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Chronic low-level Pb exposure during development decreases the expression of the voltage dependent anion channel in auditory neurons of the brainstem

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Abstract

Lead (Pb) exposure is a risk factor for neurological dysfunction. How Pb produces these behavioral deficits is unknown, but Pb exposure during development is associated with auditory temporal processing deficits in both humans and animals. Pb disrupts cellular energy metabolism and efficient energy production is crucial for auditory neurons to maintain high rates of synaptic activity. The voltage dependent anion channel (VDAC) is involved in the regulation of mitochondrial physiology and is a critical component in controlling mitochondrial energy production. We have previously demonstrated that VDAC is an in-vitro target for Pb, therefore, VDAC may represent a potential target for Pb in the auditory system. In order to determine whether Pb alters VDAC expression in central auditory neurons, CBA/CaJ mice (n=3–5/group) were exposed to 0.01 mM, or 0.1 mM Pb acetate during development via drinking water. At P21, immunohistochemistry reveals a significant decrease for VDAC in neurons of the Medial Nucleus of the Trapezoid Body. Western blot analysis confirms that Pb results in a significant decrease for VDAC. Decreases in VDAC expression could lead to an up-regulation of other cellular energy producing systems as a compensatory mechanism, and a Pb-induced increase in brain type creatine kinase is observed in auditory regions of the brainstem. In addition, comparative proteomic analysis shows that several proteins of the glycolytic pathway, the phosphocreatine circuit, and oxidative phosphorylation are also upregulated in response to developmental Pb exposure. Thus, Pb-induced decreases in VDAC could have a significant effect on the function of auditory neurons.

Keywords

Lead; voltage dependent anion channel; superior olivary complex; Medial nucleus of the trapezoid body; brain type creatine kinase; proteomics

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1. Introduction

Lead (Pb) is a naturally occurring toxic heavy metal that has been widely distributed throughout the environment due to its extensive use in a variety of industrial procedures and products. Environmental Pb enters biological systems through ingestion and respiration (Toscano and Guilarte, 2005) and continues to be a serious problem in many parts of the U.S. The U.S. Centers for Disease Control and Prevention have determined that blood Pb levels of 10 µg/dL should prompt public health actions, however recent studies in humans and animals have shown that the neurotoxic effects of Pb occurs at even lower blood Pb levels (Gilbert and Weiss, 2006). Low-level Pb exposure is a risk factor for learning disabilities and attention deficit hyperactivity disorder (ADHD) (Lidsky and Schneider, 2003; Braun et al., 2006). Many children with these behavioral syndromes also demonstrate deficits in auditory temporal processing, suggesting a disturbing link between developmental Pb exposure, behavioral dysfunction and auditory temporal processing (Gray, 1999; Otto and Fox, 1993; Lurie et al., 2006; Breier et al., 2003; Montgomery et al., 2005).

Auditory temporal processing involves the processing of central auditory neuronal signals in time and space, allowing the listener to resolve complex sounds and to recognize specific signals within a noise background. Children exposed to Pb show decreased performance in tests requiring appropriately timed reactions and demonstrate increased latencies in brainstem auditory evoked potentials (Finkelstein et al., 1998; Holdstein et al., 1986). In animals, chickens exposed to low-levels of Pb show deficits in backward masking, a test of central auditory temporal processing (Gray, 1999). We have found that mice exposed to low levels of Pb demonstrate alterations of two measures of central auditory brainstem function, the brainstem conduction time and gap encoding in the inferior colliculus (Jones et al., 2008). Taken together, these studies suggest that the auditory system is a target for Pb.

We have recently demonstrated that the voltage-dependent anion channel (VDAC) is a novel target for Pb in CNS neurons in vitro (Prins et al., 2010). VDAC is an ion channel located in the mitochondrial outer membrane that plays a central role in regulating energy metabolism in neurons by maintaining cellular ATP levels and regulating calcium buffering (Shoshan-Barmatz et al., 2006; Shoshan-Barmatz and Gincel, 2003). In vitro exposure to lower levels of Pb results in decreased VDAC transcription and expression in two different neuronal cell lines (Prins, et al., 2010). Further, the decrease in VDAC is correlated with a decrease in cellular ATP levels, suggesting a connection between decreased VDAC expression and decreased cellular ATP levels. It is not known whether developmental Pb exposure results in loss of VDAC protein in brainstem auditory neurons in vivo. Because auditory neurons have high and fluctuating energy requirements (Trussell, 1999; Hiel et al., 1996), a decrease in VDAC expression could alter the function of auditory neurons by disrupting energy buffering systems within these neurons.

In order to determine if VDAC represent a potential target for Pb in central auditory neurons, the current study examines the expression of VDAC in the brainstem following developmental Pb exposure. We find that chronic low-level Pb exposure during development results in the decreased expression of VDAC in the murine auditory brainstem. Immunohistochemical analysis of the auditory brainstem nucleus, the Medial Nucleus of the Trapezoid Body (MNTB), demonstrates that neurons in the MNTB show a significant decrease in VDAC staining following Pb exposure. In addition, western blot analysis of a ventral brainstem region (VBS) containing several auditory nuclei, including the MNTB, and the medial and lateral superior olivary nuclei, reveal a significant decrease in VDAC expression.

A comparative proteomic analysis of the VBS was then conducted to determine if other energy pathways, such as the glycolytic pathway, were upregulated in response to the Pb-induced

decrease in VDAC. If decreased VDAC levels result in the decreased production of ATP, then one would expect to see an increase in other cellular energy producing systems to compensate. We found that several proteins of the glycolytic pathway, the phosphocreatine circuit, and oxidative phosphorylation were upregulated in response to developmental Pb exposure.

2. Experimental Procedures

2.1. Chronic Pb exposure

Breeding pairs of CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and maintained in microisolator units in the University of Montana specific pathogen free animal facility. Cages, bedding, and food were sterilized by autoclaving and mice were handled with aseptic gloves. Mice were allowed food and water ad libitum. All animal use was in accordance with NIH and University of Montana IACUC (Institutional Animal Care and Use Committee) guidelines. Thirteen breeding pairs of CBA mice were randomly assigned to three groups having unlimited access to water (pH 3.0) containing 0 mM (control), 0.01 mM (low), or 0.1 mM (high) Pb acetate. Breeding pairs were given leaded water when they were paired so that offspring were exposed to Pb throughout gestation and through the dam's milk until postnatal day 21 (P21) when mice were sacrificed.

