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CHRONIC LOW-LEVEL LEAD EXPOSURE AFFECTS THE MONOAMINERGIC SYSTEM IN THE MOUSE SUPERIOR OLIVARY COMPLEX

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Low-level lead (Pb) exposure is associated with behavioral and cognitive dysfunction. It is not clear how Pb produces these behavioral changes but low-level Pb exposure and learning disabilities have been associated with altered auditory temporal processing in both humans and animals. Temporal processing is used to decode complex sounds and to detect a signal within a noise background, and it is thought that neurons of the superior olivary complex (SOC) in the brainstem play a role in sound detection in noisy environments and in selective auditory attention. The SOC receives a catecholaminergic and a serotonergic innervation from the locus coeruleus and the dorsal raphe respectively. While the physiological role of the noradrenergic input has yet to be defined, serotonin is involved in auditory temporal processing. Because Pb exposure modulates auditory temporal processing, the serotonergic system is a potential target for Pb. The current study was undertaken to determine whether developmental Pb exposure preferentially changes the expression of serotonin within the SOC. Pb-treated mice were exposed to no Pb, 0.01 mM (very low) or 0.1 mM (Low) Pb acetate throughout gestation and through 21 days postnatally. Brainstem sections from control and Pb-exposed mice were immunostained for the vesicular monoamine transporter 2 (VMAT2), serotonin, and dopamine beta hydroxylase (DβH, a marker for norepinephrine) in order to elucidate the effect of Pb on monoaminergic input into the SOC. In addition, sections were immunolabeled with antibodies to VGLUT1, VGAT and VAChT in order to determine whether Pb exposure alters the glutaminergic, gaba-ergic, or cholinergic systems. Pb exposure caused a significant decrease in VMAT2, 5HT, and DβH expression while VGLUT1, VGAT and VAChT showed no change. These results provide evidence that Pb exposure during development alters normal monoaminergic expression in the auditory brainstem.

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General Introduction

Lead in the environment

Pb is a naturally occurring metal that is most often found in the +2 oxidation state. It is found in the earth's crust at about 15-20 mg/kg and over one third of the world's stores are in North America (ATSDR 2007). The amount of Pb in the atmosphere has increased dramatically over the past hundred years due to human use. In unpopulated areas like Antarctica, the amount of Pb in the air is about $7.6x10^{-5}$ $\mu g/m^3$, but around sources such as smelting factories it can reach >10 $\mu g/m^3$. It is commonly used in industry today for leaded batteries, leaded alloys, and corrosion/acid resistant materials used in the building industry (ATSDR 2007). Common exposure routes occur through occupation and Pb paint that is still present in houses. For occupational exposures, Pb smelting and refining can lead to close proximity Pb air concentrations of 4,470 $\mu g/m^3$, and structural steel welders Pb air concentrations average 1,200 $\mu g/m^3$ in their breathing zone (ATSDR 2007). These levels are extremely high, and as a reference, the national ambient air quality standard for Pb is 1.5 $\mu g/m^3$ (ATSDR 2007). However, all of the current uses of Pb in industry do not compare to the damage done by leaded gasoline. Tetraethyl lead was an additive to gas for almost three quarters of a century until it's phase out ended in 1995. At it's peak in 1979, automobile emissions released 208 million pounds of Pb into U.S. air (ATSDR 2007). With the timed phase out of Pb in gasoline the US environmental protection agency found that from 1982 to 2002, atmospheric emissions of Pb decreased by 93%. This is a great success but the fact remains that Pb is still a readily available toxin.

In spite of these gains Pb remains a large problem in many areas of the U.S. The center for disease control and prevention set the Pb action level for children less than 7 years of age at 10 μg/dl. Between 1999 and 2002 the CDC found that approximately 310,000 children (1.6%) age 1 to 5 still had blood Pb levels above 10 μg/dl (CDC 2005). Further, the distribution of these numbers was uneven, showing children in a lower socioeconomic class to be more likely to have elevated blood Pb levels (CDC 2005). Even today Pb is still a problem in products assumed to be safe. In September of 2007 Mattel, a major children's toy manufacturer, recalled 1.5 million toys in the U.S. due to Pb concentrations reaching 200 times the legal limit (Byron 2007). Clearly, Pb remains an accessible environmental toxin.

Behavioral and cognitive impairments of Pb

Pb is not just a readily available substance but is also a toxin with far reaching effects. Numerous studies have shown a correlation between low-level Pb exposure and cognitive function. Lanphear et al. 2000 found that for every 1 μg/dL increase in blood Pb concentration, there was a 0.7-point decrease in mean arithmetic scores, a 1-point decrease in mean reading scores, a 0.1-point decrease on mean nonverbal reasoning scores, and a 0.5-point decrease in mean short-term memory scores in children exposed to low levels of Pb. In 2003, Canfield et al. showed that blood Pb concentration was inversely associated with IQ and that as lifetime average blood Pb concentrations increased from 1μg/dL to 10 μg/dL, average IQ declined by 7.4 points (Canfield, Kreher et al. 2003). The agency for toxic substances and disease registry has demonstrated that the current value of economic losses in the Unites States attributable to Pb exposure

amounts to $43.4 billion per year in each annual birth cohort when taking into consideration the loss of earning potential due to lowered IQ (ATSDR 2007).

