phosphatase (Ser/Thr) activity in mice cortices samples was done using the Ser/Thr phosphatase assay kit 1 (Millipore, CA). Protein amount of 10 μg and 200 mM peptide (KRpTIRR) substrate were used per well to determine enzyme activity after a 15 min reaction. The enzyme reaction was terminated with 100 μl Malachite Green solution and a subsequent 15 min was allowed for color development. Absorbance was measured at 650 nm with a plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA). Enzyme activity was calculated from the amount of released phosphate in pmol phosphate/min/mg based on a phosphate standard curve.

2.5. Statistical analysis

Western blot bands were quantified by using the LI-COR Odyssey infrared image system. All measurements were made in triplicate and all values are presented as mean standard error of the mean. The significance of the difference among means of the experimental groups was obtained with one-way analysis of variance, the Tukey-Kramer multiple-comparison post-hoc test, and the student Newman-Keuls comparison post-hoc test, using Graph pad Prism 3.0 computer software (La Jolla, CA, USA). The level of significance was set at $P<0.05$.

3. Results

3.1. Latent effect of Pb on total tau protein and mRNA

The protein expression of total tau was examined by Western blot analysis at different time points within the lifespan of the mice of various experimental groups. Our results revealed that compared to controls, normalized tau levels in PbE (developmental exposure) and PbEA (developmental and adult exposure) groups were slightly elevated during early life (PND 20) and remained constant during adulthood. However, a significant (*P<0.01*) increase in the tau protein expression was observed in late life (PND 700) as compared to controls (Fig. 2A&B). In comparison to the aged matched controls, aged mice exposed to Pb as adults (PbA) showed no significant change in tau protein expression (Fig. 2A&B). Our results also revealed that PbE and PbEA groups showed a significant latent upregulation in the mRNA levels of tau at PND 270 (*P<0.05*),PND 540 (*P<0.001*), and PND 700 (*P<0.001*)respectively (Fig. 2C).

3.2. Phosphorylation of tau

For tau specific phosphorylation, four different antibodies namely threonine 212 (Thr-212), threonine 181(Thr-181), serine 396 (Ser-396), and serine 235 (Ser-235) were used in Western blots of the cerebral cortex. Western blot analysis normalized against GAPDH revealed that PbE and PbEA depicted a significant (*P<0.05*) increase in the levels of (Thr-181), (Ser-396), and (Ser-235) at PND 700 as compared to aged matched control (Fig. 3 and Fig. 4). Similar increases in the levels of (Thr-212) was also observed in these mice in old age but were not statistically significant. No significant change in tau specific phosphorylation was observed in the PbA group compared to their respective aged matched controls (Fig. 3 and Fig. 4).

3.3. Effect of Pb exposure on CDK5 and p35/p25 protein levels

The elevated phosphorylation observed in aged mice at various tau residues in PbE and PbEA due to developmental Pb exposure can be associated with increases in kinases and other activators. Our results revealed that PbE and PbEA groups displayed a significant increase in the normalized protein levels of the kinase CDK5 at PND 540 *(P<0.05)* and PND 700 *(P<0.05)* (Fig. 5A&B). Furthermore a similar upregulation in the mRNA levels of cdk5 in PbE and PbEA groups at PND 180 *(P<0.05)*, PND 270 *(P<0.05)*, PND 540 *(P<0.05, P<0.01)* and PND *700 (P<0.05, P<0.01)* was seen.No change in the mRNA levels of cdk5 was observed in PbA group as compared to controls (Fig. 5C).

The alteration in cdk5 protein levels was also accompanied by a subsequent decrease in the levels of p35, a specific neuronal activator of cdk5 at PND 700 (Fig. 6A&B). Moreover, a significant *(P<0.01)* increase in the normalized protein levels of p25 (truncated form of p35) was observed at PND 500 (P<0.01) and PND 700 (P<0.01) in PbE and PbEA groups as compared to age matched control (Fig. 6A&C).

3.4. Activity of serine/ threonine phosphatase

We investigated the activity of serine/ threonine phosphatase in the cerebral cortex of the mice of all experimental groups, control as well as mice with Pb exposure at different time

points within the life span. Our results revealed that PbE and PbEA groups showed a significant *(P<0.05)* increase in the serine/ threonine phosphatase activity at PND 540 and PND 700 as compared to age-matched control. PbA group registered an insignificant increase in the activity of serine/ threonine phosphatase at old age compared to age-matched control (Fig. 7).