2.2. Blood lead levels

Blood was collected from deeply anesthetized mice by retro-orbital puncture. Blood Pb levels were measured by the Montana Health Department in Helena, MT. It should be noted that the means for the No Pb group include values of <1.0 which were included in the data set as equal to 1.0 (data not shown).

2.3. Antibodies

The polyclonal antibody against VDAC was raised by immunizing rabbits with a synthetic peptide (KLH coupled) corresponding to the amino terminus of human VDAC 1 and purified using protein A and peptide affinity chromatography (#4866, Cell Signaling Technology, Beverly, MA). VDAC detects endogenous levels of total VDAC protein that is ubiquitously expressed and located in the outer mitochondrial membrane. The staining in mouse brainstem is virtually identical to that observed in the rat cerebellum (Shoshan-Barmatz et al., 2004) and hippocampus (Jiang et al., 2007). Preadsorption with the VDAC protein (2 µg/100 µl) eliminates all immunoreactivity.

The polyclonal antibody against brain type Creatine Kinase (CKB) was raised against a KLH conjugated synthetic peptide selected within amino acid 200–300 of human CKB (ab38211, Abcam, Cambridge, MA). The CKB antibody recognizes a single band at approximately 43 kDa in our Western blots, similar to that seen in Balasubramani et al, 2006 (Balasubramani et al., 2006).

The polyclonal antibody against p44/42 MAPK(Erk1/2) was raised against a synthetic peptide (KLH-coupled) derived from a sequence in the C-terminus of rat p44 MAP kinase (Cell Signaling, Danvers, MA; product #9102). The p44/42 MAPK antibody recognizes a double band in our western blots similar to that seen in Numakawa et al, 2007 (Numakawa et al., 2007).

2.4. Immunohistochemistry

At P21, mice from the three treatment groups (n=5 to 6 per Pb treatment group) were deeply anesthetized and perfused transcardially with 4% Na-periodate-lysine-paraformaldehyde fixative (PLP, final concentrations 0.01 M sodium periodate, 0.075 M lysine, 2.1% paraformaldehyde, 0.037 M phosphate). The tissue was then processed, paraffin embedded,

and immunostained as previously described (Jones et al., 2008). For light microscopy, a “one out of six” series of 10 μ m sections of the auditory brainstem was immunostained for VDAC. The standard peroxidase anti-peroxidase procedure using the Vector ABC kit was used with appropriate secondary antibodies (Vector Laboratories, Burlingame, CA) and visualized using 3-3' diaminobenzidine (DAB, Sigma) in Tris buffer with 0.001M imidazole and 0.1% hydrogen peroxide as the chromagen. Sections were then rinsed in water, dehydrated, and coverslipped using DPX mounting media (BDH Limited, Poole U.K.). The VDAC antibody concentration used for immunohistochemistry was 1:1000 overnight at 4°C. (#4866; Cell Signaling Technology, Beverly, MA). Antibody control sections were run as described above but the VDAC antibody incubation was omitted from the procedure. Sections were also run with VDAC-specific blocking peptide (#1711B, Cell Signaling Technologies, Beverly MA) to evaluate the specificity of the antibody. Sections run with VDAC pre-incubated with blocking peptide were negative for signal (data not shown).

2.5. Tissue Analysis

VDAC stained brainstem sections from 5 to 6 mice per Pb treatment group were viewed with a Nikon Eclipse E800 microscope and a black and white Cohu (San Diego, CA) video camera connected to a PowerMac computer. Six to twelve sections per mouse were chosen from the center of each region of interest and then slides were blinded as to treatment group. Analysis of VDAC immunostaining was performed using NIH Image V1.61 as follows. Briefly, images from each region analyzed were captured at 40 $\times$. A threshold was set such that the VDAC reaction product within this area reached this threshold. Total cells in the region were counted. Cells included in the count had a well-defined cell membrane, and nucleus with a nucleolus present. All the cells in the region meeting these criteria were counted. Then, in the same area, cells that reached threshold for VDAC staining were counted. To be counted as positive, cells had to display threshold staining greater than or equal to 25% of the cell volume. A ratio of cells reaching threshold to total cells for each area measured was then calculated. Statistical significance was determined by analysis of variance followed by a Dunnett's post hoc test, $p < 0.05$. Regions analyzed for VDAC staining were the spherical cell areas of anterior ventral cochlear nucleus (AVCN), lateral superior olive (LSO), medial nucleus of the trapezoid body (MNTB), and the motor trigeminal nucleus (Mo5).

2.6. Isolation of auditory brainstem regions

The auditory region of the mouse brainstem was isolated using a 1mm mouse brain matrix. A 2mm thick section was cut and then flash frozen on a microscope slide using liquid nitrogen and dissected into three separate regions: the cochlear nucleus (CN); the ventral brainstem region (VBS), containing the superior olivary complex (SOC) which consists of three principle auditory nuclei, the lateral superior olive (LSO), medial superior olive (MSO), and the medial nucleus of the trapezoid body (MNTB); and the dorsal brainstem region (DBS) (Figure 1A). The three fractions were placed in separate microfuge tubes, flash frozen with liquid nitrogen, and stored at -80°C .

2.7. Western Blots for Individual Mice

VBS brainstem sections from each Pb treatment group ($n=4$ animals per group) were dissected from individual CBA mice as described above. For our western blot analysis, total protein from one VBS section per Pb treatment group was extracted by homogenizing in a lysis buffer containing 20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1mM Na_2EDTA , 1 mM EGTA, 1% triton $\times$, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate and 1 mM Na_3VO_4 (Cell Signaling Technology, Beverly, MA). Additions were made giving final concentrations of 0.5% Na-deoxycholate, 0.5% SDS, 1 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL benzamidine, 8 $\mu\text{g}/\text{mL}$ calpain inhibitors I and II and 1 $\mu\text{g}/\text{mL}$ each

