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EARLY POSTNATAL LEAD EXPOSURE INDUCES TAU PHOSPHORYLATION IN THE BRAIN OF YOUNG RATS

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Cognitive impairment is a common feature of both lead exposure and hyperphosphorylation of tau. We, therefore, investigated whether lead exposure would induce tau hyperphosphorylation. Wistar rat pups were exposed to 0.2% lead acetate via their dams' drinking water from postnatal day 1 to 21. Lead in blood and brain were measured by atomic absorption spectrophotometry and the expression of tau, phosphorylated tau and various serine/threonine protein phosphatases (PP1, PP2A, PP2B and PP5) in the brain was analyzed by Western blot. Lead exposure significantly impaired learning and resulted in a significant reduction in the expression of tau but increased the phosphorylation of tau at Ser199/202, Thr212/Ser214 and Thr231. PP2A expression decreased, whereas, PP1 and PP5 expression increased in lead-exposed rats. These results demonstrate that early postnatal exposure to lead decrease PP2A expression and induce tau hyperphosphorylation at several serine and threonine residues. Hyperphosphorylation of tau may be a mechanism of Pb-induced deficits in learning and memory.

Keywords: Tau – hyperphosphorylation – lead-protein phosphatases – PP2A

INTRODUCTION

Lead (Pb) is a known neurotoxicant which affects the central nervous system even at low level of exposure [3]. Exposure to low levels of Pb (as low as 7 μg/dL) is associated with impairment of learning and memory in children [7, 32] and in young adults [8]. Pb accumulates in the brain, and preferentially in the hippocampus – an area that is associated with learning and memory [25, and our unpublished data]. The developing brain is a primary target for lead-induced neurotoxicity. However, the mechanism of lead-induced neurotoxicity and deficits in learning and memory is not well understood.

Neurite growth and synaptogenesis require an intact microtubule structure, and the disruption of microtubules has been shown to be associated with memory loss and neuronal death [19]. The microtubule network is stabilized by several microtubule-associated proteins (MAP). Tau is one of the MAPs which can be phosphorylated at several serine (Ser) or threonine (Thr) residues [12]. Tau promotes the assembly and

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maintenance of the microtubule structures. However, abnormally hyperphosphorylated tau (HPT) has less affinity for binding with microtubules. HPT also sequesters normal tau and other microtubule-associated proteins (MAP1 and MAP2) and causes disassembly of microtubules [2]. HPT forms paired helical filaments (PHF) and accumulates as neurofibrillary tangles in neurons. Accumulation of NFTs in neurons have been implicated in dementia in many tauopathies like Alzheimer's disease and frontotemporal dementia [14, 15, 39].

The phosphorylation state of tau determines its biological activity [27]. Phosphorylation of tau at a stoicheometry of 2–3 mole phosphates per mole of protein is required for promoting assembly and stability of the microtubule, whereas, its hyperphosphorylation (9–10 mole of phosphate per mole of tau) results in the disruption and disassembly of microtubule and subsequently in memory loss and neuronal death [1, 2].

Since cognitive impairment is a common feature of both lead exposure and hyperphosphorylation of tau, we were interested whether lead exposure would induce tau hyperphosphorylation. We have previously reported that exposure of primary human fetal neurons in culture to Pb results in hyperphosphorylation of tau at Ser199/202 and Thr231 [36]. However, the effect of Pb exposure on tau hyperphosphorylation *in* vivo remains poorly investigated.

Ser/Thr protein phosphatases are among the major regulators of tau phosphorylation state [4, 11]. Impairment of protein phosphatases not only directly affects the dephosphorylation of tau but may also up-regulate the activity of some protein kinases, which are phosphorylation dependent [21, 35, 45]. The major Ser/Thr phosphatases in the brain are PP1, PP2A and PP2B [25]. It is therefore, relevant to investigate whether the expression pattern of these phosphatases would be altered by Pb exposure.

Therefore, we used a rat model where animals were exposed to Pb during the early postnatal period and investigated the effect of Pb exposure on tau hyperphosphorylation and the expression of different serine/threonine phosphatases.