4.0. Discussion

The involvement of environmental factors in disease etiology is becoming increasing noticeable. Heavy metal such as Pb have been found to pose an environmental concern and its consistent presence in the environment is a health risk to human populations (Tong et al., 2000). Studies have also revealed that cognitive deficit caused by childhood Pb exposure can prevail in adulthood (Mazumdar et al., 2011). Although Pb exposure has been projected as a risk factor for the health of adults and young ones, studies carried out in our lab on rodents and primates have provided strong proof indicating that early life exposure is associated with latent effects contributing to the neurodegenerative process (Bihaqi et al., 2011, Bihaqi et al., 2013).

The protein tau is one of the microtubule associated proteins, mainly found in neuronal axons and is thought to have a role in the stabilization of neuronal microtubules; which in turn supports intracellular transport (Mandelkow and Mandelkow, 1994). The failure of regular tau function can be catastrophic, and its consequences can promote neurodegenerative disease (Gendron and Petrucelli, 2009). *In vitro* investigations have also indicated that overexpression of tau leads to altered cell shapes and reduced cell growth which eventually results in a dramatically redistribution of various organelles transported by microtubule-dependent motor proteins (Ebneth et al., 1998). Recent studies from our lab have shown that aged primates with developmental Pb exposure showed a significant increase in the tau protein and mRNA levels. Tau immunoreactivity was also found to be increased in these primates compared to control (Bihaqi and Zawia, 2013).Consistent with our primate findings mice exposed to Pb during the developmental phases showed a drastic increase in the protein and mRNA levels of tau in their old age.

A primary hallmark of tau in AD is the presence of abnormal phosphorylation at serine and threonine sites compared to normal adult brain tau, which leads to the formation of neurofibrillary tangles (NFT) (Lee et al., 2004). Aberrant phosphorylation of tau is responsible for compromising microtubule stability and function, resulting in a loss or decline in axonal or dendritic transport in disease (Alonso et al., 1996, Salehi et al., 2003). Seminal work carried out by Iqbal and co-workers observed impaired microtubule assembly in AD brain extracts was associated with tau hyperphosphorylation (Iqbal et al., 2010). Recently studies carried out in our lab showed that 23 year-old primates with developmental exposure to Pb exhibit an increase in phosphorylation at Thr 181, Thr 212, Ser 396, and Ser 235 (Bihaqi and Zawia, 2013). Similar to our previous findings, our present study revealed that mice with developmental exposure to Pb displayed increased phosphorylation at serine and threonine sites.

Phosphorylated sites of tau found in AD brains are proline-directed (Thr-Pro or Ser-Pro), suggestive of the involvement of kinases such as cdk5 (Imahori and Uchida, 1997). Studies have shown cdk5 to co-localize with the NFT-containing neurons in the AD brains (Takahashi et al., 2000). Interactions of cdk5 with proteins p35 or p39, which are present in abundance in post mitotic neurons, are pivotal for its activation (Tomizawa et al., 2002). The transformation of p35 to p25 is guided by calpain which delocalizes and deregulates cdk5, and over-activation of cdk5 due to p25 has been associated with NFT (Cruz et al., 2003).

Primate studies undertaken in our lab have shown increased cdk5 and p25 levels in aged primates with developmental exposure to Pb (Bihaqi and Zawia, 2013). Consistent with these results, we observed an upregulation of protein and mRNA levels of cdk5 accompanied by a decrease in p35 and a subsequent increase in p25 in aged mice with developmental Pb exposure. These observation indicate that the post-translational regulators of tau may be also candidate for epigenetic reprogramming which can have an impact on the tau gene expression, which can result not only in an increase of total tau, but also in enzymes which are involved in its phosphorylation.