leupeptin, pepstatin A and aprotinin. Homogenates were sonicated and vortexed for approximately 30 seconds before centrifugation at $12,000 \times g$ for 20 minutes. Protein concentrations for the whole cell lysate were quantified using the BCA Protein Assay kit (Pierce) and 20 μg of protein/sample was used for immunoblotting of brain type creatine kinase (CKB), total VDAC, and ERK. Proteins were separated by SDS-PAGE and transferred to PVDF membranes for immunoblotting. Briefly, proteins were suspended with an equal volume of Laemmli buffer, heated at 95°C for 5 min, loaded, and separated on 18-well Criterion Bis-Tris 4–12% pre-cast gels (Bio-Rad Laboratories, Hercules, CA, USA). The MagicMark XP western standard (Invitrogen Corp., Carlsbad, CA) was used to determine the approximate molecular weight of each protein. Gels were transferred to Immun-Blot PVDF membrane (Bio-Rad Laboratories, USA) using Criterion transfer cell with plate electrodes (Bio-Rad Laboratories, USA) for 60 minutes at 100 V and blocked in 5% milk in TBST ($1 \times$ Tris-buffered saline, 0.1% Tween 20, 5% dry milk). Membranes were washed in TBST, and then incubated overnight at 4°C with antibodies directed against total VDAC (1:1000), and CKB (1:400). Membranes were then washed in TBST and incubated with 2° -antibody (Vector Laboratories, INC., Burlingame, CA; HRP-conjugated Anti-Rabbit, PI-1000; 1:2000) for two hours. Membranes were then washed in TBST before imaging with the Fuji LAS-3000 CCD based imaging system (FujiFilm Life Science, Valhalla, NY, USA). Membranes were re-probed with total ERK (1:1000) to confirm equal protein loading. Band pixel intensity was measured using the Fuji LAS-3000 software. Band intensities from control and treated groups were normalized to ERK, and plotted using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA). Statistical significance was determined by a t-test, $p < 0.05$ ($n = 4$ animals per group).

2.8. Proteomic Analysis

2.8.1. Pooled Sample preparations—Auditory brainstem sections from the VBS and DBS regions for control and Pb treated mice were dissected from individual CBA mice as described above. The 0.1 mM (high) Pb group was chosen for the proteomic analysis in order to reduce the number of animals needed and maximize any Pb-induced changes in protein expression. The proteomic analysis was performed on two duplicate pooled samples (10 mice per duplicate for a total of 20 mice per Pb treatment group) from each brainstem region. Pooled samples were collected from three different litters and the average litter size was approximately 4–6 mice. Figure 1B summarizes the procedure from protein extraction through protein identification.

In order to simplify the proteome and increase the sensitivity of the assay, proteins were extracted according to their subcellular localization. Ten VBS (or DBS) brainstem regions from each Pb treatment group were pooled and added to ice cold fractionation buffer, (ice-cold PBS containing 3 mM EDTA and a protease/phosphatase inhibitor cocktail: 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptine, 1 μM Okadaic Acid (phosphatase inhibitor) and tissue was sheared using a 1ml pipette tip. The sample was then centrifuged at $1,000 \times g$ for approximately 10 minutes and the supernatant was discarded. Proteins from the remaining cell pellet were extracted using a Calbiochem Proteoextract subcellular proteome extraction kit (Merk Biosciences, USA). The subproteome extraction separates the proteins into cellular fractions based on the proteins solubility in different detergents. After extraction, the protein concentration was determined for each fraction using a BCA protein assay and samples were stored at -80°C . To concentrate proteins and remove any impurities such as salts and detergents that may interfere with 2-D SDS PAGE separation, protein was precipitated using a Calbiochem Proteoextract protein precipitation kit (Merk Biosciences, USA). Precipitated protein are then prepared for isoelectric focusing (IEF) by solubilizing proteins in 7 M urea, 2 M thiourea, 1% (w/v) ASB-14 detergent, 40 mM tris base, and 0.001% Bromophenol Blue.

2.8.2. Two-dimensional gel electrophoresis—In order to compare spot densities and identify protein expression changes between Pb-treatment groups (no Pb and high Pb), 75 µg of total protein from each treatment group was loaded onto a non linear 11-cm Immobilized pH Gradient (IPG) strip, pH 3–10 (Bio-Rad Laboratories, USA). Separate gels were run for each protein fraction and for each treatment group. Proteins were loaded onto IPG strips by placing protein samples in a 12-well rehydration tray and protein samples were allowed to rehydrate overnight (approximately 16 h). After rehydration, isoelectric focusing was performed as follows: 250 V for a 15 minute warm up and then ramped to 8,000 V; voltage was limited by a 50 µA/gel current restriction; after reaching 8000 V, the samples were focused for an additional 5 hours. In preparation for the second dimension, all sulfhydryl groups were reduced and alkylated. Briefly, the IPG strips were first placed in a 11 cm rehydration tray containing approximately 1 ml of reducing solution (3.6 g Urea, 0.2 mg DTT, 2.0 ml 10% SDS, 2.5 ml 1.5 M Tris (pH 8.8), 2.0 ml Glycerol, and 1.0 ml H₂O) for 30 minutes. After 30 minutes the reducing solution was decanted and channels were refilled with an alkylating solution (3.6 g Urea, 0.25 g Iodoacetamide, 2.0 ml 10% SDS, 2.5 ml 1.5 M Tris (pH 8.8), 2.0 ml Glycerol, 1.0 ml H₂O, and a very small amount of Bromophenol blue) for 30 minutes. After IPG strips had been equilibrated, they were positioned in a precast 12.5% Tris-HCl criterion gel (Bio-Rad Laboratories, USA) and overlaid with a layer of molten agarose. Gels for all treatment groups were run simultaneously in a Bio-Rad criterion dodeca cell (Bio-Rad Laboratories, USA), which is capable of running up to 12 gels simultaneously. Gels were run for approximately 1.5 hours at 20°C at a constant voltage of 150 V.

2-D gels were removed from the gel cassette, and the comparative gels were stained with SYPRO ruby fluorescent gel stain (Bio-Rad Laboratories, USA). Gels were washed for 30 min in 10% methanol, 7% acetic acid. After 30 minutes the wash solution was removed and gels were covered with SYPRO Ruby protein gel stain. Gels were stained for approximately 3 hours with gentle agitation and rinsed in 10% methanol, 7% acetic acid for 60 minutes to decrease background fluorescence. Finally, gels were washed in water for 15 minutes before imaging. Gel images were recorded using a Bio-Rad Versa Doc CCD based imaging system (Versadoc imaging system, Model 300; Bio-Rad Laboratories, USA). Gel images were analyzed using Bio-Rad PDQuest 2-D Gel Analysis Software Version 6.2. The average spot density for each protein spot was then used to compare the spot density among no Pb, and low Pb treated animals. Protein spots that exhibited a greater than ± 1.4 fold change between no and low Pb treated animals on the SYPRO ruby gels were identified by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

