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Lead-induced Alterations of Apoptosis and Neurotrophic Factor mRNA in the Developing Rat Cortex, Hippocampus, and Cerebellum

Shirley L. Chao[1], **Jason M. Moss**[2], and **G. Jean Harry**[2]

1*Department of Natural Sciences, Fayetteville State University, Fayetteville, NC 28301*

2*Laboratory of Neurobiology, NIEHS, RTP, NC 27709*

Abstract

Previous reports have recently shown the prototypic neurotoxicant, lead, to induce apoptosis in the brains of developing organisms. In the current study, timed-pregnant rats were exposed to lead acetate (0.2% in the drinking water) 24 hrs following birth at postnatal day 1 (PND 1). Dams and pups were continuously exposed to lead through the drinking water of the dam until PND 20. Postnatal exposure in the pups resulted in altered mRNA levels of the following apoptotic and neurotrophic factors: caspase 2 and 3, bax, bcl-x, and brain-derived neurotrophic factor (BDNF). Ribonuclease protection assays were conducted to measure the factors simultaneously at the following postnatal time points: 9, 12, 15, 20, and 25 days. Our results suggest a brain region- and time-specific response following lead acetate exposure. The region most vulnerable to alterations occurs in the hippocampus with alterations beginning at PND 12 in which caspase 3, bcl-x, and BDNF increase with lead exposure. Significant treatment effects were not observed for both the cortex and cerebellum.

Keywords

lead acetate; apoptosis; neurotrophic factor; cortex; hippocampus; cerebellum; caspase 2; caspase 3; bax; bcl-x; bdnf

INTRODUCTION

The prototypic neurotoxicant, lead acetate, has long been recognized to exert neuronal damage in several regional brain sites that are associated with functional manifestations of lead neurotoxicity [1,2]. While prenatal exposure to lead contribute to lifetime exposure, evidence points to postnatal exposure in which the predominant source of children's body lead burden is from lead-contaminated dust, soil, and water during the first five years of life. [3]. One specific morphological outcome of low level lead neurotoxicity is an alteration in the normal dendritic field and a decrease in synaptic density and development [1,4,5,6]. This may reflect either a direct action on dendritic arborization and synaptic connectivity or a secondary effect due to loss of target sites or trophic factor influences.

The final outcome of brain development is dependent upon a critical sequence of events that occur under strict temporal and spatial regulation. While the process of apoptosis is one of such events that occur throughout development the consequence of such events is also dependent upon trophic factors such as NGF, BDNF, and NT3 for the maturation and survival of neuronal

Correspondence to: Dr. Shirley L. Chao, Department of Natural Sciences, Fayetteville State University, 1200 Murchison Rd., Fayetteville, NC 28301, 910-672-1427 phone, 910-672-4599 fax, E-mail: schao@uncfsu.edu.

populations [7]. The pivotal role of neurotrophic factors in neuronal survival has been demonstrated in mice deficient for trk B and C receptors with affinity for brain-derived neurotrophic factor (BDNF). While these mice are viable at birth, massive neuronal cell death occurs during postnatal development in hippocampal and cerebellar granule neurons [8]. Apoptotic cell death is determined by the balance between the expressions of pro-and anti-apoptotic genes [9]. The perturbation of this balance results in activation of the caspase family, specifically caspase 3, and the most frequently involved in apoptosis [9].

Recently, lead has been shown to induce apoptosis during development [10,11,12]. Evidence for lead-induced apoptosis was provided in rats where six weeks of lead exposure produced retinal degeneration characterized by morphological features of apoptosis in both rods and bipolar cells [11]. The rate of apoptosis occurring in the hippocampus following lead acetate exposure is increased in rats and this morphological demonstration occurs in conjunction with an increase in protein for the mitochondrial apoptotic factor bax [12]. Lead -induced cell death has been demonstrated in the hippocampus in vivo in which 2-4 week old rats had higher expression of Bax proteins compared to 12-14 week old rats [12]. This indicated a higher sensitivity in younger animals with more apoptotic cells. Similar observations were made in which Bax expression was also measured in the cortex and cerebellum on neonatal rats and downregulated in adults [13].

The current study examined alteration in the developmental profile of mRNA levels for various apoptotic factors and neurotrophins developing in rats exposed to lead acetate (0.2% in drinking water) during lactation via nursing dams. Each brain region showed a distinct developmental profile for bcl-x, BDNF, bax, caspase 2, and caspase 3 mRNA levels and the effects of lead exposure were also region specific. A delay in the normal developmental pattern was seen in the cortex. Elevated levels of BDNF and bcl-xL mRNA were seen in the hippocampus. And lead exposure resulted in slightly higher levels of bax, and caspase 3 mRNA in the developing cerebellum. In summary, our study demonstrates altered developmental patterns for vital pro-and anti- apoptotic factors for the hippocampus, cortex, and cerebellum following lead acetate exposure during lactation in rats from birth to 20 days.