Protein Phosphatase like protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B) are the major phosphatases found in the brain (Liu et al., 2007), with PP2A constituting around 90% of the total protein phosphatase activity (Gong et al., 1995). Several lines of evidence have shown over expression of PP1, PP2A and PP2B in human primary neuron culture are associated with Pb exposure, and these imbalance in the activity of phosphatases have also been associated with cognitive dysfunction (Rahman et al., 2011). Seminal studies by Bihaqi et al have also shown that aged primates with infantile exposure to Pb display an increase in the activity of the Ser/Thr activity thus resulting in variable changes in the activity of cdk5. In agreement with our earlier findings, the present data also reveal that mice with developmental Pb exposure pose a latent over-activation of Ser/Thr protein phosphatase activity at old age. We have also previously explained that the increase in protein phosphatase activity was to compensate for the increasing activity of kinase levels. However the process seemingly advocates more kinase activity leading to over-burden of tau phosphorylation.

In conclusion our study suggests that early life exposure to the environmental toxicant Pb up-regulates tau protein and mRNA, as well as tau phosphorylation levels late in life. These changes were also related to an imbalance in the kinase-phosphatase equilibrium leading to over abundance of phosphorylated tau.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Developmental exposure to lead (Pb) alters expression of the tau gene later in life.
- Prior Pb exposure increased cdk5 levels and enhanced tau phosphorylation.
- Increased Ser/ Thr Phosphatase activity due to developmental exposure to Pb.

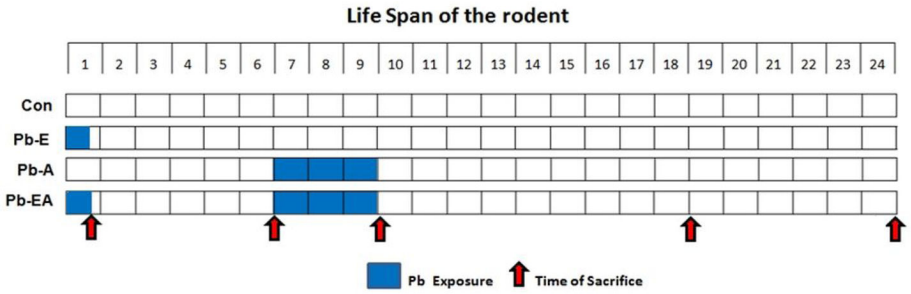

Figure 1. Exposure protocol for wild type murine animals

The table shows the lifetime scale of the mice and the Pb exposure scenario. The red arrowheads indicate the various time points of sacrifice and tissue collection. Control (Con) group**,** Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and adult Pb exposure group (Pb-EA).

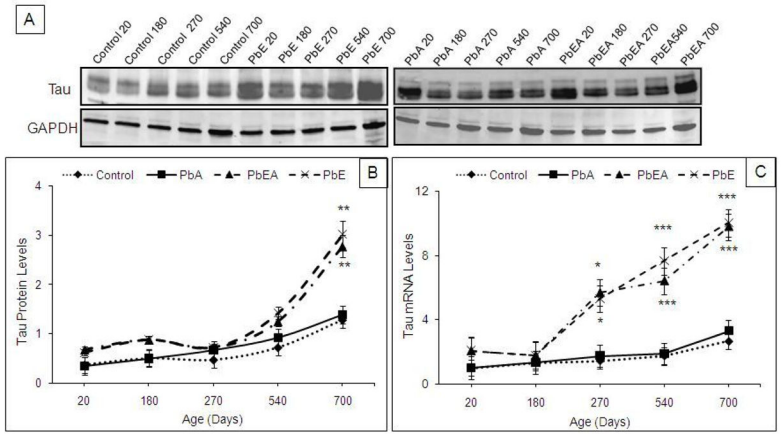

Figure 2. Total Tau expression across the lifespan and following developmental exposure to Pb

(A) Changes in the developmental profiles of tau protein expression in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and adult Pb exposure group (Pb-EA). (B) Quantification by normalization to GAPDH. (C) mRNA levels of Tau relative to GAPDH. Each data point in the curve is the mean ± SD. *p<0.05, **p< 0.01, ***p<0.001 compared to the control group.