2.8.3. In gel trypsin digestion—Protein spots were excised from gels using a ProteomeWorks™ Spot Cutter (Bio-Rad Laboratories, USA) and gel plugs transferred to a 96 well plate. The proteins were enzymatically digested and the tryptic peptides were ZipTip purified (ZipTip® Pipette Tips, P10 C18; Millipore, Billerica, MA). Briefly, gel plugs were destained by washing plugs for 30 minutes in 200 µl of 50% H₂O/50% acetonitrile, dried in a non-humidified oven (37°C) for approximately 2 hours, and then trypsinized using a 50 µl trypsin solution (12.5 ng trypsin/1 25mM NH₄HCO₃ in 50% acetonitrile). The sample was then incubated at 4°C in the trypsin solution for 20 minutes, excess trypsin was removed and the sample overlaid with 30 µl of 25 mM NH₄HCO₃ and allowed to incubate overnight at 37°C. The following morning the supernatant containing peptides was removed and placed in a fresh 0.5 ml microfuge tube. The sample was then extracted two times with 200 µl 0.1% trifluoroacetic Acid/60% acetonitrile and reduced to approximately 10 µl. The extraction was ZipTip purified following the manufacturer's protocol. After ZipTip purification, the tryptic peptides were eluted from the ZipTip with 4 µl 60% Acetonitrile/0.2% formic acid. For analysis by MALDI-TOF mass spectrometry, 10 µg-cyano hydroxycinnamic acid is dissolved in 1 ml of 50% acetonitrile and then spiked with 2 µl of Bruker peptide calibration standard (#206195

Bruker Daltonics Inc., Billerica, MA). The MALDI-TOF matrix is mixed 1:1 with peptide sample before they are spotted on the MALDI-TOF sample plate.

2.8.4. Mass Spectrometry—Peptide mass spectra were recorded in positive ion mode on a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Mass spectra were obtained by averaging 500 individual laser shots. A calibration standard mixture (Bruker peptide calibration standard) containing 7 different peptide standards was used as internal calibration standards. The selected proteins were identified by MALDI-TOF mass spectrometry and Mascot protein database search. In order to accept a Mascot database hit as a correct protein identification, several criteria had to be met. First, the protein match had to have a MOWSE (Molecular Weight Search) score of at least 62 to be considered significant ($p < 0.05$). In addition, most of the identified proteins had average sequence coverage of greater than 25%. Finally, the experimental molecular weights and isoelectric points (pIs) had to be a close approximation of the protein's theoretical molecular weight and/or pI.

2.8.5. Database search—The peptide mass data was used to identify proteins using a Mascot protein database search engine against the MSDB database (MSDB is a non-identical protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College London. MSDB is designed specifically for mass spectrometry applications) (<http://www.matrixscience.com/>). The following search parameters were used: MSDB database; Taxonomy, *mus musculus* (house mouse); maximum missed cleavages, 1; variable modifications, carbamidomethyl (C) and oxidation (M); and peptide tolerance, ± 0.2 Da. The Mascot search engine was used to calculate a theoretical molecular weight, isoelectric point, and search (MOWSE) score for each experimental peptide mass spectra (Protein score in $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 62 were considered significant ($p < 0.05$)).

3. Results

3.1. Mouse blood lead levels

The present study used three different doses of Pb in the drinking water; the no Pb control, low Pb (0.01 mM), and high Pb (0.1 mM). Blood lead levels (mean $\pm$ SEM) of the mice were as follows: No Pb controls ($\leq 1.38 \pm 0.14$ $\mu\text{g/dL}$), low Pb (8.0 ± 0.4 $\mu\text{g/dL}$), and high Pb (42.3 ± 1.97 $\mu\text{g/dL}$). Neither the low nor the high Pb dose resulted in any change in body size and weight, and thus were considered sub-toxic doses. In addition, the blood lead levels of our high Pb animals have been commonly used to demonstrate the neurotoxic effects of Pb in rodents (Gilbert et al., 1999; Lasley and Gilbert, 2000, 2002). The low dose is below the established guidelines for blood lead levels in humans.

3.2. Pb decreases VDAC expression in MNTB neurons

Immunohistochemistry was first used to characterize the effects of Pb on VDAC expression levels in auditory neurons. Neurons within the superior olivary complex (SOC) as well as the anterior ventral cochlear nucleus (AVCN) were all immunopositive for VDAC in control animals, however the neurons of the medial nucleus of the trapezoid body (MNTB) were more darkly labeled compared to the other brainstem auditory nuclei (data not shown). The greatest change in VDAC immunoreactivity in the Pb exposed central auditory nuclei was observed in MNTB neurons. Representative micrographs of the MNTB from no, low, and high Pb-treated brains are shown in Figure 2 and illustrate a large decrease in VDAC immunostaining as a result of Pb exposure. Quantification of the VDAC immunolabel in MNTB demonstrated that this is a significant decrease (Figure 3A). It is important to note that the decrease in VDAC immunostaining was not the result of neuronal cell death. The Pb concentrations used in this

study have been previously shown to not cause cell death within murine brainstem auditory nuclei (Jones et al., 2008).

In contrast to the MNTB, there was no observed change in VDAC immunostaining in the lateral superior olive (LSO) and AVCN from the control and Pb-treated animals (Figure 3B & 3C). Although there appeared to be small decreases in VDAC immunostaining in the LSO, this decrease was not statistically significant (Figure 3B). Additionally, we examined VDAC immunostaining in neurons of the motor trigeminal nucleus in order to determine if the changes in VDAC immunoreactivity were specific to central auditory neurons. The motor trigeminal nuclei are non-auditory brainstem nuclei consisting almost entirely of motor neurons located within the VBS fraction. No change in VDAC immunostaining was observed in motor trigeminal neurons (Figure 3D). In addition, we also investigated the effect of Pb on VDAC immunostaining in hippocampal neurons in the forebrain, but found no significant change in VDAC expression following Pb exposure (Data not shown).

3.3. Pb decreases VDAC and increases CKB in the VBS region of the brainstem

The Pb-induced downregulation of VDAC would be expected to result in the suppression of mitochondrial uptake of ADP and inorganic phosphate (Pi), necessary for the synthesis and release of ATP. This in turn would lead to decreased levels of cytosolic ATP, and may result in the stimulation of energy producing systems, like glycolysis and possibly the phosphocreatine circuit (Lemasters and Holmuhamedov, 2006). The end result would be an upregulation of other cellular energy producing systems in response to decreases in cellular ATP. In order to further investigate this possibility, we utilized western blot analysis to document the effects of Pb on VDAC and CKB expression in the VBS region of the brainstem. CKB is part of a cellular energy buffering system, the creatine-phosphocreatine circuit, catalyzing the transfer of intracellular energy between ATP consuming and ATP generating sites in the cell, which is essential for maintaining ATP levels during high synaptic activity (Dzeja and Terzic, 2003; Andres et al., 2008).