MATERIALS AND METHODS

Animals

Timed-pregnant Long Evans hooded rats (Charles River Laboratories; Portage, MI) were individually housed in polyethylene cages in rooms maintained on a 12:12 hour photoperiod, L:D (0700: 1900), temperature (70E± 2E F), and humidity (50% ± 5%). Twenty-four hours following birth, postnatal day 1 (PND 1), all pups were pooled and new litters consisting of eight pups, a minimum of 7 males, were randomly selected and placed with each dam. Dams and litters were randomly assigned to experimental exposure conditions. Pb-exposure was initiated on PND1 with the addition of Pb acetate (0.2%, Sigma Chemical Co., St. Louis, MO) to the deionized boiled drinking water of the dam and continued until PND 20. Control animals received deionized boiled drinking water. This exposure equated to an average daily dose to the dam of 23 mg/kg body weight. This exposure level was chosen based upon previous studies demonstrating alterations in neuronal arborization, astrocyte structures, and associated developmentally regulated genes in the cortex, hippocampus, and cerebellum (14, 15). This exposure did not produce signs of maternal toxicity; growth and weight gain of pups were within normal range and no differences were seen in regional brain weights. Food and water were available *ad libitum* throughout the study.

Male pups within each litter were randomly assigned for examination at PND 9, 12, 15, 20, or 25. At the removal of a pup from each litter, a filler pup was added to maintain a litter size of eight throughout the study. At each time point, animals were lightly anesthetized with CO_2,

decapitated, brain excised, and the cerebellum, hippocampus, and neocortical tissue were dissected and stored at -80EC. All experiments were conducted in compliance with animal protocols approved by the NIEHS/NIH Animal Care and Use Committee.

Lead Determination

On PND 15 and 20, one previously randomly selected male pup per litter was used to determine lead levels in the cortex, hippocampus, and cerebellum. At each time, animals were deeply anesthetized with pentobarbitol and perfused with sterile saline via cardiac puncture; the brain excised, brain regions dissected using EDTA rinsed teflon coated forceps. Each standard and sample was analyzed with an atomic absorption spectroscopy graphite furnace (Perkin-Elmer 3030: wavelength 283.3 nm; argon flow, atomization at 2300°C). Each sample was sequentially wet ashed by microwave in nitric acid followed by 30% hydrogen peroxide, filtered through Whatman 541 filters, brought to a final volume of 15 ml with deionized water and a 20ul aliquot was used to determine lead concentration. The concentration of lead in each sample was calculated by the analysis of the calibration standards. The mean value of three absorbance readings for the standards and the mean value of two absorbance readings for each sample were used to calculate the concentration of lead. Based on the addition of external lead standards to the samples at various stages of the assay, overall lead recoveries from the samples averaged 104% (83 - 125%). Method spike recoveries ranged from 90.6 to 105%. (Radian Corp., Research Triangle Park, NC). Trunk blood was collected following decapitation for determination of blood lead levels at PND 20. The lower limit of detection was 2 ug Pb/dl blood.

Ribonuclease Protection Assay (RPA)

Total RNA was isolated from cortical, hippocampal, or cerebellar tissue from individual animals by the Trizol method (Gibco BRL, Frederick, MD). Ten μg aliquots from each sample were subjected to ribonuclease protection assays using commercially available probe sets for brain specific growth factors and apoptosis factors (Pharmingen; San Diego, CA). The first set contained probes for Fas Ligand Receptor, bcl-x, Fas ligand, caspase 1, caspase 3, caspase 2, bax, and bcl-2 (rAPO-1). The second set contained probes for IL-1α, bcl-x, BDNF, caspase 3, caspase 2, bax, and TNFα. Each set contained probes for two housekeeping gene products, ribosomal fraction (L32) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A 1 μl aliquot of an equimolar pool of plasmid templates was used for the synthesis of ^{32}[P]-labeled cRNA probe set using a T7 RNA polymerase. Briefly, a 10 μg aliquot of total RNA was hybridized overnight at 56°C with 8 μl of hybridization buffer and 4×10^5 cpm of ^{32}[P] labeled probe set. Single-stranded RNA was digested with Ribonuclease (1:100; Promega, Madison, WI) in 10 mM Tris, 300 mM MgCl, and 5 mM EDTA, pH 7.5. The protected fragments were extracted and precipitated and separated by gel electrophoresis on a 5% acrylamide/8M urea sequencing gel. Radioactivity in each fragment was visualized by autoradiography and quantitation of radioactivity in each fragment was conducted by phosphorimaging with the relation volume of each determined using Imagequant (Molecular Dynamics, Sunnyvale, CA) with the addition of a background subtraction. No significant differences as a function of age or exposure were demonstrated for either L32 or GAPDH. Given the potential for biological variance due to the developmental process, each individual RPA gel represented 3 full replicates of control and lead dosed samples over the developmental period. Thus, each brain region was analyzed on a minimum of 2 RPA gels.