It is evident that low levels of Pb cause behavioral deficits in children but a "behavioral signature" has yet to be identified. One particular area of interest in behavioral studies has been attention. Bellinger et al. found significant correlations for low level Pb exposure, focus, and executive function (Bellinger and Dietrich 1994). Mendelsohn et al. showed Pb exposed children were more hyperactive, impulsive, and easily frustrated (Mendelsohn, Dreyer et al. 1998). Another study showed deficits in attention areas such as executive function, off-task behaviors, and withdrawn behaviors with Pb exposures <10 μg/dl (Chiodo, Jacobson et al. 2004). However, the effects of environmental Pb exposure does not rely just on ambiguous observances, but also can be clinically classified as disorders. Braun et al. found environmental exposure to Pb increased the incidence of ADHD and that Pb exposure accounts for 290,000 cases of ADHD in U.S. children (Braun, Kahn et al. 2006). Further, Glotzer et al. has analyzed the relationship between reading disabilities and the costs associated with reading disabilities in Pb exposed children (Glotzer, Freedberg et al. 1995). Even though a significant link between Pb exposure and behavioral deficits has been found, the underlying cause for attention deficits remains unknown.

One potential underlying cause for the behavioral deficits is change in auditory processing. Finkelstein et al. reviewed the link between Pb exposure in children and auditory deficits. The review included altered auditory processing, and decreased performance in tests requiring appropriately timed reactions (Finkelstein, Markowitz et al. 1998). In addition, Holdstein et al. found Pb exposed children showed increased

latencies in brainstem auditory evoked potentials, in peak V and interpeak I-V (Holdstein, Pratt et al. 1986). These auditory deficiencies are mirrored in several animal studies. A study conducted by Gray et al. showed that chicks exposed to low levels of Pb are deficient in a test of central auditory temporal processing called backward masking. Further, our lab demonstrated that Pb exposed CBA mice displayed increased latencies in the interpeak interval between peaks I and V with presentation of acoustic stimuli (Jones, Prins et al. 2007). Taken as a whole, this body of literature indicates that there are deficits in auditory temporal processing following Pb exposure.

Auditory temporal processing and the SOC

Temporal processing is used to decode complex sounds and to detect a signal within a noise background. There are several regions of the brain that are involved in auditory temporal processing but the superior olivary complex (SOC) is of primary interest in the current study. The SOC is the first site of binaural auditory processing in the brainstem and is thought to be important in using temporal aspects of sound to determine stimuli location (Squire 2003).

Further, neurons of the SOC are thought to play a role in sound detection in noisy environments and in selective auditory attention (Mulders and Robertson 2005). Previous studies have shown that removal of the olivocochlear bundle alters the ability to discriminate vowels in noise. In addition, recordings of primary auditory afferents in the area have demonstrated enhancement of responses to transient signals in the presence of noise background (Giraud, Garnier et al. 1997). The SOC has the ability to modify

sound but how the structure and the function of the SOC is altered with Pb exposure has not been studied.

Serotonin is linked to auditory temporal processing, attention, and arousal

The connection between auditory temporal processing and Pb exposure could in part be explained by alterations in the serotonergic system. Serotonin plays an important role in attention and cognitive function (Schmitt, Wingen et al. 2006). Further, studies have shown it has the ability to direct and focus cognitive activity or alertness for specific stimuli over a prolonged period of time (Schmitt, Wingen et al. 2006). Interestingly, serotonergic neurons in the dorsal raphe nuclei, have their highest activity levels in awake and alert animals further linking the dorsal raphe nuclei to attention (Hurley and Pollak 2005). Alterations in the serotonergic system are related to ADHD and other learning disabilities (Hawi, Dring et al. 2002; Oades 2007). The functions of the serotonergic system show a link with the deficits seen in auditory processing by Pb.

The link between serotonin and auditory temporal processing is enhanced by the fact that the system innervates most nuclei in the ascending auditory system including the superior olivary complex (SOC) (Woods and Azeredo 1999; Thompson and Schofield 2000; Behrens, Schofield et al. 2002; Schofield 2002; Horvath, Ribari et al. 2003; Thompson and Hurley 2004; Hurley and Pollak 2005; Hall and Hurley 2007; Hurley 2007). The superior olivary complex consists of the lateral superior olive, medial superior olive, and medial nucleus of the trapezoid body. A large number of neurons from the LSO ascend and synapse in the inferior colliculus, but some neurons project efferently to inner hair cells providing a pathway of descending input. The functional role

of this efferent system remains unknown but it has been hypothesized to modify or control binaural interactions, reduce the masking effects of background noise, protect the cochlea from noise-induced trauma, and alter the response of the cochlea to sound with changes in attention (Woods and Azeredo 1999; Darrow, Maison et al. 2007).