MATERIALS AND METHODS

Pb exposure protocol

Wistar rat pups were culled to 10 per litter and exposed to 0.2% Pb acetate dissolved in distilled water via their dams' drinking water from postnatal day (PND) 1 to PND21. The control group received regular tap water in a similar manner. The animals were housed at constant temperature (21 ±2 °C) and relative humidity (50 ± 10%) with a 12-hour light/dark cycle (07:00–19:00 h). The animal exposure and maintenance was according to the approved protocol of the Institutional Animal Care and Use Committee of Kuwait University.

Collection of blood and tissue samples

At PND21 animals were anesthetized with CO_2 and weighed. Blood was drawn from the right ventricle for Pb analysis. Animals were subsequently decapitated, brain was removed, weighed, snap-frozen in liquid nitrogen and stored at –70 °C till further analyses.

Lead analysis

Pb levels in blood and brain tissues of control and Pb-exposed rats were measured by a Varian Graphite Furnace Atomic Absorption Spectrophotometer (Varian, Spectra AA 220, Australia). For wet digestion, approximately 150 mg of homogenized brain tissue and 1 ml of blood samples were incubated overnight in 5 ml nitric acid for pre-digestion. Samples were then gently heated at 50 °C for 30 minutes and boiled at 90 °C for 15 minutes until a clear solution was obtained. Samples were diluted with double de-ionized water (Millipore). Lyophilized bovine liver (National Bureau of Standards, USA) and Serenorm (Lyophilized serum, SERO AS, Norway) were used as reference materials while analyzing brain and blood samples, respectively. The

Table 1
Setting of graphite furnace

Wave length	283.3 nm
Slit	0.5 nm
Lamp current	10 mA
Background correction	On
Sample volume	15 µL (10 µL sample + 5 µL modifier)
Replicates	2 (both for standards and samples)
Re-slope rate	10

Furnace program

Temperature	Time (sec.)	Read
85	5	No
95	40	No
120	10	No
400	5	No
400	1	No
2100	1	Yes
2100	2	Yes
2100	2	No

Table 2
Primary antibodies used in this study

Antibody	Type	Antigen/epitope	Dilution	Source
Tau (clone BT2)	m	Total tau	1:2000	Thermo Scientific, Rockford, IL, USA
AT8	m	pS199/pS202	1:1000	Thermo Scientific, Rockford, IL, USA
AT180	m	pT231	1:1000	Thermo Scientific, Rockford, IL, USA
AT100	m	pT212/pS214	1:1000	Thermo Scientific, Rockford, IL, USA
Anti-calcineurin (clone CN-A1)	m	PP2B catalytic subunit	1:5000	Sigma-Aldrich, St. Louis, MO, USA
Anti-PP5	p	Amino acids 71-240 of human PP5	1:1000	Santa Cruz, CA, USA
Anti-beta-actin	m	Beta-actin	1:2000	Sigma-Aldrich, St. Louis, MO, USA
Anti-PP1	m	Catalytic subunit of PP1	1:500	Santa Cruz, CA, USA
Anti-PP2A (clone 1D6)	m	PP2A catalytic subunit	1:2000	Millipore, Oak Drive, USA

m – monoclonal; p – polyclonal

analytical values deviated by ±4% from the certified values. Duplicate standard reference samples of lyophilized bovine liver and serum samples were used for spike recovery test while standardizing the method. Five hundred µL of lead standard solution (10 µg/L) was added to approximately 150 mg of bovine liver and 1 ml of serum samples, and subjected to wet digestion as described above and analyzed using graphite furnace. Final recovery of added Pb from both liver and serum samples ranged from 96–98%. The intra-assay variation ranged from 3–4%. Pb values were expressed as µg/dL of whole blood or µg/g wet brain tissue. Setting of the graphite furnace is given in Table 1.

SDS-PAGE and Western blotting

Brain tissues were homogenized in RIFA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 50 nM NaF and protease inhibitor cocktail (Roche Diagnostics, Castle Hill, NSW, Australia). Protein concentration in each sample was determined by the Bradford method. Western blot was performed to measure the expression levels of total and phosphorylated tau at different epitopes. β-Actin was used as a loading control. Expression level of various protein phosphatases was determined by Western blot using antibodies to PP1, PP2A, PP2B and PP5. Details of the primary antibodies used are given in Table 2.