Statistical Analysis

The response of each mRNA was calculated relative to corresponding L32 mRNA level for 5-6 animals per group. Litter was the unit of measure and the minimum sample size for each age and treatment was 5. The experimental design for the mRNA levels was a randomized

block design, with dams as blocks, and age (5 levels) and treatment (2 levels) as main factors in the design. For each brain region, data for mRNA transcripts were analyzed by an analysis of variance (ANOVA). Given the high degree of variability that can be inherent to developmental ontogenic data, RPA data for each specific mRNA transcript relative to L32 were logarithmically transformed and analyzed by ANOVA to assess main effect of age and treatment and interactions between these factors. Following determinations of significant main effects for each region ($p<0.05$), multiple comparisons by a Fisher's LSD procedure were performed to determine significant effects for each transcript in a given region. All data are reported as mean ± SEM. Brain and blood lead levels were analyzed by an ANOVA followed by a Fisher's LSD. In all cases the level of statistical significance was set at $p<0.05$.

RESULTS

Brain and Blood Lead Levels

At PND 15 and 20, mean lead levels for control tissues were 0.10 ± 0.01μg/g brain tissue in all brain regions examined. The mean lead levels (μg/g brain tissue) for lead-exposed tissue at PND 15 consisted of the following: cortex, 0.16 ± 0.05; hippocampus, 0.30 ± 0.06; cerebellum, 0.28 ± 0.05 with elevations in both the hippocampus and the cerebellum reaching statistical significance. At PND 20 lead levels were more consistent across brain regions and consisted of the following: cortex, 0.22 ± 0.04; hippocampus, 0.31 ± 0.17, cerebellum, 0.25 ± 0.06. All regions contained significantly ($p<0.05$) higher mean lead levels in lead-exposed animals at this time-point compared to controls as determined by Fisher LSD following an ANOVA. At PND 20 blood lead levels in the exposure groups were significantly elevated compared to controls with controls measuring at <10μg/dl compared to lead exposed groups measuring at 80 ± 20 μg/dl.

Cortex

While the data was highly variable, a pattern of mRNA for caspase 2, caspase 3, and bax was evident in the cortex during normal postnatal development suggesting a dynamic process occurring around PND 15. The pattern was similar for each transcript in control animals with a transient increase in levels at PND 15 returning to a lower level of expression after weaning at PND 25 (Figure 1). A similar transient increase was evident in mRNA levels for bcl-x and BDNF in control animals also increased at PND 15 (Figure 2). With lead exposure, the general pattern suggested that the elevation at PND 15 seen in the controls was delayed with any indication of an elevation not occurring until PND 20. There were no overall significant treatment effects however; significant effects were observed for the main effect of PND for bcl-x ($F=3.173$, $df=4$) and BDNF ($F=6.616$, $df=4$). The mRNA levels for Fas Ligand, Fas Ligand Receptor, and caspase 1 were not detected in the cortex.

Hippocampus

No distinct pattern of mRNA levels over time or significant differences between treatment groups were observed for caspase 2 or bax. For caspase 3 mRNA levels, overall main effects for treatment were observed ($F=8.125$, $df=1$). In both the control and lead exposed groups, the profile for caspase 3 mRNA for controls showed a gradual decrease from PND 9 to PND 25 (Figure 3). In the lead exposed animals, the transient elevation in mRNA levels for caspase 3 at PND 12 was significantly higher ($p<0.05$) as compared to the decreasing levels observed in the controls (Figure 3).

In control animals, the general pattern for both bcl-x and BDNF displayed a relatively uniform level of transcript expression over the postnatal time period examined (Figure 4).

For bcl-x significant main effects were observed for PND ($F=4.463$, $df=4$) and treatment ($F=30.541$, $df=1$). For BDNF, significant main effects were observed for PND ($F=7.931$, $df=4$) and treatment ($F=9.584$, $df=1$). As compared to controls, mRNA levels in lead exposed animals for BDNF were significantly elevated (Fisher's LSD, $p<0.05$) at PND 12. Fas Ligand, Fas Ligand Receptor, and caspase 1 mRNA transcripts were not detected in the hippocampus.

Cerebellum

In general, data acquired from the cerebellum showed less variability than that seen in the cortex as would be expected given the defined postnatal development of this specific brain region. In the control cerebellum, the mRNA levels for caspase 2, caspase 3, and bax did not display an ontogenic pattern but were rather consistent from PND12 - PND 25 (Figure 5). The mRNA levels for bcl-x displayed a slight elevation with age however, these changes failed to reach statistical significance and no differences were evident as a function of lead exposure. BDNF mRNA levels were increased at PND 20 and 25 as compared to levels at PND 9 with no differences evident between the control and lead exposed animals (Figure 6). Fas Ligand, Fas Ligand Receptor, and caspase 1 mRNA transcripts were not detected in the cerebellum.