Serotonin is thought to alter auditory responses to acoustic stimuli. A study done by Cransac et al. found that if experimentally controlled white noise increased, 5-HT content in the dorsal cochlear nucleus and posteroventral cochlear nucleus also increased. They also found that application of 5-HT to cochlear neurons inhibited acoustically evoked neuronal firing, indicating that 5-HT could be used to eliminate background noise(Cransac, Cottet-Emard et al. 1998). Hurley et al. found that serotonin altered tone-evoked responses in 66% of inferior colliculus neurons sampled and that it had the ability to depress or facilitate neuronal response depending on the nature of the acoustic stimulus (Hurley and Pollak 1999). Hurley et al. further showed that serotonin significantly altered first-spike latencies in response to tones in IC neurons and that the size of the serotonin-evoked latency shifts were dependent on the intensity and frequency of the stimulus. They concluded that serotonin could alter spike count, first spike latency, variation of first-spike latency and the interspike interval of responses (Hurley and Pollak 2005). These alterations are significant because they represent features of sensory stimuli and alterations in serotonin has the potential to effectively change features of sound. A study in Mexican free-tailed bats showed that serotonin application altered the neuronal population response to species-specific vocalizations. Serotonin created a unique spatio-temporal pattern of activity among neurons in the inferior colliculus in response to specific vocalizations (Hurley and Pollak 2005). These studies show that serotonin could

cause both alterations of focused attention to auditory stimuli and changes in the ability to decipher an auditory signal in a background of ambient noise.

In order to determine whether the serotonergic system is preferentially altered by developmental Pb exposure, the expression of the vesicular transporters for glutamate, GABA, acetylcholine, and the monoamines were examined within the murine SOC by immunocytochemistry. The goal was to identify those neurotransmitter systems that might be affected by Pb exposure. The auditory system uses all of the neurotransmitters mentioned above, with glutamate being the primary neurotransmitter used by ascending auditory pathways. The CBA mouse was chosen because this strain does not exhibit degeneration of the auditory system at early ages. In addition, our lab has previously shown that Pb affects the CBA mouse SOC at low levels of exposure (Prins et al, submitted). The mouse model also allows us to explore a chronically-treated, developmental Pb exposure model in a moderately short time frame. Further, the serotonergic system is well defined in the auditory brainstem of mice and has been shown to innervate our regions of interest. We focused our attention on the LSO and MNTB because of their relevance to auditory temporal processing and because the LSO receives auditory input directly from the dorsal raphe nuclei. The current study quantifies protein expression levels for VGLUT1, VACHT, VGAT and VMAT2 in the LSO and MNTB of mice chronically exposed to low levels of Pb.

Introduction

Lead (Pb) has long been recognized as a toxic agent that has a significant impact on human health (Tong, von Schirnding et al. 2000; Sanborn, Abelsohn et al. 2002; Mannino, Albalak et al. 2003; Mannino, Homa et al. 2005; Toscano and Guilarte 2005). The neurotoxic effects of low doses of Pb have been shown to result in behavioral and cognitive deficits and in 1991, The Centers for Disease Control and Prevention set the acceptable blood Pb level at 10 µg/dL. However, an increasing body of evidence has demonstrated that blood Pb levels below 10 µg/dL produce many behavioral deficits including lowered IQ, attention deficit hyperactivity disorder, and dyslexia (Glotzer, Freedberg et al. 1995; Lanphear, Dietrich et al. 2000; Canfield, Henderson et al. 2003; Canfield, Kreher et al. 2003; Kamel, Ramadan et al. 2003; Chiodo, Jacobson et al. 2004; Braun, Kahn et al. 2006; Gilbert and Weiss 2006; Chen, Cai et al. 2007).

It is not clear how Pb produces these behavioral changes but both low-level Pb exposure (Mulders and Robertson 2005) and learning disabilities have been associated with altered auditory temporal processing in both humans and animals (Finkelstein, Markowitz et al. 1998; Gray 1999; Lurie, Brooks et al. 2006). Temporal processing is used to decode complex sounds and to detect a signal within a noise background, and it is thought that neurons of the superior olivary complex (SOC) in the brainstem play a role in sound detection in noisy environments and in selective auditory attention (Mulders and Robertson 2005). The SOC receives a catecholaminergic and a serotonergic innervation from the locus coeruleus and the dorsal raphe respectively (Mulders and Robertson 2005); (Thompson and Hurley 2004).

While the physiological role of the noradrenergic input to olivocochlear neurons has yet to be defined (Mulders and Robertson, 2005), serotonin has been shown to alter auditory temporal processing in the mammalian auditory brainstem (Hurley, Thompson et al. 2002; Hurley and Pollak 2005; Hall and Hurley 2007; Hurley 2007). Olivocochlear neurons project to the inferior colliculus (IC), and serotonin alters both the magnitude and the latency of neuronal responses to auditory stimuli within the IC (Hurley 2007). Serotonin has been shown to modulate neuronal spike count, first-spike latency, temporal precision, and the interspike interval, all of which may alter temporal processing (Hurley and Pollak 2005). Because Pb exposure modulates auditory temporal processing, the serotonergic system is a potential target for Pb within auditory brainstem nuclei.