An aliquot of lysate (20 µg protein) was resolved on 10% SDS-PAGE (NuPAGE, Invitrogen, Carlsbad CA, USA). Protein was transferred onto PVDF membranes; the membranes were blocked with 5% milk in TBS for one hour and incubated with primary antibody overnight at 4 °C. After incubation, the membranes were rinsed several times, washed with TBS-tween three times (10 minutes each), and then twice with TBS only. The membranes were then incubated with the secondary antibody for 4 hours at room temperature, washed as before and developed using ECL kit (Thermo Scientific, Rockford, USA). Blots were scanned and quantified using the software ImageJ from NIH (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2007). For quantitation, the signal for total tau was normalized to the signal for β-actin, and the signal for phosphorylated tau was normalized to the signal for total tau. Similarly, the expression of various phosphatases was quantitated by normalizing the blot signal for each phosphatase to the signal for β-actin.

Spatial learning testing

The influence of drinking the affected milk on spatial learning was tested using the Morris water maze (MWM) system as outlined by Terry Jr. 2009 [38]. AT PND 21 rats were randomly selected for the water maze test. MWM consisted of a circular, black fiberglass tank (200 cm diameter, 50 cm height) that was filled up to 40–43 cm

height with clear water (27 ± 2 °C). Four locations around the edge of the pool were defined as starting points. A black escape platform (13 cm in diameter) was submerged 1 cm below the surface of the water in a fixed location of the pool. The platform remained in the same zone during the entire experiment. The maze was located in a quiet test room, surrounded by many visual cues hung on drapes around the maze. These were visible from within the pool and could be used by the rats for spatial orientation. Locations of the cues were not changed throughout the period of testing. Animal's performance was recorded automatically by EZVideo™ – Digital Video Tracking, an automated tracking system (Accuscan Instruments, Inc. Columbus, OH, USA). Two separate teams blinded to the animal groups were designated to conduct training sessions and test sessions randomly. To familiarize the animals to the pool and experimental settings, they were allowed to swim freely from four different starting points relative to the platform for 60 s before being assisted onto the platform, on which they were allowed to rest for 20 seconds. Four training sessions were conducted each day for two days. For the spatial learning experiments, animals were given four trials (one from each starting position) per session for 7 days, with each trial having a cut-off time of 60 s and a trial interval of 30 s. After climbing onto the platform, the animal remained there for 20 s before the commencement of the next trial. Recording was automatically terminated when the animal found the target. The time required to reach the platform is known as the escape latency. If rats could not escape to the platform within 60 s by themselves, they were placed onto the platform and allowed to remain there for 20 s and their