In agreement with our immunohistochemical analysis, the western blot analysis of the VBS fraction from individual animals confirmed that total VDAC is significantly decreased by 19% (Figure 4A) in high Pb treated animals. VDAC expression was also decreased by 18% in the low Pb treated animals however this decrease was not statistically significant. In contrast, the expression of total CKB was significantly increased by 20% in the VBS fraction of high Pb animals (Figure 4B) supporting our hypothesis that the creatine-phosphocreatine circuit is upregulated in response to the Pb-induced decrease in VDAC.

3.4. Pb alters proteins involved in energy homeostasis and metabolizing pathways in the VBS

In order to determine whether Pb upregulates other energy metabolism proteins in brainstem auditory nuclei, a comparative proteomic analysis using 2-D SDS PAGE was performed. Representative 2-D gel maps of overall protein expression from the cytosolic, membrane, and cytoskeletal fractions of the VBS from control mice are shown in Figure 5A-C. It is important to note that there is no significant difference in the technical variability between control sample replicates. The representative gels demonstrate that proteins are widely distributed across the entire pI range of the gel and the majority of the proteins are located between 20 and 120 kDa. A total of 187 protein spots were identified in the three cellular fractions, with an average of approximately 60 protein spots identified per fraction. Our comparative proteomic analysis of the VBS following Pb exposure revealed an increased expression of proteins involved in several diverse cellular systems including energy homeostasis and metabolizing pathways in the VBS. These systems included glycolysis (Aldolase A & C, Alpha enolase, and GAPDH), oxidative phosphorylation (Isovaleryl coenzyme A dehydrogenase, NADH dehydrogenase

(Ubiquinone) Fe-S protein 1), the phosphocreatine circuit (CKB) (Wallimann et al., 1992), and VDAC1 (Figure 5).

Representative 2-D gel maps from control and Pb treated animals are shown in Figure 6. Several proteins show increases in expression following Pb treatment (circled in Figure 6), including CKB (Figure 6C) which was increased by 1.5 fold in high Pb animals.

One of the largest decreases in protein expression identified by the 2-D gel analysis was VDAC1. VDAC1 decreased by 2.1 fold in the VBS cytoskeletal fraction following Pb exposure (Table 3). Thus, the decrease in total VDAC protein that we observed by western blot is largely due to decreases in VDAC1. These findings are in agreement with our in vitro studies that found VDAC1 transcription is decreased by Pb exposure (Prins et al., 2010). Another important observation is that the decrease in VDAC1 expression is not a general response of the brain to Pb. There was little change in VDAC1 expression in the non auditory (DBS) fraction following Pb exposure.

3.5. Pb induced changes in energy metabolism are specific for auditory areas

In order to identify those protein changes that were specific to the auditory region and were not a general effect of Pb on the brain, an analysis of the DBS brainstem fraction (non-auditory region) was compared to the VBS brainstem fraction (Tables 1–3). The majority of the proteins that changed expression following Pb exposure were specific to the VBS fraction and did not change within the DBS fraction. These proteins fell into five major categories - energy metabolism, molecular chaperones, synaptic proteins, glutamate turnover, and cytoskeletal proteins. This suggests that the brainstem area containing auditory nuclei appears more vulnerable to the effects of Pb compared to the rest of the brainstem. The analysis also demonstrated that chronic low-level Pb exposure has a very large effect on proteins involved in energy metabolism (Figure 7).

4. Discussion

The current study demonstrates that VDAC1 is a novel target of Pb in central auditory neurons and shows a decrease (2.1 fold by proteomic analysis) with Pb exposure. In addition, Pb appears to primarily affect energy buffering systems within the auditory region of the brainstem. Our immunohistochemical analysis reveals a significant decrease in VDAC expression in medial nucleus of the trapezoid body (MNTB) neurons following Pb exposure. Our western blot analysis demonstrates that decreased VDAC expression corresponds with an increase in levels of CKB as a result of Pb exposure in the VBS. Finally, a comparative proteomic analysis of the VBS brainstem fraction suggests that Pb has a large impact on proteins involved in energy producing systems, such as VDAC, glycolysis and the phosphocreatine circuit.

4.1. Effect of Pb exposure on VDAC expression in auditory neurons

In order to determine whether a developmental Pb induced decrease in VDAC expression occurs in auditory brainstem neurons, brainstem sections containing the anterior ventral cochlear nucleus (AVCN) and the superior olivary complex (SOC) were immunolabeled for VDAC and the expression quantified. Binaural auditory signals from the two ears converge in the SOC and sound localization within the brainstem occurs within this complex (Nothwang et al., 2006). The SOC consists of three principal nuclei, the Medial Superior Olive (MSO), the Lateral Superior Olive (LSO), and the MNTB. The MSO is associated with processing of interaural time differences, and the LSO and MNTB are associated with the processing of interaural intensity differences (Schneggenburger and Forsythe, 2006; Nothwang et al., 2006).

Brainstem sections containing the AVCN and the SOC were immunolabeled for VDAC and the expression quantified. We found that exposure to Pb significantly decreased VDAC expression in the MNTB of the murine auditory brainstem. Because the MNTB is associated with the processing of interaural intensity differences (Schneggenburger and Forsythe, 2006; Nothwang et al., 2006), Pb exposure during development may preferentially impact this aspect of auditory processing. It will be of interest to determine whether this indeed occurs physiologically. In contrast, there was no significant decrease in VDAC expression in the AVCN, LSO, or the motor trigeminal nuclei (non-auditory brainstem nuclei), suggesting that MNTB neurons may be particularly vulnerable to the Pb-induced decrease in VDAC. Why MNTB neurons are susceptible to Pb-induced decreases in VDAC remains to be elucidated. One reason could be the specialized nature of these neurons and the fact that MNTB neurons contain a large number of mitochondria, suggesting a large energy requirement for this brain region. In support of this, we found that MNTB neurons are very darkly immunolabeled for VDAC compared to other brainstem nuclei, including AVCN and LSO. Therefore, it may be that changes in VDAC expression are particularly noticeable in MNTB following Pb exposure, particularly if Pb affects the rate of VDAC transcription. Future studies will address this issue.