The current study was undertaken to determine whether developmental Pb exposure changes the expression of serotonin within the superior olivary complex (SOC). Brainstem sections from control and Pb-exposed mice were immunostained for the vesicular monoamine transporter 2 (VMAT2), serotonin, and dopamine beta hydroxylase (DβH, a marker for noradrenalin) in order to elucidate the effect of Pb on monoaminergic input into the SOC. Control and Pb-exposed brainstem sections were also immunolabeled for the vesicular glutamate transporter 1 (VGLUT1) , the vesicular acetylcholine transporter (VAChT), and the vesicular GABA transporter (VGAT) in order to determine the effect of Pb on glutaminergic, gaba-ergic, and cholinergic neurotransmitter systems. Glutamate is considered to be the primary neurotransmitter for ascending auditory information (Thompson and Hurley 2004) but GABA, acetycholine, and glycine are also important neurotransmitters within the auditory brainstem

(Thompson and Hurley 2004); (Thompson and Hurley 2004); (Maison, Adams et al. 2003)).

We found that developmental Pb exposure reduces immunoreactivity for VMAT, serotonin, and DβH within the Lateral Superior Olive (LSO) but has no effect on VGLUT 1 and 2, VAChT, or VGAT. This affect appears to be specific to the LSO, as western blot analysis did not reveal decreased monoamergic expression within the whole brainstem. These findings demonstrate that developmental Pb exposure has a significant effect on the monoamergic innervation to the LSO.

Materials and Methods

Animals

Breeding pairs of CBA mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). All mice used in these studies were maintained in micro isolator units in the University of Montana specific pathogen free animal facility. The animal care facility housed all mice on a 12 h light/dark cycle in a temperature-controlled environment. 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 procedures were in accordance with NIH and the University of Montana IACUC guidelines using approved animal protocols.

Lead Exposure

Breeding pairs were randomly assigned to three groups and given unlimited access to water containing 0 mM (control) , (0.01 mM) (very low), or 0.1 mM (low) Pb

acetate. Breeding pairs were given Pb in their drinking water at the time of pairing and offspring were exposed to Pb throughout gestation and through the dam's milk until postnatal day 21-24 (P21). At this time mice were sacrificed as described below.

Blood Lead Levels

Blood was collected from anesthetized mice by retro-orbital puncture. Blood Pb^{2+} levels were measured by the Montana Health Department in Helena, MT. The means for the no Pb group included values of <1.0 which were included in the data set as equal to 1.0.

Western Blot Analysis

Animals were sacrificed by cervical dislocation and the brainstem removed (n=16 brains per treatment group). The auditory region of the mouse brainstem was located by cutting a 2mm section out of a 1 mm mouse brain matrix. The 2 mm section was snap frozen in liquid nitrogen on a microscope slide and the cochlear nucleus removed. The central brainstem was discected into two sections consisting of the ventral brainstem (VBS), containing the SOC, and dorsal brainstem (DBS) followed by storage at –80 C.

Pooled samples containing four brains each were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate and 1 mM Na_3VO_4. (Cell Signaling Technology, Beverly, MA) for a total of n=4 separate homogenates per treatment group. Additions were made giving final concentrations of 0.5% Na-deoxycholate, 0.5% sodium dodecyl sulfate, 1μM okadaic acid, 1 mM

phenylmethylsulfonyl fluoride, 0.1 mg/ml benzamidine, 8 µg/ml calpain inhibitors I and II and 1 µg/ml each leupeptin, pepstatin A and aprotinin. Tissue was homogenized on ice in .6 ml of ice-cold lysis buffer, incubated on ice for 30 minutes followed by 30 seconds of sonication and centrifugation at 50,000 rpm for 20 minutes at 4˚C. The supernatant was assayed for protein concentration using the Bio-Rad Protein Assay (Bio-Rad #500-0001, Hercules CA) and aliquots were stored at -80°C for use in Western analysis. Protein separation was done by SDS-PAGE using NuPAGE® 4-12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA). Alliquated samples were mixed with distilled deionized water and NuPAGE® LDS sample buffer followed by 10 minutes at 70˚C on a standard VWR® heatblock (West Chester, PA). Gels were loaded with 20 µg of denatured protein per well along with 10 µl MagicMark™ XP Western Protein Standard (Invitrogen, Carlsbad, CA) and 5 ul of Kaleidoscope™ Western Protein Standard (Bio-Rad). Gels were run in NuPAGE® MOPS SDS Running Buffer (Invitrogen) with 500 µl of NuPAGE® Antioxidant (Invitrogen) for 55 min. Nitrocellulose membranes were pretreated in methanol for 15 seconds, washed in distilled water for three minutes and soaked in transfer buffer for 5 minutes prior to transfer. Gels were transferred for 1 hr on ice at 100 V in a cold room using a Bio-Rad Power Pac 200 power supply (Bio-Rad). Post-transfer membranes were blocked in 5% dried nonfat milk, 0.1% tween and TBS for 1.5 hours at room temperature. Membranes were washed in TBST 3 times for 10 minutes and incubated with primary antibody in blocking buffer overnight at 4˚C. The VMAT2 antibody was used at a dilution of 1to 500. Membranes were washed in TBST for 5 minutes and the secondary antibody (GAPDH) was applied at 1:2000 for 1 hr at room temp. Membranes were washed three

times for 10 minutes in TBST and then visualized using an electrochemiluminescence western blotting detection reagents (Amersham Biosciences , Piscataway NJ). Exposures were taken in a Fuji film Intelligent Darkbox using a Fujifilm LAS-3000 camera (Fujifilm, Valhalla NY).