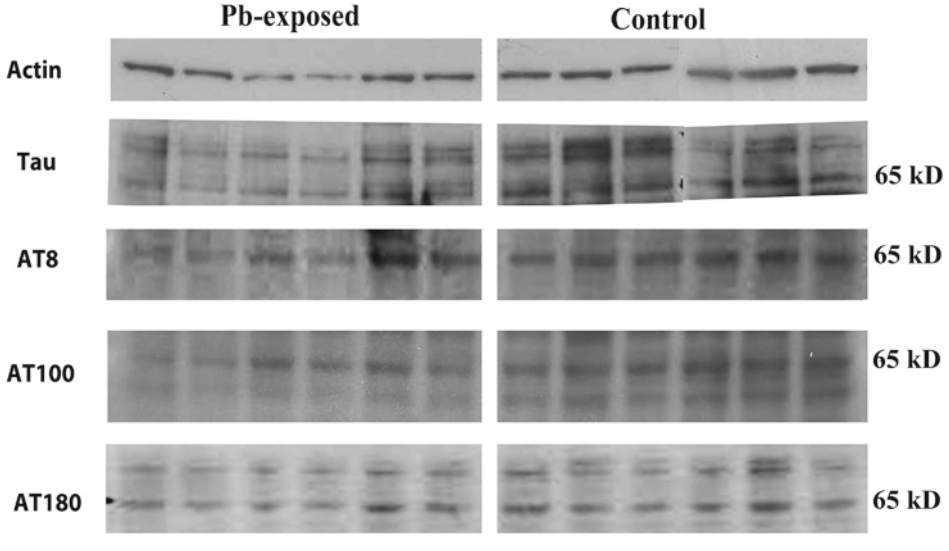

Fig. 1. Pb induces tau hyperphosphorylation in the brain of young rats. Brain of rats at PND21 were lysed in RIFA buffer and Western blot was performed using antibodies towards total tau (tau), tau phosphorylated at Ser199/202 (AT8), Thr212/Thr214 (AT100) and Thr231 (AT180). Beta-actin antibody was used as loading control. Antibody dilution is shown in Table 2

Table 3
Accumulation of Pb in the blood and brain of rats

	Control (mean ± SD)	Pb-exposed (mean ± SD)[a]
Blood Pb (μg/dL)	1.35 ± 0.49	12.40 ± 2.68*
Brain Pb (μg/g)[b]	0.21 ± 0.04	0.48 ± 0.11**

[a]Data represent the mean of 6 rats in each group. [b]Pb level in the brain is expressed per gram of wet weight of tissue. * Significantly different from control ($p < 0.0001$). ** Significantly different from control ($p < 0.01$).

escape latency was accepted as 60 s. The mean latency of finding the invisible platform was measured for individual animals on each day. The trials were started at PND21 and were completed at PND28.

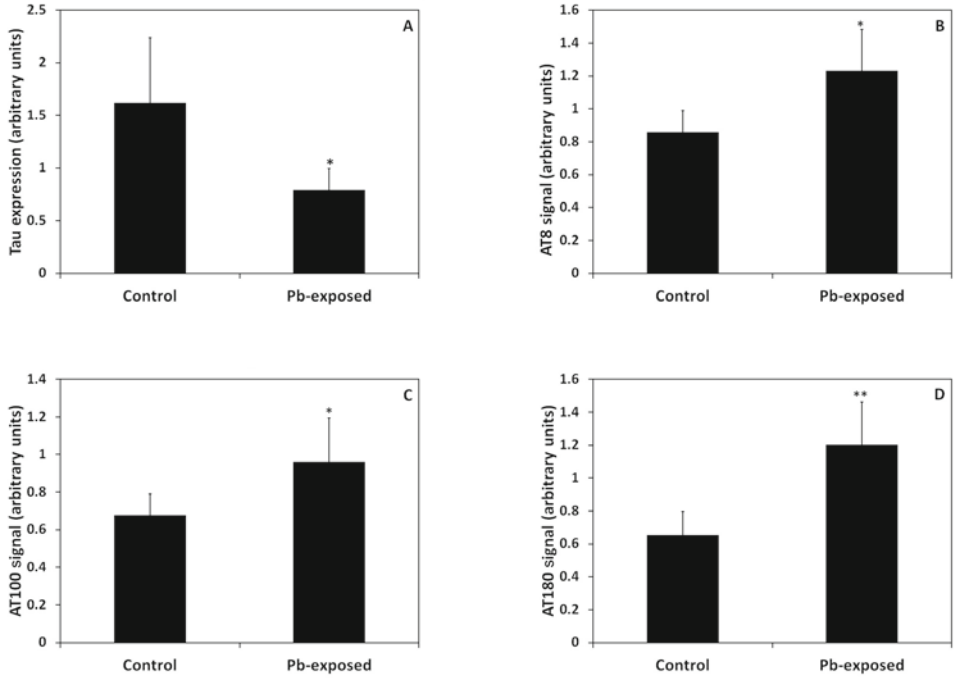

Fig. 2. Quantitation of Pb-indecued tau hyperphosphorylation. Sixty-five kD bands on the blots shown in Fig. 1 were quantified after scanning the image and analyzing the band densities with the ImagJ software. Data are presented as mean ± SD (n = 6). Signal for total tau was normalized to signal for beta-actin (loading control). The signal for AT8, AT100 and AT180 was normalized to the signal for total tau and is presented as arbitrary units. (A) total tau (tau), (B) tau phosphorylated at Ser199/202 (AT8) and (C) Thr212/Ser214 (AT100) and (D) Thr231 (AT180). * Significantly different from control ($p < 0.05$), ** Significantly different from control ($p < 0.01$) using *t*-test for two independent samples