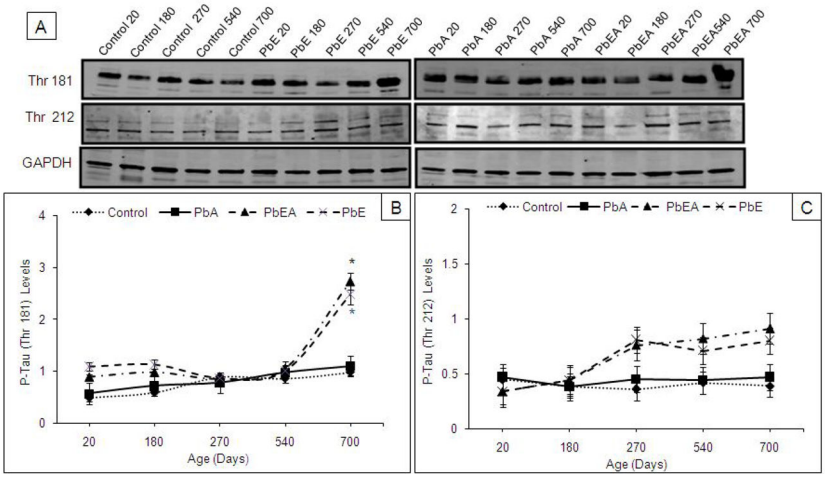

Figure 3. Protein expression of Phosphorylated Tau Thr 181 and Thr 212 across the lifespan and following developmental exposure to Pb

(A) Changes in the developmental profiles of tau Thr 181 and 212 protein expression in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and adult Pb exposure group (Pb-EA). (B &C) Quantification by normalization to GAPDH. Each data point in the curve is the mean ± SD. *p< 0.05 compared to the control group.

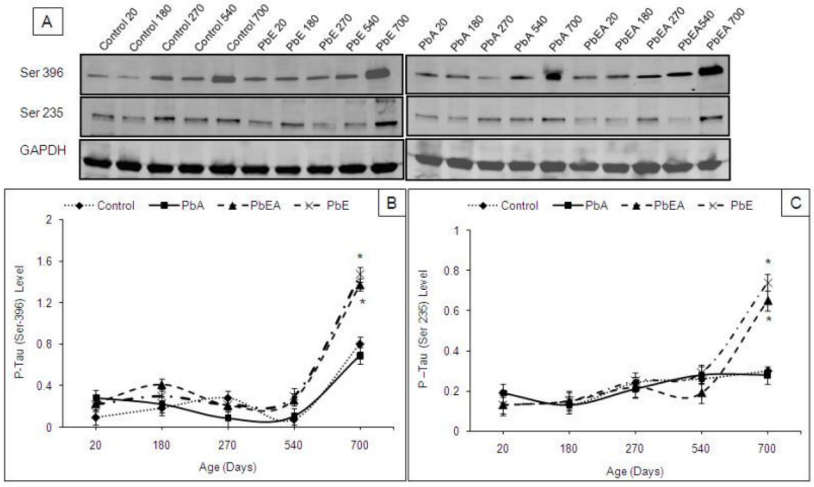

Figure 4. Protein expression of Phosphorylated Tau Ser 396 and Ser 235 across the lifespan and following developmental exposure to Pb

(A) Changes in the developmental profiles of Ser 396 and 235 protein expression in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and adult Pb exposure group (Pb-EA). (B&C) Quantification by normalization to GAPDH. Each data point in the curve is the mean ± SD. *p< 0.05 compared to the control group.

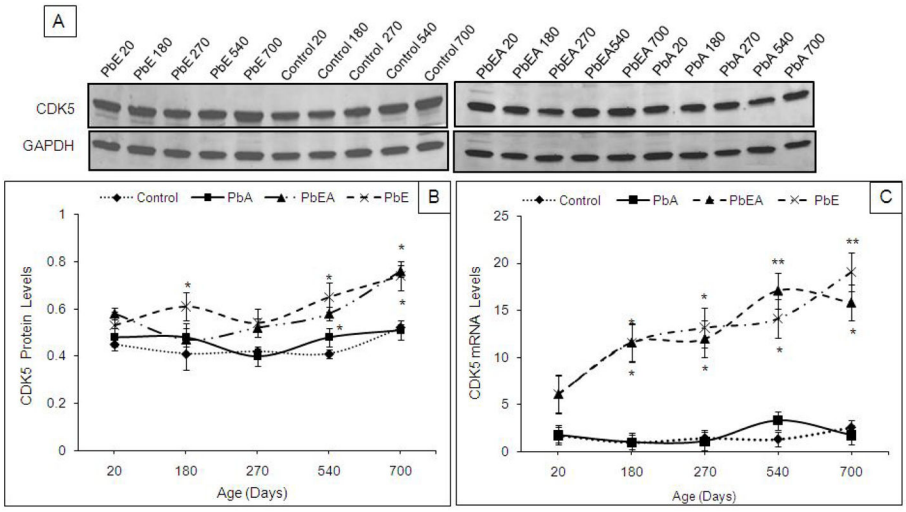

Figure 5. cdk5 expression across the lifespan and following developmental exposure to Pb