Immunohistochemistry

At P21, mice from all three treatment groups (n=5 per group) were deeply anesthetized and perfused transcardially with 4% Na-periodate-lysine-paraformaldehyde fixative (PLP, final concentrations 0.01M sodium periodate, 0.075M lysine, 2.1% paraformaldehyde, 0.037M phosphate). Brains were removed and post-fixed for 2 hours at 4°C in PLP, rinsed 3 times for 10 minutes each in PBS and transferred to a 30% sucrose solution in PBS overnight at 4°C. Brains were bisected between the forebrain and brainstem and tissue was embedded cut-side down into 1.5 cm square embedding cups filled with optimal cutting temperature (O.C.T.) compound. Brains were then frozen in liquid nitrogen and stored at -20°C. Ten micron tissue sections were cut on a Thermo Shandon Cryotome Cryostat (Thermo Shandon, Pittsburgh PA) and a one in three series of sections was collected for each brain.

Sections were rinsed in PBS, permeabilized for 30 minutes with 0.5% Triton X-100 in PBS and then immunostained as previously described (Lurie and Durham, 2000; Wishcamper et al, 2001). Briefly, tissue sections were blocked for 20 minutes with either 4% Normal Goat Serum in TAB or 4% Normal Rabbit Serum and incubated with primary antibody for 24 hours in a humid chamber at 4° C. The tissue was rinsed and then stained with the appropriate secondary antibody for 1 hr. at room temperature (Alexa

Fluor 488--1:400, or 488 Avidin biotin complex-1:500). Sections were then rinsed and the slides coversliped with FluorSave™ (Calbiochem®, San Diego CA) and stored at 4° C. Antibody concentrations used for immunohistochemistry (IHC) were as follows: VGLUT1 1:1000, VGAT 1:200, VAChT 1:2000, DβH 1:800, TH 1:1200, 5-HT 1:10,000 (please see below for detailed descriptions of the antibodies).

For light microscopy and DAB immunohistochemistry, an additional set of control and Pb exposed brains were paraffin embedded as previous described (Lurie and Durham, 2000; Wishcamper et al., 2001) and a one in six series was collected and mounted onto slides. Tissue was then immunostained for synaptophysin (1:200) using 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.).

Antibodies

The mouse monoclonal against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mouse MAB374, Chemicon, Billerica, MA) was raised against rabbit muscle. The antibody recognizes a single band of approximately 37 kD in western analysis in human, rat, mouse, chicken, frog, and fish {Phillips, 1997 #207}.

The rabbit polyclonal against Dopamine β-Hydrroxylase (DβH; Rabbit ab43868, Abcam, Cambridge MA) was raised from cow adrenal medulla. In western analysis the

antibody detects a band of approximately 72 kDa (predicted molecular weight: 68 kDa) and reacts with mouse, rat and cow. It exists in soluble form in chromaffin granules and also exists in a membrane bound form.

The rabbit polyclonal against serotonin (5-HT) was raised in rabbit against serotonin coupled to bovine serum albumin with paraformaldehyde (Cat. No. 20080, Lot No. 541317, ImmunoStar Inc., Hudson WI). No cross-reactivity of serotonin antisera was seen with 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's specifications).

The rabbit polyclonal antibody against vesicular acetylcholine transporter (VAChT) was raised in rabbit against Strep-Tag fusion protein containing C-terminal amino acid residues 475-530 of VAChT from rat (Anti-VAChT, Cat. No. 139103, Synaptic Systems, Gottingen, Germany). Specificity for VACht was tested by the manufacturer in rat and was characterized by Arvidsson et al 1997.

The rabbit polyclonal antibody against vesicular glutamate transporter 1 (VGLUT1) was raised in rabbit against Strep-Tag fusion protein containing amino acid residues 456-560 of VGLUT1/BNPI from rat (Anti-VGLUT1, Cat. No. 135303, Synaptic Systems, Gottingen, Germany). The antibody labels a band at 60 kDa in western blot as demonstrated by the manufacturer. Specificity for VGLUT1 was tested by the manufacturer and is referenced in Redecker et al 2003, Sherry et al 2003, Montana et al 2004, Prange et al 2004, Dal Bo et al 2004.