Another interesting difference among auditory brainstem nuclei is that Pb decreased both serotonin and the vesicular monoamine transporter 2 (VMAT2) in LSO but not MNTB (Fortune and Lurie, 2009). However, the Pb-induced changes in the serotonergic system appear to be due to alterations in development rather than the result of an acute effect of Pb (observations of Park and Lurie). Our studies showing an effect of Pb on VDAC expression in vitro suggest that Pb causes an acute decrease in VDAC protein expression due to decreased transcription of VDAC1 (Prins et al., 2010). This indicates that Pb is not interfering with the VDAC protein directly, but is altering gene transcription. Thus, there could be a critical threshold at which Pb interferes with VDAC1 transcription, and that is why we observe a dose-independent decrease in VDAC in vivo. This threshold may be lower in MNTB neurons and thus they are particularly vulnerable to an acute effect of Pb. It will be very interesting to determine if VDAC expression in MNTB neurons continues to be decreased in vivo once Pb is removed from the system.

Further, MNTB neurons appear to be particularly sensitive to acute Pb exposure since no change in VDAC immunostaining was observed for the motor trigeminal nucleus or the hippocampus, another brain area linked to Pb-induced cognitive deficits (White et al., 2007).

4.2. VDAC, CKB, and cellular energy metabolism

Our immunochemical, proteomic, and in vitro (Prins et al., 2010) studies all show that Pb results in very significant decreases in VDAC expression (specifically VDAC1), demonstrating that VDAC is a target for Pb. VDAC is primarily located in the mitochondrial outer membrane, where it regulates mitochondrial permeability to anions, cations, creatine phosphate, Pi, ATP, ADP, and other metabolites into and out of the mitochondria (Lemasters and Holmuhamedov, 2006). In addition, VDAC modulates the Ca^{2+} dependent enzymes pyruvate dehydrogenase and iso-citrate dehydrogenase in the mitochondria (Shoshan-Barmatz and Israelson, 2005). As a result, disrupted VDAC function has been shown to compromise energy metabolism as well as Ca^{2+} homeostasis within the cell (Lemasters and Holmuhamedov, 2006) (Shoshan-Barmatz and Gincel, 2003). Because VDAC plays a central role in regulating cellular energy metabolism, the Pb-induced decrease in VDAC could have a significant impact on the function of auditory neurons. The Pb-induced downregulation of VDAC would be expected to result in the suppression of mitochondrial uptake of ADP and Pi, necessary for the synthesis and release of ATP. This would lead to decreased levels of cytosolic ATP, resulting in the stimulation of energy producing systems, like glycolysis and possibly the phosphocreatine circuit (Lemasters and Holmuhamedov, 2006). The end result would be an upregulation of other cellular energy

producing systems in response to decreases in cellular ATP. Indeed, we found an upregulation of proteins such as CKB in response to Pb.

CKB is a cytosolic enzyme that plays an important role in the energy homeostasis of cells with high-energy requirements (Wallimann and Hemmer, 1994; Andres et al., 2008). CKB is part of the creatine-phosphocreatine circuit, a cellular energy buffering system, that catalyzes the transfer of intracellular energy between ATP consuming and ATP generating sites in the cell; essential for maintaining ATP levels during high synaptic activity (Dzeja and Terzic, 2003; Andres et al., 2008; Hiel et al., 1996). Its function is to reversibly catalyze the conversion of creatine phosphate to creatine, regenerating ATP from ADP (Wallimann et al., 1992). CKB is able to replenish ATP levels in areas of high demand at a faster rate than glycolysis or oxidative phosphorylation in mitochondria (Wallimann et al., 1992).

Of particular relevance to the current study, CKB is thought to directly interact with VDAC in order to efficiently shuttle newly synthesized ATP from the mitochondria to areas of high demand (Wallimann et al., 1992). Therefore, if decreased VDAC expression results in decreased cellular ATP levels, CKB levels would be expected to increase in order to attempt to compensate for decreases in mitochondrial ATP production (Rafalowska et al., 1996; Bessman and Carpenter, 1985). Results from our study suggest that this indeed occurred following Pb exposure in our in vivo system. Our western blot analysis demonstrated that decreased VDAC expression corresponds with an increase in levels of CKB as a result of Pb exposure.

In addition, our proteomic analysis shows that Pb treatment resulted in the increased expression of several proteins involved in various cellular energy homeostasis and metabolizing pathways in the VBS, including glycolysis (Aldolase A, Alpha enolase, and GAPDH), oxidative phosphorylation (Ubiquinol-cytochrome c reductase core protein 1, Isovaleryl coenzyme A dehydrogenase, NADH dehydrogenase (Ubiquinone) Fe-S protein 1), and the phosphocreatine circuit (CKB) (Wallimann et al., 1992). Taken together, these results demonstrate that Pb significantly reduces VDAC expression in central auditory neurons, and several pathways involved in cellular energy homeostasis are upregulated. Further experiments are needed to confirm that these pathways are upregulated as a direct result of decreases in VDAC.

4.3. The auditory region of the brainstem is vulnerable to Pb

Performing a rigorous proteomic analysis of the murine auditory brainstem was beyond the scope of this investigation. However, since very little is known about the cellular systems affected by Pb in the auditory system, we used a global comparative proteomic approach to identify proteins that changed expression in the VBS compared to the rest of the brainstem (dorsal brainstem (DBS)) following Pb exposure. Since the amount of available tissue in the murine auditory brainstem is very limited, we were required to pool brainstem sections from multiple animals in order to obtain enough tissue for the sub proteome extraction, (10 animals per treatment group/duplicate), therefore care needs to be taken when interpreting this information.

However, by categorizing the proteins altered by Pb into groups based on their cellular function, we can begin to uncover the primary cellular systems affected by Pb treatment. For this study, the proteomic analysis is essentially used as a very general screen to identify proteins that changed expression in the VBS compared to the DBS. Energy metabolism proteins accounted for 34% of the proteins that were changed by Pb, and represented the largest class of proteins. This was followed by cytoskeletal proteins (29%), synaptic proteins (20%), and molecular chaperones (17%). That Pb alters a number of cytoskeletal proteins specifically within the auditory region of the brainstem is not surprising, we have previously reported that Pb increases the phosphorylation of neurofilament within auditory brainstem nuclei (Jones et al., 2008).