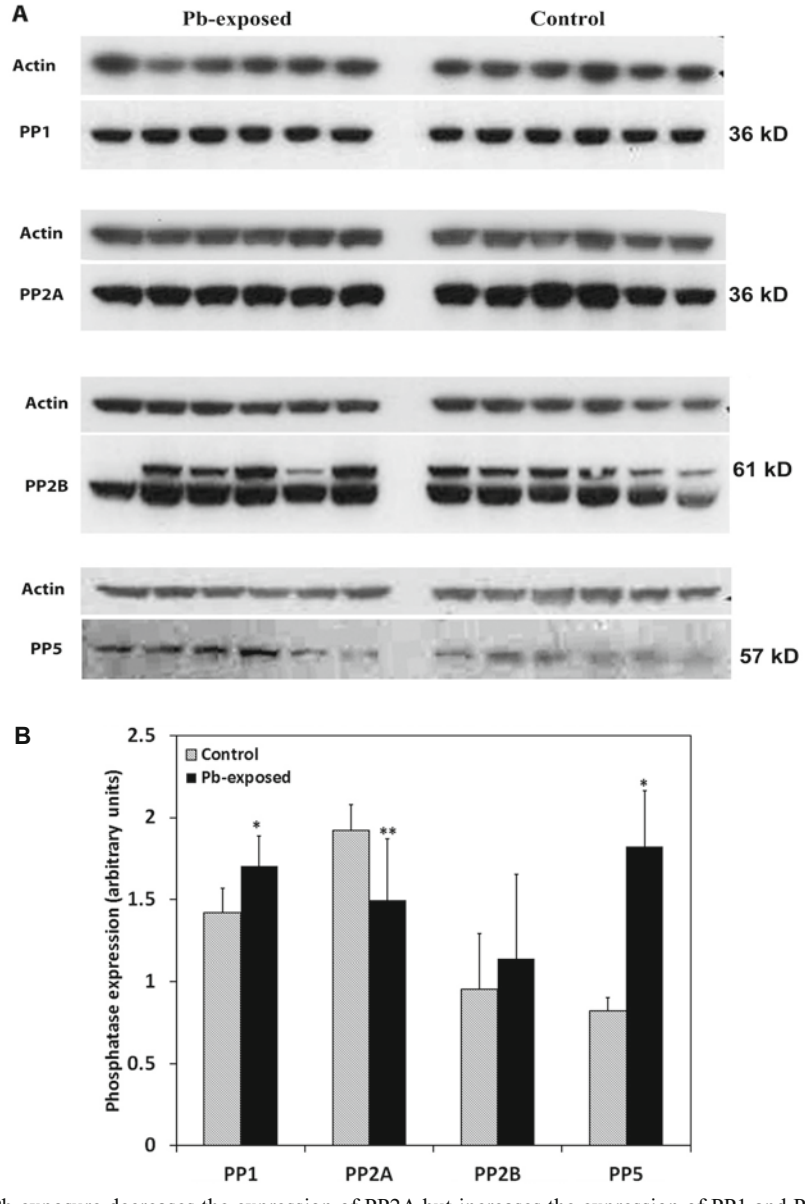

Fig. 3. Pb exposure decreases the expression of PP2A but increases the expression of PP1 and PP5: (A) 20 μg brain lysate protein was resolved on 10% SDS-PAGE, and immunoblotted with antibodies to PP1, PP2A, PP2B and PP5 (Table 1). For loading control, the same membranes were stripped and re-probed with anti-actin antibody. (B) For quantitation the blots were scanned and analyzed by the ImageJ software. The signal for each phosphatase was normalized to the signal for actin. For PP2B quantitation, the upper 61 kD band was quantitated. The data (as arbitrary untis) are presented as mean ±SD (n=6). * Significantly different from control ($p<0.001$), ** Significantly different from control ($p<0.01$) using *t*-test for two independent samples

Statistical methods

Data are expressed as mean ± SEM or SD as appropriate. Means of the two groups were compared with *t*-test for two independent samples with unequal variances. For the spatial learning test, we averaged the escape latencies across the four sessions for each rat each day. These means were then analyzed across the seven days of testing. A two-way repeated measures ANOVA was used for main effect (treatment comparisons) with days as the repeated measure and escape latency as the dependent variable. A p-value of <0.05 was considered statistically significant. Data were analyzed by SPSS for Windows version 18 (SPSS Inc., Chicago, IL, USA).