(A) Changes in the developmental profiles of cdk5 protein expression in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and adult Pb exposure group (Pb-EA). (B) Quantification by normalization to GAPDH. (C) mRNA levels of CDK5 relative to GAPDH. Each data point in the curve is the mean ± SD. *p< 0.05, ** p<0.01 compared to control group.

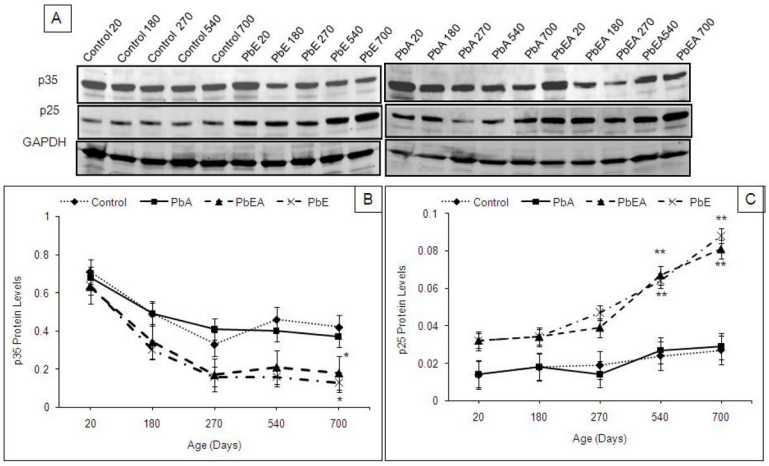

Figure 6. Protein expression of p35 and p25 across the lifespan and following developmental exposure to Pb

(A) Changes in the developmental profiles of p35 and p25 protein expression in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and Adult Pb exposure group (Pb-EA). (B) Quantification by normalization to GAPDH. Each data point in the curve is the mean ± SD. *p< 0.05, ** p<0.01 compared to the control group.

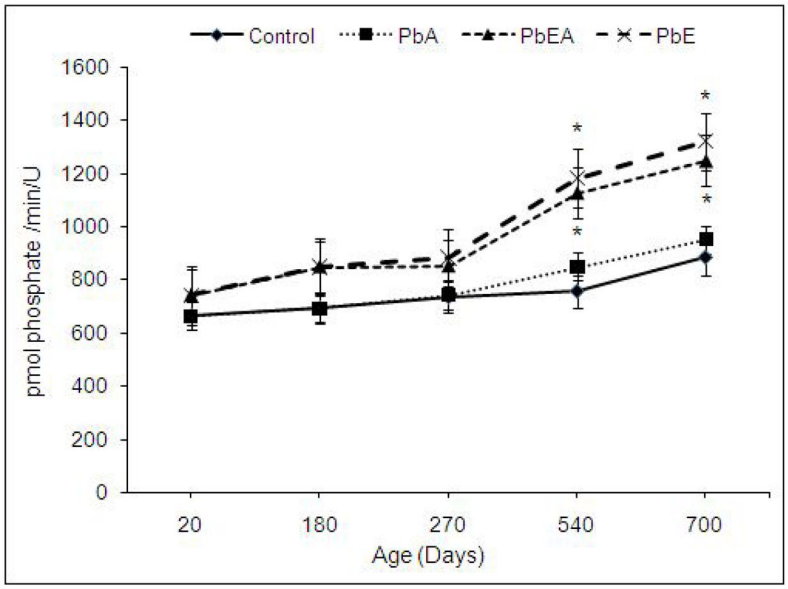

Figure 7. Ser/Thr protein phosphatase levels across the lifespan and following developmental exposure to Pb

Changes in the developmental profiles of Ser/Thr protein phosphatase levels in the cerebral cortex of control, Early Pb exposure group (PbE), Adult Pb exposure group (PbA) and Early and Adult Pb exposure group (Pb-EA). Each data point in the curve is the mean ± SD. *$p < 0.05$ compared to the control group.