The rabbit polyclonal antibody against vesicular glutamate transporter 2 (VGLUT2) was raised in rabbit against Strep-Tag fusion protein containing amino acid residues 510-582 of VGLUT1/BNPI from rat (Anti-VGLUT2, Cat. No. 135402, Synaptic

Systems, Gottingen, Germany). The antibody labels a band at 65 kDa in western blot as demonstrated by the manufacturer. Specificity for VGLUT2 was tested by the manufacturer and has been referenced in multiple papers (Land et al 2003, Redecker et al 2003, Chen et al 2004, Hrabovszky et al 2004, Wojcik et al 2004, Dal bo G et al 2004, Montana et al 2004).

The rabbit polyclonal antibody against vesicular monoamine transporter 2 (VMAT2, C-terminus) was raised in rabbit against Synthetic peptide comprising the cytoplasmatic C-terminus of rat VMAT2. (Anti-VMAT2, Cat. No. 135402, Synaptic Systems, Gottingen, Germany). The antibody labels a band at 65 kDa in western blot as demonstrated by the manufacturer. Specificity for VMAT2 was tested by the manufacturer and described in multiple studies (Liu et al 1994, Nirenberg et al 1995, Nirenberg et al 1997, Colliver et al 2000, Holtje et al 2000, Jakobsen et al 2001).

The rabbit polyclonal antibody against vesicular GABA transporter (VGAT) was raised in rabbit against synthetic peptide AEPPVEGDIHYQR (amino acid residues 75-87 in rat) coupled to key-hole limpet hemocyanin via an added N-terminal cysteine (Anti-VGAT, Cat. No. 131002, Synaptic Systems, Gottingen, Germany). The antibody labels a band at 57 kDa in western blot as demonstrated by the manufacturer. Specificity for VGAT was tested by the manufacturer, and the antibody has been used in multiple studies (Takamori et al 2000, Geigerseder et al 2003, Wojcik et al 2004, Prange et al 2004, Saito et al 2004, Harman et al 2004).

The mouse monoclonal antibody against synaptophysin was raised in mouse against the SY38 epitope. The antibody labels a band at 38 kDA in western blot as demonstrated by the manufacturer and is referenced in (Provoda 2000)

Tissue Analysis

All fluorescent slides were viewed at 60x magnification using a Nikon Eclipse TE 300 confocal microscope and the BioRad Radiance 2000 Laser Scanning System connected to a Dell PC. Images were collected and then converted from color tiff files to black and white, 12 bit tiff files. Two to five sections per animal were analyzed and the integrated optical density of the immunostaining was measured using MediaCybernetics Image-Pro software (Bethesda MD). Integrated optical density measurements were used for quantification of immunostaining because it analyzes both the area of immunostained tissue that met threshold as well as the intensity of the immunostaining. In summary, a threshold of immunostaining for each antibody was obtained for the appropriate area of interest (either LSO or MNTB) in two-five no Pb animals. This set a control threshold that was unique for each antibody and was used as a comparison to the Pb treatment groups. Immunostaining within three random areas (225 square microns - 225 square microns) within LSO and MNTB in control and Pb-exposed mice was then quantified and averaged. Statistical differences in immunostaining between control and Pb exposed animals were analyzed using Synergy Software's KaleidaGraph software (Reading PA)

Light microscopic brainstem sections (synaptophysin immunoreactivity) were viewed at 40x magnification with a Nikon E-800 attached to a CRI Nuance multi spectral imaging system (CRI, Inc., Woburn PA). Images were analyzed with NIH image 1.62 in order to quantify the density of DAB staining. Images containing the entire LSO and MNTB were captured on the screen and the entire LSO and MNTB within each section was analyzed. In control sections of LSO and MNTB, a threshold was set such that the reaction product for synpatophysin reached this threshold. The number of pixels that

reached threshold was then calculated by the computer and the mean pixel count was calculated to determine a single value for all control and Pb exposed brains examined. Measurements were performed on two to three sections per animal.

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed using one-way analysis of variance with Dunnet's and/or Tukey's post-hoc analyses where appropriate; $p<0.05$ was considered significant.

Results

Blood Lead Levels

The current study uses three different doses of Pb in the drinking water, the no Pb control, very low Pb (0.01mM), and low Pb (0.1mM). The blood Pb levels (mean $\pm$ SEM) for these mice are as follows: No Pb control (1.36 $\pm$ 0.14 µg/dL), very low Pb (8 $\pm$ 0.45), and low Pb (42.26 $\pm$ 1.97 µg/dL). None of our doses of Pb produced changes in size or body weight (data not shown) and the animals appeared unaffected by the Pb, indicating that the Pb doses used in this study can be considered a sub-toxic dose.

Pb exposure results in decreased expression of VMAT-2, serotonin, and DβH

In order to determine whether developmental Pb exposure results in alterations in monoaminergic neurotransmitters, control and Pb-exposed brainstem sections were immunolabeled for the monoaminergic vesicular transporter, VMAT 2. In control animals, VMAT 2 immunoreactivity is abundant in LSO (Figure 1A) but very little immunoreactivity is found in the medial nucleus of the trapezoid body (MNTB; data not

shown). This is in agreement with previous studies, which have reported the presence of many 5-HT immunoreactive fibers in the murine LSO, and relatively sparse numbers of 5-HT immunopositive fibers in MNTB (Thompson and Hurley 2004).