DISCUSSION

Data from the current study suggest that developmental exposure to lead acetate results in the activation of several molecular processes in various brain regions possibly to retain a balance for proper development. Previous studies have already established cells undergoing apoptosis following lead exposure in the hippocampus and cerebellum [10,12]; however, specific pro- and anti-apoptotic factors involved have not been well studied. Our results demonstrate that certain essential factors in neurodevelopment are altered following lead acetate exposure. These key factors include apoptotic factors such as caspase 3, bcl-x, and a neurotrophin, BDNF and that the changes may be subtle and differentially involve brain regions.

Cell death during development can be influenced by several factors during critical stages of neuronal maturation: decrease of anti-apoptotic or neurotrophic factors; loss of afferent/efferent connections; and altered energy metabolism and membrane integrity due to mitochondrial dysfunction. Our study demonstrates a delay in the normal upregulation of both apoptotic (pro- and anti-) factors and BDNF in the cortex following lead exposure although significance was not reached for pro-apoptotic factors. Similarly the cerebellum did not exhibit significant changes in both pro- and anti-apoptotic factors following lead exposure as indicated by the high variability in the data. However, in the hippocampus the anti-apoptotic factor bcl-x and BDNF mRNA were significantly increased after increases of the pro-apoptotic factor, caspase 3, following lead exposure. We believe that the initial increase of caspase 3 at PND 12 in the hippocampus activated a compensatory response by increasing survival factors, bcl-x and BDNF mRNA from PND 12 until weaning. This finding agrees with a previous report of BDNF supporting post-mitotic neurons in the dentate gyrus [8]. In fact, the rat dentate gyrus is unique in that most of its neurons are born postnatally [16] and that substantial amounts of pyknotic cells are present throughout the dentate gyrus during the first postnatal week. Furthermore, our results agree with previous reports of apoptosis of cells in the hippocampus of 2-4 week old rats exposed to lead [12]. Therefore, the significant increase in factors regulating survival such as bcl-x and BDNF would indicate the system's need to balance any perturbations produced by lead.

With respect to the loss of afferent/efferent connections initiating apoptosis, lead acetate has been shown to reduce dendritic branching and decrease synaptic density and development [17]. Thus the absence of synaptic or dendritic target sites could also reflect the delay in the upregulation of survival factors, bcl-x and BDNF needed at PND 15 following peak level of apoptosis reported during the first postnatal week [18]. Similarly, lead acetate has been shown

to alter neurotransmitter systems [19,20] and calcium channels [21]; both of which are influenced by each other and by the presence of neurotrophins [22].

In PC12 cells, lead has been shown to induce apoptosis with accompanying increases in bax and caspase 3 [23]. Normal peak levels of apoptosis have been previously shown in the external granule layer of the cerebellum at PND 9 [24] and lead-induced apoptosis has also been demonstrated in cultured cerebellar granule cells [10]; however, while our data clearly demonstrated changes in the hippocampus, significant increases in apoptotic-related factors such as bax and caspase were not detected in the cerebellum as a result of developmental lead exposure. Nonetheless, the body of existing data suggests the need for further examination at the age of weaning for any changes related to apoptosis within this region.

Despite reductions in exposure, lead remains a dangerous environmental agent for childhood exposure. While lead exposure has been associated with many changes in neurochemical processes, altered brain morphometrics, and deficits in learning and memory, there is no general agreement for a mechanism of action underlying the behavioral and cognitive dysfunction observed. Our results indicate a specific response in the hippocampus in which the mRNA levels for caspase 3 were significantly increased followed by increases in bcl-x and BDNF mRNA after lead exposure possibly as a mechanism to compensate for the caspase activation. If an alteration in the general apoptotic process in the developing nervous system or an induction of such processes underlies many of the effects of developmental lead neurotoxicity, gaining an understanding of the timing of these effects will be a critical component to understanding the specific developmental effects of lead exposure. Understanding the mechanisms responsible for lead-induced apoptosis is complicated by the fact that there are numerous players in the possible cascade of events leading to neuronal apoptosis and that apoptosis is a phenomenon regulated by pro- and anti-apoptotic genes.