4.4. Pb and VDAC

This study is the first to demonstrate that Pb exposure results in decreased expression of VDAC in auditory neurons, however, several studies have found that low levels of Pb can interfere with mitochondrial energy metabolism, resulting in decreased levels of ATP (Verity, 1990; Holtzman et al., 1978; Gmerek et al., 1981; Rafalowska et al., 1996). Because the synthesis and translocation of ATP within cells of the CNS is a highly regulated and organized process, even small interruptions in cellular ATP producing processes could compromise neuronal activity and interfere with normal brain function (Ames, 2000). Our previous in vitro studies found that Pb results in decreased gene expression for VDAC 1 (one of the three known VDAC isoforms in mice (VDAC1, VDAC2, and VDAC3)) in vitro (Prins et al., 2010). The knockout of VDAC1 and VDAC2 in mice has been demonstrated to result in reductions of mitochondrial respiratory capacity (Wu et al., 1999), suggesting that VDAC1 plays a role in cellular energy homeostasis. Supporting this idea, a recent study has shown that VDAC is important for maintaining the photoreceptor response during prolonged bright light stimulation, a process thought to involve high levels of energy production (Lee et al., 2007).

Studies in the murine brainstem are currently underway to confirm that ATP levels decrease in vivo in response to the Pb-induced decrease in VDAC. Our finding that VDAC is a target for Pb is exciting and could explain the multiple effects of Pb on CNS function.

5. Conclusions

The current study identifies VDAC as a novel target of Pb in auditory brainstem neurons. The decrease in VDAC expression is particularly notable since the protein plays a central role in regulating cellular energy metabolism and VDAC is thought to play an important role in neurons with high-energy requirements. Further, these results support the hypothesis that Pb preferentially impacts energy production systems in the auditory brainstem. The physiological consequences resulting from disrupted energy metabolism may be decidedly more pronounced in the auditory system since auditory neurons require tightly regulated and efficient energy production in order to correctly maintain the precise timing and large number of action potentials necessary for central auditory processing. This in turn, appears to leave auditory neurons particularly vulnerable to the effects of Pb. The results from both the present in vivo study and our previous in vitro work (Prins et al., 2010) demonstrate that VDAC represents a new and important target for Pb, and auditory neurons may be exquisitely sensitive to small changes in VDAC expression. Future studies will fully elucidate the effects of Pb on auditory neurons and further clarify the physiological consequences of decreased VDAC expression on central auditory processing.

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Figure 1.

A) Illustration of brainstem area from which proteins were extracted. A 2mm brainstem slice is obtained and cochlear nucleus sections containing the anteroventral cochlear nucleus (AVCN) are removed and the remaining tissue separated into a ventral brainstem region (VBS) and a dorsal brainstem region (DBS). The VBS (outlined in the circle) is isolated in order to obtain tissue enriched with auditory nuclei, and then compared to the DBS region. The DBS is used to represent the non-auditory brainstem. The VBS contains the Superior Olivary Complex (SOC), which is composed of several principle auditory nuclei including the lateral superior olivary nuclei (LSO), medial superior olivary nuclei (MSO), superior olivary nuclei (SPO), and the medial nucleus of the trapezoid body (MNTB). B) Summary of the various steps of the proteomic analysis. Briefly, proteins were extracted from VBS and DBS tissue according to cellular localization. Proteins from the different cellular fractions were then

separated by 2-D SDS-PAGE. Comparative gels were stained with SYPRO ruby and protein expression from the Pb treatment group was compared to the no Pb control. Proteins displaying a significant expression change (± 1.4 fold) were selected for identification by MALDI-TOF MS and a Mascot peptide mass fingerprint database search.

Figure 2.

VDAC immunoreactivity in MNTB neurons. Pb exposure results in a significant decrease of VDAC immunostaining in the MNTB as demonstrated in representative micrographs from control and Pb-treated brains. A) Immunolabeled MNTB neurons in control mice. MNTB neurons are darkly immunostained for VDAC (black arrows). Pb exposure results in decreased VDAC immunostaining in many MNTB neurons in both the low (B) and high (C) Pb mice (gray arrows). Bar=20 μ m.

Figure 3.

Quantification of staining for VDAC. Six to twelve sections per mouse were chosen from the center of each region of interest and then slides were blinded as to treatment group. A) Density of VDAC immunostaining in MNTB. Pb exposure results in a significant decrease for VDAC immunostaining in the MNTB. In contrast, there is no significant difference in VDAC immunostaining in the B) LSO, C) AVCN, or D) the motor trigeminal nucleus, a non-auditory nucleus located in close proximity to MNTB. Note that the overall level of VDAC immunoreactivity is higher in the MNTB compared to the LSO, AVCN, and the motor trigeminal nucleus. Graphs illustrate mean $\pm$ the standard error of the mean (SEM) (n=5 or 6). (One-way ANOVA with a Dunnett's Post-hoc test, $P < 0.05$).

Figure 4.

Western blots of VBS fractions from individual mice demonstrate the Pb induced changes in protein expression for VDAC and CKB in the VBS fraction. A) Total VDAC significantly decreases by 19% in the high Pb group and shows a decreased trend in the low Pb group (n=4 mice). B) CKB significantly increases by 20% in the high Pb group (n=4 mice). Results are normalized to total ERK and are displayed as normalized spot density. Data are expressed as the mean $\pm$ SEM. (* One-way ANOVA with a Dunnet's Post-hoc test, $P < 0.05$).

Figure 5.

Representative two-dimensional gel maps of overall protein expression in VBS from No Pb (Control) mice. Protein expression from (A) cytosolic fraction, (B) membrane fraction, and (C) cytoskeletal fractions are shown. Proteins from several energy homeostasis and metabolizing pathways in the VBS are circled because Pb impacts energy metabolism. These systems include glycolysis (Aldolase A & C, Alpha enolase, and GAPDH), oxidative phosphorylation (Isovaleryl coenzyme A dehydrogenase, NADH dehydrogenase (Ubiquinone) Fe-S protein 1), the phosphocreatine circuit (CKB).

Figure 6.

Representative 2-D gel maps of the VBS membrane fraction from (A) No Pb control, (B) Pb treated animals. Several proteins that were differentially expressed in the VBS membrane fraction following Pb treatment have been circled, including Heat Shock Protein 60 (HSP 60), Heat Shock Protein 84 (HSP 84), glutamate dehydrogenase, and Creatine Kinase B (CKB). (C) Expanded view of CKB illustrates an increase in protein expression with Pb exposure. Quantification of this spot reveals that Pb induces a +1.5 fold expression change in CKB in the membrane fraction.

Figure 7.

VBS protein groups regulated by Pb exposure. Pie chart shows upregulated and down-regulated proteins (+ and -, respectively), following chronic Pb exposure during development. The modified proteins are organized into five main categories- proteins involved in energy metabolism, molecular chaperones, synaptic proteins, glutamate turnover, and cytoskeletal proteins. Most of the proteins that changed expression following Pb exposure are specific to the VBS fraction and did not change within the DBS fraction, suggesting that Pb has a greater effect on the auditory region of the brainstem.