RESULTS

Exposure of rat pups to Pb through their dams' milk resulted in significant accumulation of Pb in blood and brain of the pups (Table 3). There was 9-fold increase in blood Pb and a more than 2-fold increase in brain Pb. Pb exposure resulted in a significant reduction (~50%) in the expression of tau in the brain (Figs 1 and 2). On the other hand, the phosphorylation level of tau at all the epitopes tested was significantly increased. The phosphorylation of Ser199/202 and Thr212/Ser214 increased by ~40%, whereas, the phosphorylation of Thr231 increased by almost 80%. This increased tau phosphorylation was accompanied with a significant 20% decrease in

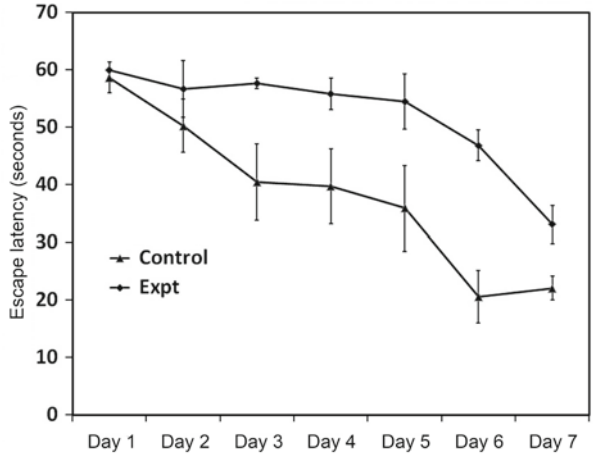

Fig. 4. Spatial learning is affected by Pb. Control and Pb-exposed rats were tested for spatial learning in Morris water maze as described in Material and Methods at PND 21 over a 7-day training protocol. Mean (±SD) escape latency of control and Pb-exposed rats at PND21 is shown across the 7-day training period. The difference between the two groups was significant across the days ($F=29.1$; $p<0.001$). Similarly, latencies on different days in both groups were significantly different ($F=108.2$; $p<0.001$) and the interaction of days and groups was also significant ($F=6.48$; $p=0.014$). Six from the control and 10 from the Pb-exposed rats were tested for spatial learning

the expression of PP2A (Fig. 3). The expression of PP1 was increased by 20%, whereas, the expression of PP5 was increased by more than twofold. PP2B expression was not affected by lead exposure.

Figure 4 shows the learning performance of control and Pb-exposed rats in the MWM test during the training period across the 7-day training protocol. Repeated measure ANOVA was performed to evaluate the learning performance of control and Pb-exposed groups across the days of testing (repeated measurements). Both the days and groups effects were significant (group effect: $F = 29.10$, $p < 0.001$ and days effect: $F = 108.21$, $p < 0.001$), suggesting that escape latencies (time to find the visible platform) of both control and Pb-exposed rats progressively became shorter over the training period. Furthermore, the escape latency across the days was significantly lower in the control group as compared to that of Pb-exposed group. The interaction between groups and repeated measures (days) was also significant ($p = 0.014$), suggesting that the rate of learning was not the same for both groups. Control rats learned faster than the Pb-exposed rats.

DISCUSSION

We report here that Pb exposure during early postnatal neuronal development produce biochemical changes (tau hyperphosphorylation) in the brain. Such changes have been implicated in learning and memory deficits. Pb exposure induces tau hyperphosphorylation at several Ser and Thr residues in the brain of rats, and this hyperphosphorylation of tau is associated with a significant decrease in the expression of PP2A. We have confirmed the previously reported findings that similar Pb exposure protocol results in substantial impairment of learning, and thus it appears that the two events (tau hyperphosphorylation and cognitive impairment) are associated.