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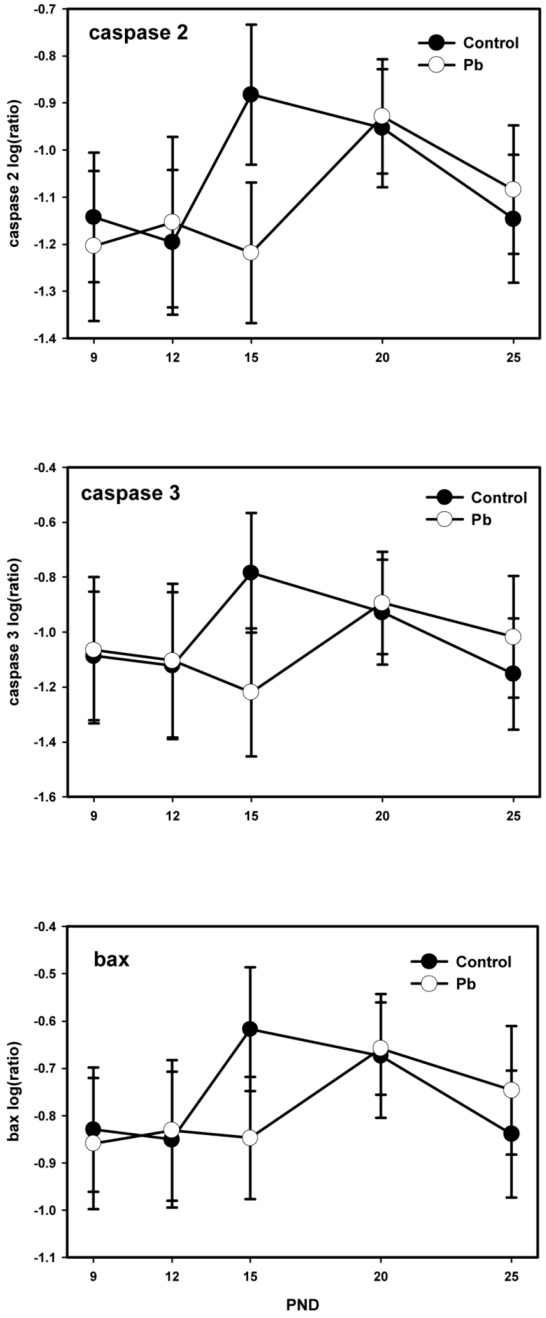

Figure 1.

Profile of mRNA levels of caspase 2, caspase 3, and bax in the cortex following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls, were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6).

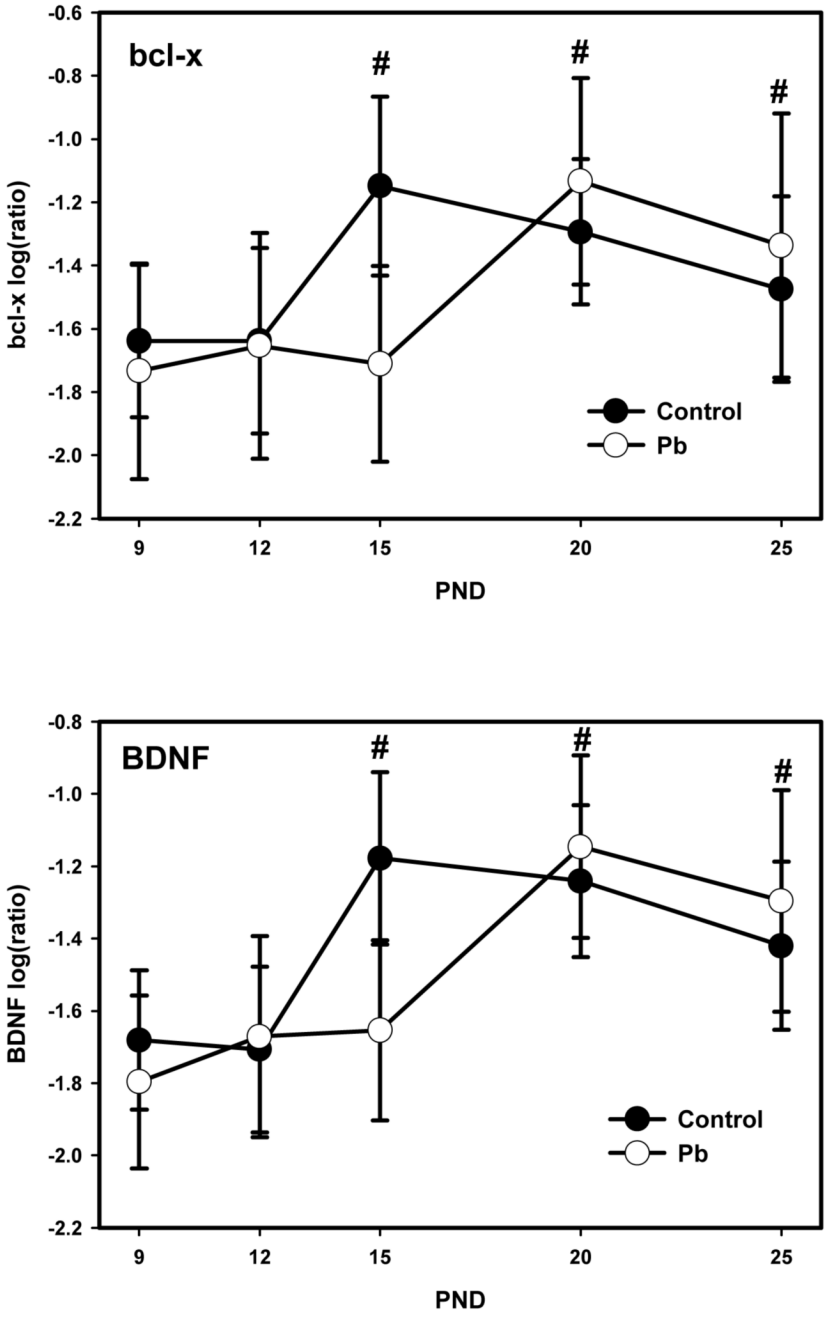

Figure 2.