Table 1
Cytosolic protein changes in VBS and DBS following Pb exposure during development

Protein Name	VBS Protein (fold change)	DBS Protein (fold change)	Mr/pl Theoretical	Mr/pl Calculated	Mascot Score	Sequence Coverage	Function
<u>No Pb²⁺ vs. High Pb²⁺</u>							
Dihydropyrimidinase-related protein 2 (Similarity)	+1.6	NC	62/6.0	64/5.2	92	28%	Neuronal development
ATPase, H ⁺ transporting, V1 subunit E isoform 1	+1.5	NC	26/8.4	32/9.5	81	31%	Vesicular proton pump subunit
ATPase, H ⁺ transporting, V1 subunit A isoform 1	-1.4	-1.5	68/5.5	65/5.2	120	25%	Vesicular proton pump subunit
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	+1.7	NC	36/8.4	36/9.5	78	39%	Energy generation, Glycolysis
Fructose-bisphosphate aldolase A	+1.6	NC	39/8.3	42/9.5	111	43%	Energy generation, Glycolysis
Fructose-bisphosphate aldolase C (Aldolase 3)	-1.6	NC	39/6.8	41/6.2	114	49%	Energy generation, Glycolysis
14-3-3 zeta protein	-1.5	NC	28/4.7	30/4.7	89	39%	Synaptic plasticity
Guanosine diphosphate (GDP) dissociation inhibitor	-1.4	NC	51/5.0	59/4.9	125	42%	Neurotransmitter release
Heat shock protein, 60kD	+1.6	NC	61/5.7	61/5.2	81	15%	Molecular chaperone

A protein fold change of ± 1.4 or greater was considered to reflect a significant effect of Pb on protein expression within the cytosolic fraction. Energy metabolism proteins are highlighted in gray. Theoretical and calculated molecular weight and isoelectric point (Mr/pl) are shown for each protein. Mascot search scores (MOWSE score) of greater than 62 are considered significant.

Table 2
Membrane protein changes in VBS and DBS following Pb exposure during development

Protein Name	VBS Protein (fold change)	DBS Protein (fold change)	Mr/pI Theoretical	Mr/pI Calculated	Mascot Score	Sequence Coverage	Function
<u>No Pb²⁺ vs. High Pb²⁺</u>							
Fructose Biphosphate (aldolase A)	+1.5	NC	39/8.4	42/9.5	73	32%	Energy generation, Glycolysis
Alpha enolase	+1.8	NC	47/6.4	49/5.6	71	24%	Energy generation, Glycolysis
Creatine Kinase B	+1.5	NC	43/5.4	46/5.3	112	39%	Energy transduction, Mitochondrial
Ubiquinol-cytochrome c reductase core protein 1	+1.4	NC	53/5.8	49/5.2	86	29%	Energy transduction, Mitochondrial
ATPase, H ⁺ -transporting, V1 subunit A	+2.4	+1.4	56/5.6	68/5.3	79	20%	Vesicular proton pump subunit
Isovaleryl coenzyme A dehydrogenase	+1.4	NC	46/8.3	43/6.0	94	22%	Energy transduction, Mitochondrial
Glutamate dehydrogenase	+1.4	NC	61/8.1	55/6.7	86	24%	Glutamate turnover, glutamate breakdown
Glutamine synthetase	+1.5	NC	42/6.6	45/6.0	64	16%	Glutamate turnover, inactivates glutamate
Beta tubulin	-1.5	+1.4	50/4.8	54/4.8	142	32%	Structural protein
Beta actin	+1.4	-1.6	39/5.8	45/5.0	105	33%	Structural protein
Heat shock protein 8 (Hsc70)	+1.8	NC	71/5.2	70/5.1	126	26%	Molecular chaperone
	-1.8	NC	71/5.2	69/5.1	235	41%	
Heat shock protein 60	+1.4	NC	61/5.7	60/5.0	116	25%	Molecular chaperone
Heat shock protein 84	-1.4	NC	83/5.0	94/4.9	91	21%	Molecular chaperone
Glucose-regulated protein 58 (GRP58)	+2.3	NC	57/5.8	59/5.8	107	28%	Molecular chaperone

A protein fold change of ± 1.4 or greater was considered to reflect a significant effect of Pb on protein expression within the membrane fraction. Energy metabolism proteins are highlighted in gray. Theoretical and calculated molecular weight and isoelectric point (Mr/pI) are shown for each protein. Mascot search scores (MOWSE score) of greater than 62 are considered significant.

Table 3
Cytoskeletal protein changes in VBS and DBS following Pb exposure during development

Protein Name	VBS Protein (fold change)	DBS Protein (fold change)	Mr/pl Theoretical	Mr/pl Calculated	Mascot Score	Sequence Coverage	Function
<u>No Pb²⁺ vs. High Pb²⁺</u>							
Neurofilament light polypeptide	+1.4	NC	61/4.6	65/4.6	182	37%	Structural protein
	+1.6	NC	61/4.6	66/5.1	117	24%	
	+1.5	NC	61/4.6	67/5.2	153	27%	
Neurofilament medium polypeptide	+2.0	NC	96/4.8	97/4.7	164	28%	Structural protein
	-1.7	+2.0	96/4.8	98/4.6	206	38%	
	+2.5	+1.8	96/4.8	99/5.3	106	27%	
Neurofilament High polypeptide	+2.5	NC	115/5.7	110/5.2	98	15%	Structural protein
Neurofilament 3, medium	+1.5	+2.0	64/4.8	56/5.3	82	25%	Structural protein
Guanine nucleotide binding protein G(o) subunit alpha 1	+1.5	NC	40/5.3	39/5.1	73	28%	Neurotransmitter release
NADH dehydrogenase (Ubiquinone) Fe-S protein 1	+1.5	NC	80/5.5	75/5.2	104	24%	Energy transduction, Mitochondrial
Voltage dependent anion channel 1	-2.1	NC	31/7.7	33/9.5	122	50%	Energy transduction, Mitochondrial
Cyclic nucleotide phosphodiesterase	-1.4	-1.6	45/8.7	47/9.5	107	28%	Synaptic plasticity

A protein fold change of ± 1.4 or greater was considered to reflect a significant effect of Pb on protein expression within the cytoskeletal fraction. Energy metabolism proteins are highlighted in gray. Theoretical and calculated molecular weight and isoelectric point (Mr/pl) are shown for each protein. Mascot search scores (MOWSE score) of greater than 62 are considered significant.