Our Pb exposure protocol resulted in significant accumulation of Pb in the blood and brain of Pb-exposed rats, and our results are in agreement with the previously reported Pb levels in similar Pb exposure protocols [17, 20, 41]. The mechanism by which Pb gets into the brain is not clear. One potential mechanism is the disruption of the blood-brain barrier (BBB) by Pb. However, the reports on the effects of Pb on the integrity of BBB are conflicting. Some investigators have reported no effect of Pb on BBB [30, 31], while others have reported significant disruption of BBB by Pb [37, 41]. It has also been postulated that Pb might be transported into the brain by the divalent metal ion transporter 1 (DMT1), which is localized in astrocytes and neurons in the normal brain [18, 34, 42, 43].

In our experimental model, Pb substantially increased phosphorylation of tau at Ser199/202 (AT8 epitope), Thr212/Ser214 (AT100 epitope) and Thr231 (AT180 epitope). We have previously reported a similar hyperphosphorylation of tau at Ser199/202 and Thr231 in cultured human neurons treated with Pb [36]. Increased phosphorylation of tau at Ser404 has also been reported in the hippocampus of Pb-exposed mouse pups [26]. Our current findings in the rat model, therefore, suggest that the effect of Pb on tau phosphorylation is not species-specific.

Tau can be phosphorylated at multiple Ser and Thr residues. At least 30 Ser/Thr phosphorylation sites have been identified in tau that has been shown to be hyperphosphorylated in different taupathies [12]. Hyperphosphorylated tau is assembled into paired helical filaments (PHF) of neurofibrillary tangles [14, 15] and results in the disruption and disassembly of microtubule and subsequently in memory loss and neuronal death [1, 12, 19]. The disruption of microtubules in neurons by the hyperphosphorylated tau is one of the fundamental aspects of dementia in Alzheimer's disease and other tauopathies [14, 15, 39].

The phosphorylation of different Ser/Thr residues of tau exerts different effects on tau function with respect to microtubule stability. For example, phosphorylation of tau at Ser199, Ser202, Thr205, Thr212, Ser235, Ser262 and Ser404 is required for the sequestration of normal tau, and the further phosphorylation at Thr181, Thr217, Thr231 and Ser396 is needed for self-assembly [1]. Tau sites that are apparently hyperphosphorylated by Pb-exposure in this study are all associated with the gain of toxic function of tau and thus can potentially be implicated in Pb-induced neurotoxicity. Whether other Ser or Thr residues that are usually phosphorylated in taupathies are also phosphorylated by Pb exposure remains to be investigated.

The role of tau phosphorylation in Pb-induced deficits in learning in young children is still unknown. However, several studies have shown that phosphorylated tau is responsible for memory deficits in many models of tauopathies (other than Alzheimer's disease) including Drosophila [24], mouse [6] and rat [5]. Furthermore, the accumulation of NFT, similar to the ones seen in Alzheimer's disease, in the brain has been reported from a human patient who suffered from severe Pb encephalopathy at 2 years of age and died of severe mental deterioration at the age of 42 [33]. Based on these reports it would not be surprising if such a mechanism also affects young children exposed to Pb.

Tau can be hyperphosphorylated by either an increase in the activities of Ser/Thr kinases or reduced activity of Ser/Thr protein phosphatases. It has been suggested that the hyperphosphorylation of tau in Alzheimer's disease is mainly the result of reduction in phosphatases rather than increase in protein kinases [4, 11, 28]. Impairment of protein phosphatases not only directly affects the dephosphorylation of tau but may also up-regulate the activity of some protein kinases, which are phosphorylation dependent [21, 35, 45]. The major Ser/Thr phosphatases in the brain are PP1, PP2A and PP2B [27]. PP2A, PP1, PP5 and PP2B account for ~71%, ~11%, ~10% and ~7%, respectively, of the total tau phosphatase activity of human brain. PP1 and PP2A together account for over 90% of the total mammalian brain protein phosphatase activity and any alteration in the expression of these two phosphatases may significantly affect the phosphorylation status of tau [28].