Profile of mRNA levels of bcl-x and BDNF in the cortex following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6) (# denotes $p<0.05$ as determined by Fisher's LSD post-hoc analysis following ANOVA significant main effect of PND (for bcl-x ($F=3.173$, $df=4$) and BDNF ($F=6.616$, $df=4$).

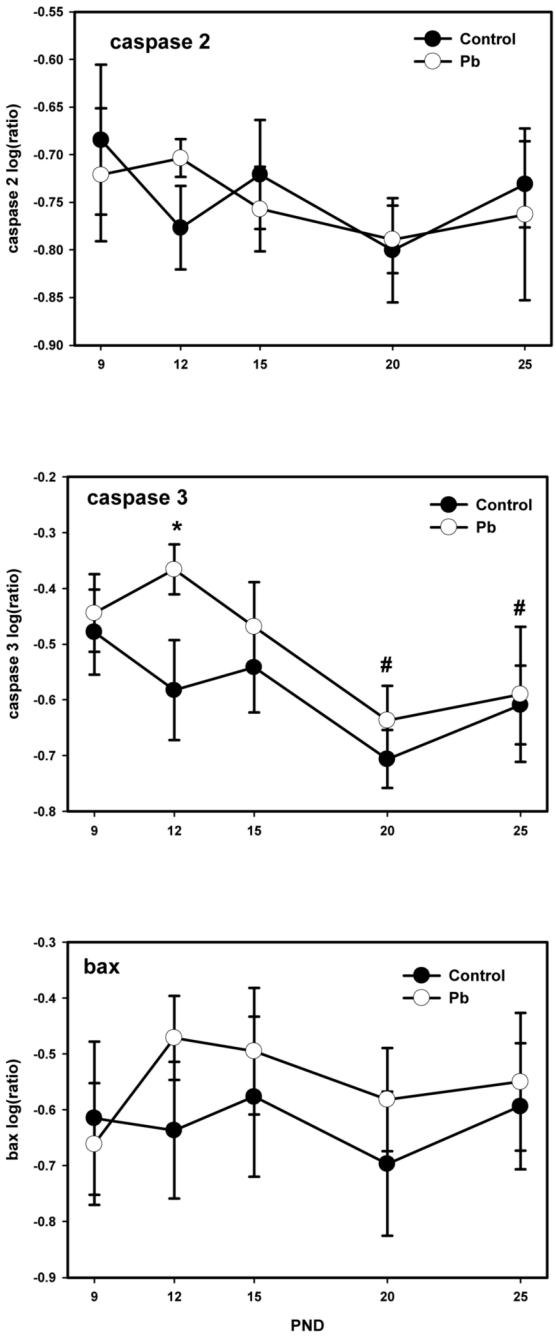

Figure 3.

Profile of mRNA levels of caspase 2, caspase 3, and bax in the hippocampus following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6). Asterisk (*) and number sign (#) denote significance at $p<0.05$ based on Fisher's LSD post-hoc analysis following ANOVA significant main effect of treatment ($F=8.125$, $df=1$) and PND ($F=3.858$, $df=4$). For the main effect of PND, PND 20 and PND 25 were significantly decreased compared to other time points.