Developmental Pb exposure results in a significant decrease in VMAT 2 immunoreactivity in both the very low and low Pb mice. Figure 1 illustrates the dramatic decrease in VMAT immunoreactivity within the LSO in Pb-treated mice compared to controls, and quantification of the immunostaining reveals a 33% decrease in the VMAT 2 staining. However, Pb exposure does not result in a significant decrease of VMAT 2 within the entire brainstem. Western analysis reveals a trend towards decreased VMAT 2 protein in the brainstem but this decrease is not significant (Figure 2).

In order to determine if Pb exposure changes the expression of other neurotransmitter transporters such as VGLUT 1, VGAT, and VAChT, brainstem sections were immunolabeled with antibodies to the appropriate transporter protein. Glutamate is the major excitatory neurotransmitter that is used by the auditory system and we found robust immunostaining for VGLUT 1 in both the LSO and MNTB in control mice (Figure 3). This immunostaining was not changed by Pb exposure (Figures 3 and 5), indicating that Pb does not significantly alter glutamate expression within the auditory brainstem. Similarly, immunostaining for VGAT and VAChT was not changed with Pb exposure, suggesting that both gaba-ergic and cholinergic expression levels remain unaffected by Pb (Figures 4 and 5). It should be noted that VGAT expression is high in MNTB and not detectable in LSO, while VAChT expression is found in LSO (Yao and Godfrey 1998).

In summary, Pb exposure decreased the expression of VMAT 2 within LSO but not VGLUT 1, VGAT, or VAChT. Therefore, it appears that monoaminergic neurotransmitters systems within brainstem auditory nuclei are particularly vulnerable to low levels of Pb. Monamines such as dopamine, serotonin and norepeniphrine are transported into synaptic vesicles by VMAT 2 (Gopalakrishan et al, 2007). In order to determine whether Pb decreases expression of all monoamines, tissue sections were immunolabeled with antibodies to tyrosine hydroxylase (TH, a marker for dopamine), dopamine beta-hydroxylase (DβH, a marker for noradrenalin) and sertonin (5-HT). Tyrosine hydroxylase converts tyrosine to L-DOPA, a precursor for dopamine, and is also the rate-limiting step in dopamine synthesis (Pan, Berman et al. 2006; Kaushik, Gorin et al. 2007). TH immunostaining is commonly used as a marker for dopamine. Dopamine beta hydroxylase converts dopamine to noradrenalin in synaptic vesicles and is routinely used as a marker for noradrenalin expression within the brain, and has been shown to label varicosities within auditory pathways (Behrens, Schofield et al. 2002).

Figure 1. Pb treatment decreases VMAT2 expresssion in the LSO.