The major Ser/Thr protein kinases that have been reported to be mainly responsible for tau phosphorylation are glycogen synthase kinase (GSK-3β), cyclin-dependent kinase 5 (cdk5), cAMP-dependent protein kinase (PKA), and calcium-calmodulin-dependent kinase II (CaMK-II) [12]. Whether Pb-induced tau phosphorylation involves over-activation or over-expression of any of these kinases has poorly been investigated. In one study no effect of Pb on the activity of PKA was found [9].

Conflicting reports exist in the literature on the effect of Pb on CaMK-II. Toscano et al. [40] reported a decrease in the activity of CaMK-II, no effect on the expression of CaMK-IIα, reduction in the expression of CaMK-IIβ and no effect on the phosphorylation of Thr286 of CaMK-II in rats developmentally exposed to Pb. On the other hand, Zhang et al. [44] reported a decreased in the phosphorylation of Thr286 of CaMK-II but no effect total CaMK-II following acute exposure of rats to Pb. We focused on Ser/Thr protein phosphatases for two reasons: First, none of the kinases have been consistently shown to be activated in Alzheimer's disease which is characterized by abnormal hyperphosphorylation of tau, and the hyperphosphoryaltion of tau is mainly believed to be due to decreased activity of phosphatases [12]. Second, Ser/Thr protein phosphatases have been directly implicated in the learning and memory impairment [10, 13, 16, 22, 23].

As a mechanism of Pb-induced tau hyperphosphorylation, we measured the expression level of PP1, PP2A, PP2B and PP5 in the brain of Pb-exposed rats. We observed a significant reduction in the expression of PP2A. These data suggest that the observed increase in tau phosphorylation in the brain is due to a decrease in the expression of PP2A, which is the major tau phosphatase. The expression of PP1 and PP5, on the other hand, was significantly increased. This increased expression of PP1 and PP5 could be a compensatory mechanism for the significant decrease in PP2A expression. Similar decrease in the expression of PP2A and increase in the expression of PP1 has been reported by us previously in cultured human neurons exposed to Pb [36]. Our current *in vivo* results are, however, discordant with the *in vitro* results in the expression of PP2B and PP5. In the cultured human neurons Pb-exposure resulted in increased expression of PP2B and decreased expression of PP5. These differences could be explained by the fact that the *in vitro* environment is very distinct from the *in vivo* environment. Unlike the population of almost pure neurons in culture, brain has a number of support cells like astrocytes, and microglia, which can respond differently to Pb insult. A number of regulatory mechanisms exist in the brain which may be lacking in the pure culture of neurons.

The immunoblot with anti-calcineurin antibody (anti-PP2B) produced two bands. Similar double bands of PP2B have previously been reported by us from cultured human neurons [36]. The upper band (at 61 kD) which corresponds to the catalytic subunit of PP2B was quantitated and reported in this study. There was a non-significant 20% increase in the expression of this band in Pb-exposed rats. The lower band at ~57 kD appears to correspond to the truncated product of PP2B, which is produced by removing the autoinhibitory domain from the C-terminal end of calcineurin. This truncated product has been reported to maintain calcium/calmodulin dependence for phosphatase activity and has higher phosphatase activity than the full-length calcineurin [29]. Quantitation of this band produced results similar to the upper band (20% non-significant increase).

Increased expression of PP1 in Pb-exposed rats has also implication in learning and memory deficits. PP1 is a suppressor of learning and memory [10, 13, 16, 22, 23]. However, the role of PP5 in modulating learning and memory is not known. These results suggest two possible mechanisms for Pb-induced impairment of learning and

memory. First, Pb-induced overexpression of PP1 may directly impair learning and memory. Second, Pb-induced hyperphosphorylation of tau may result in cytoskeletal disruption in neurons and the subsequent loss of learning and memory.

In summary, we report here that early postnatal exposure of rats to Pb induces tau hyperphosphorylation at several serine and threonine residues. This tau hyperphosphorylation appears to be due to a decrease in the expression of PP2A. Hyperphosphorylation of tau (and the subsequent disruption of microtubule structure) may be one of the mechanisms of Pb-induced deficits in learning and memory.

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