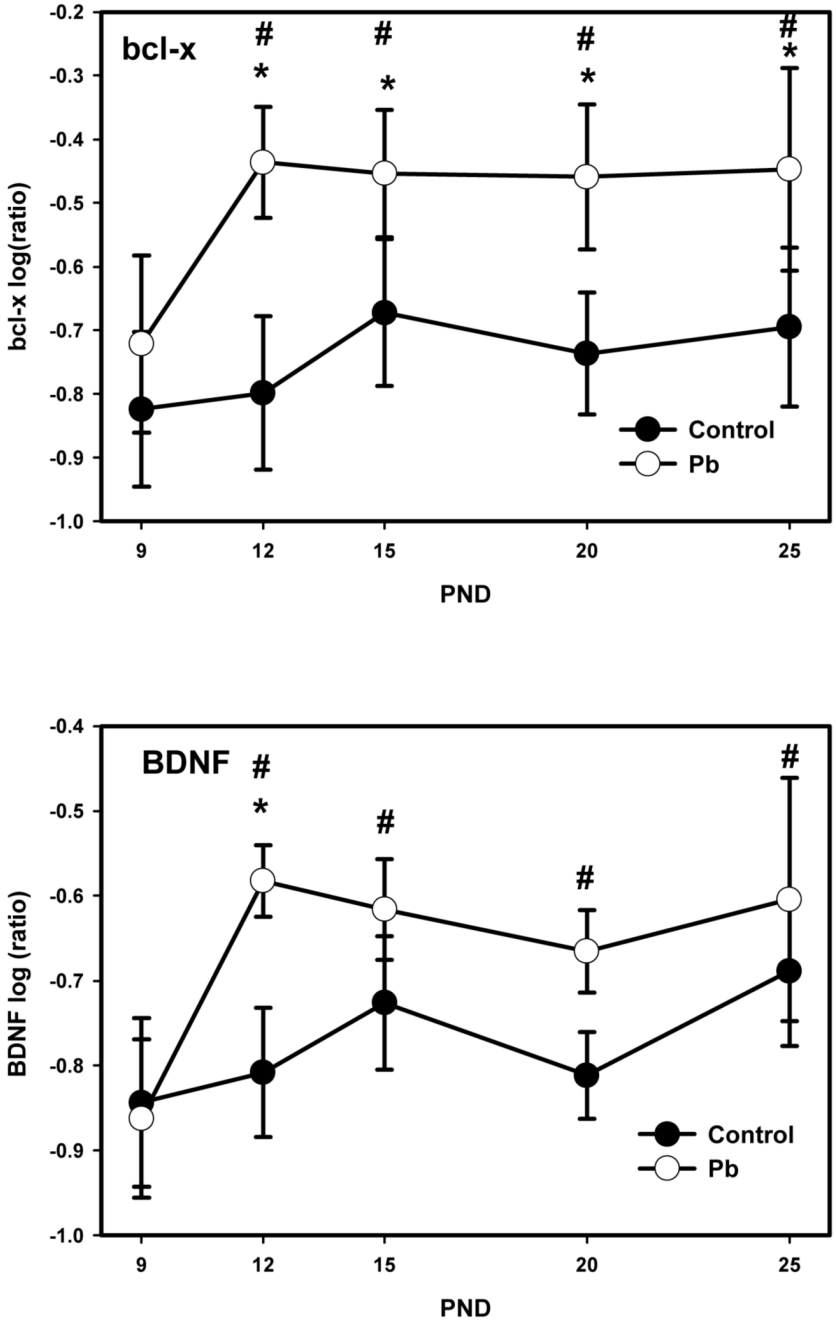

Figure 4.

Profile of mRNA levels of bcl-x and BDNF in the hippocampus following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6). Asterisk (*) and number sign (#) denote significance at $p<0.05$ based on Fisher's LSD following ANOVA main effect of treatment (for bcl-x $F=30.541$, $df=1$; for BDNF $F=9.584$, $df=1$) and PND (for bcl-x $F=4.463$, $df=4$; for BDNF $F=7.931$, $df=4$) respectively. For the main effect of PND, PND 9 was significantly lower than any other timepoint for both bcl-x and BDNF.

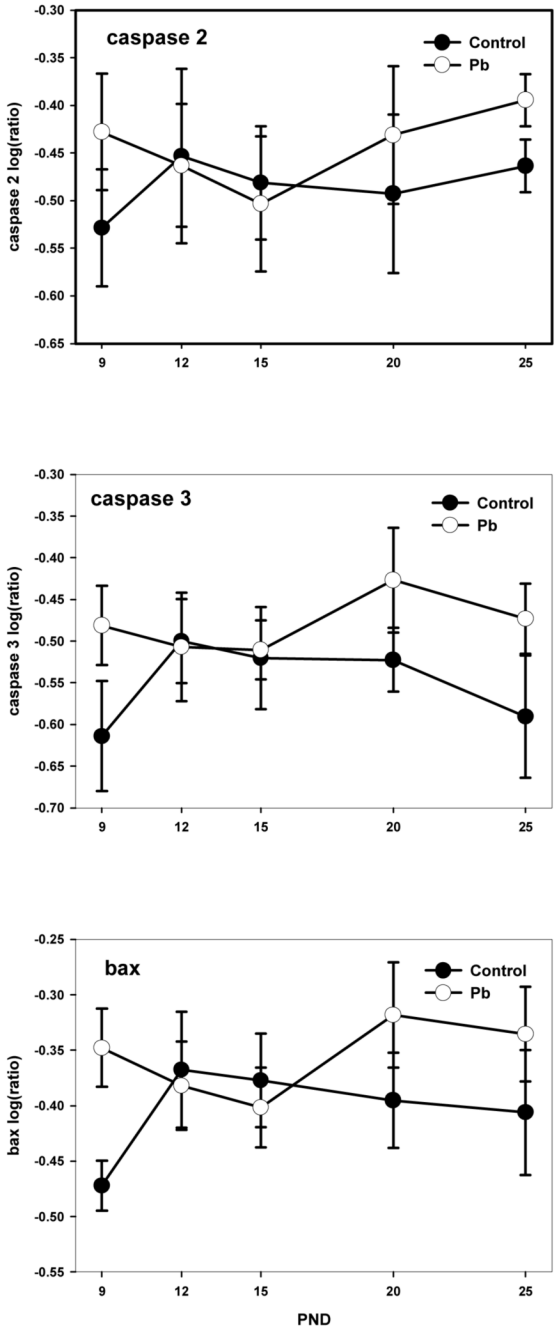

Figure 5.
Profile of mRNA levels of caspase 2, caspase 3, and bax in the cerebellum following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls, were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6).

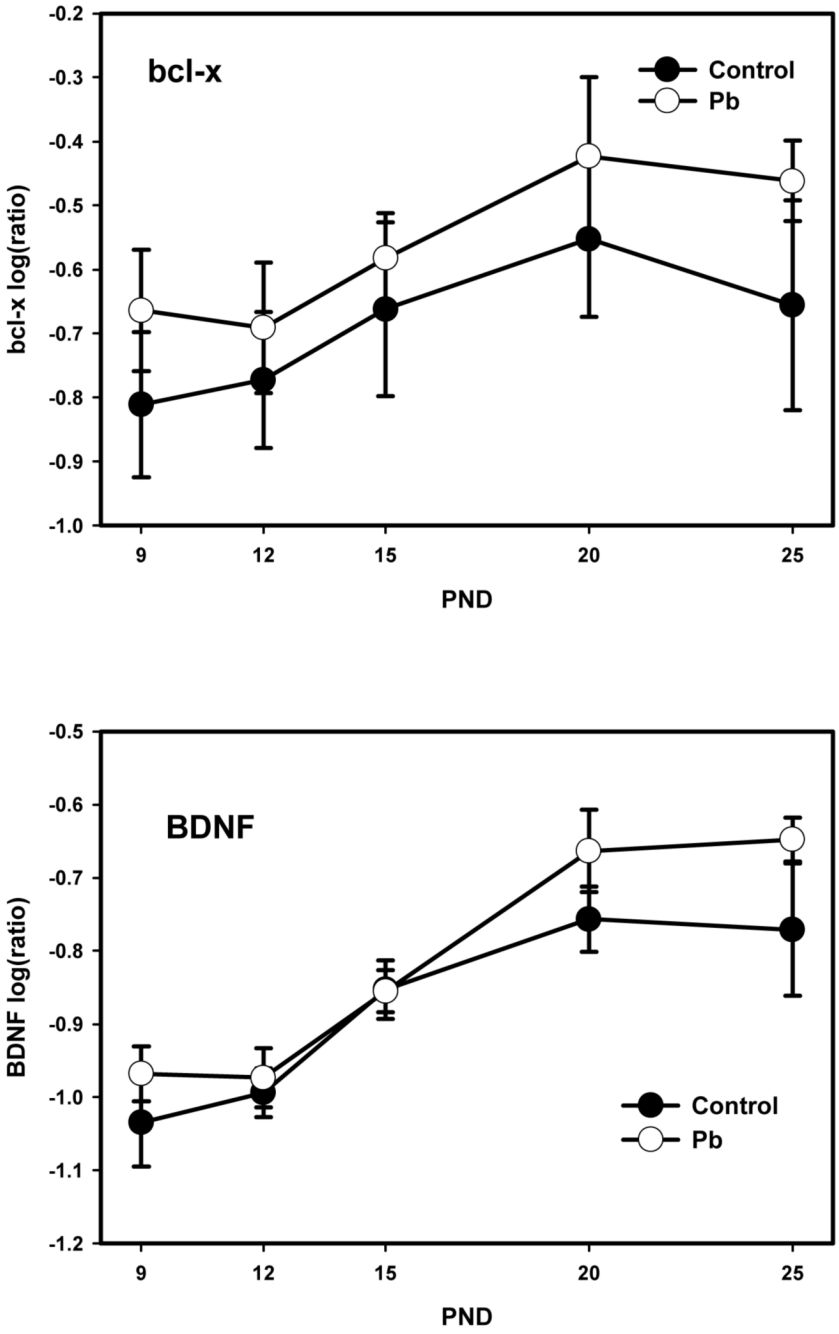

Figure 6.

Profile of mRNA levels of bcl-x and BDNF in the cerebellum following postnatal lead acetate exposure (0.2% in the drinking water). Samples from 5-6 individual animals per group, both Pb exposed and controls, were examined at postnatal day (PND) 9, 12, 15, 20, and 25. Data are expressed as means ± SEM (n=5-6).