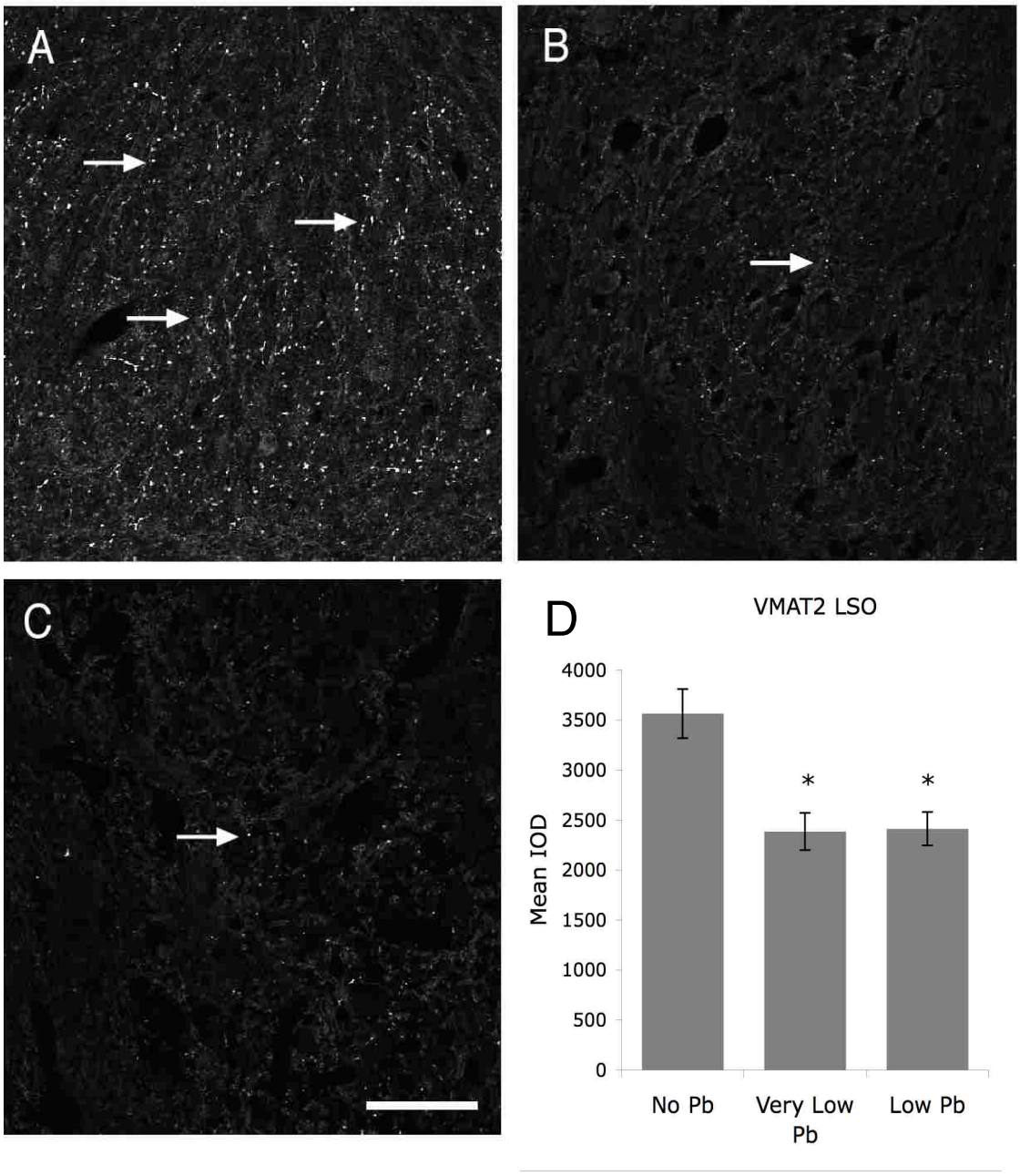

Figure 1. Pb treatment decreases VMAT2 expresssion in the LSO. A-C) Representative micrographs of immunofluorescent staining for VMAT2 in the LSO in No (A), Very Low (B), and Low (C) Pb mice reveal a decrease in immunoreactivity with Pb treatment (Arrows). Quantification of staining for VMAT2 in the LSO reveals that this decrease is statistically significant (D). Graphs illustrate mean + the standard error of the mean (SEM). (n=5 per group) *P < 0.05, One-way Anova with Tukey's all pairs comparison. Bar = 50 μm for panels A-C.

Figure 2. Pb exposure does change VMAT2 expression levels in the entire ventral brainstem.

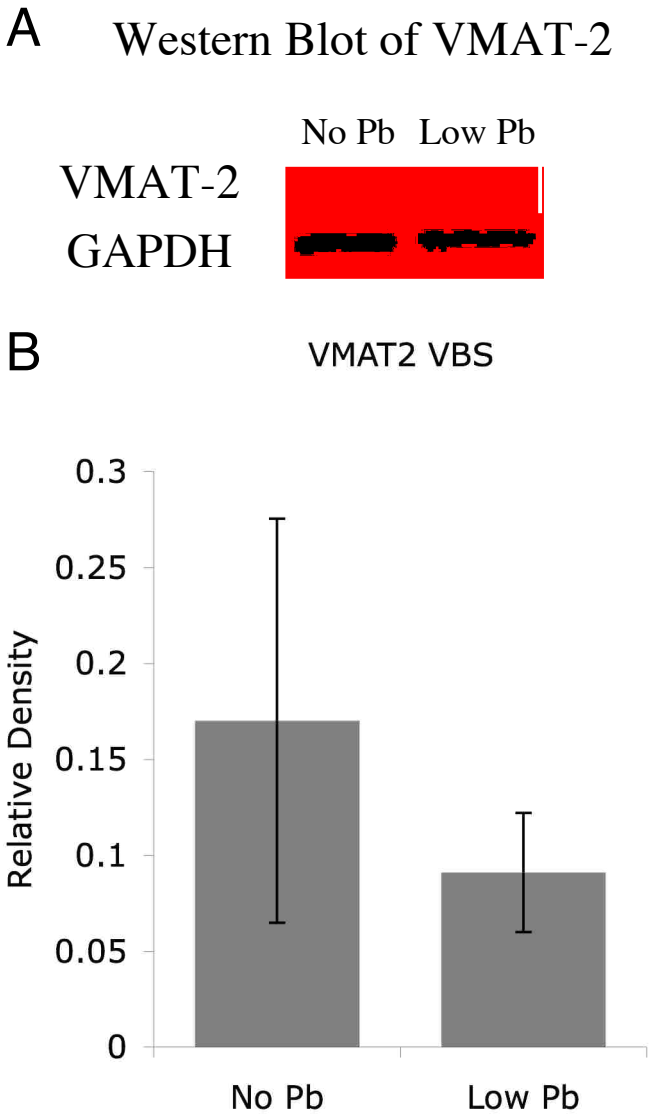

Figure 2. Pb exposure does change VMAT2 expression levels in the entire ventral brainstem. A) Western analysis demonstrates no significant change in VMAT2. B) Quantification of the western blots reveals no significant change in VMAT 2 with Pb exposure. Representative blots are shown above the quantification (n=4 separate homogenates; 4 brainstem/homogenate). The graphs illustrate mean + the standard error of the mean (SEM).

Figure 3. Pb exposure does not affect VGLUT1 expression levels in either LSO or MNTB.

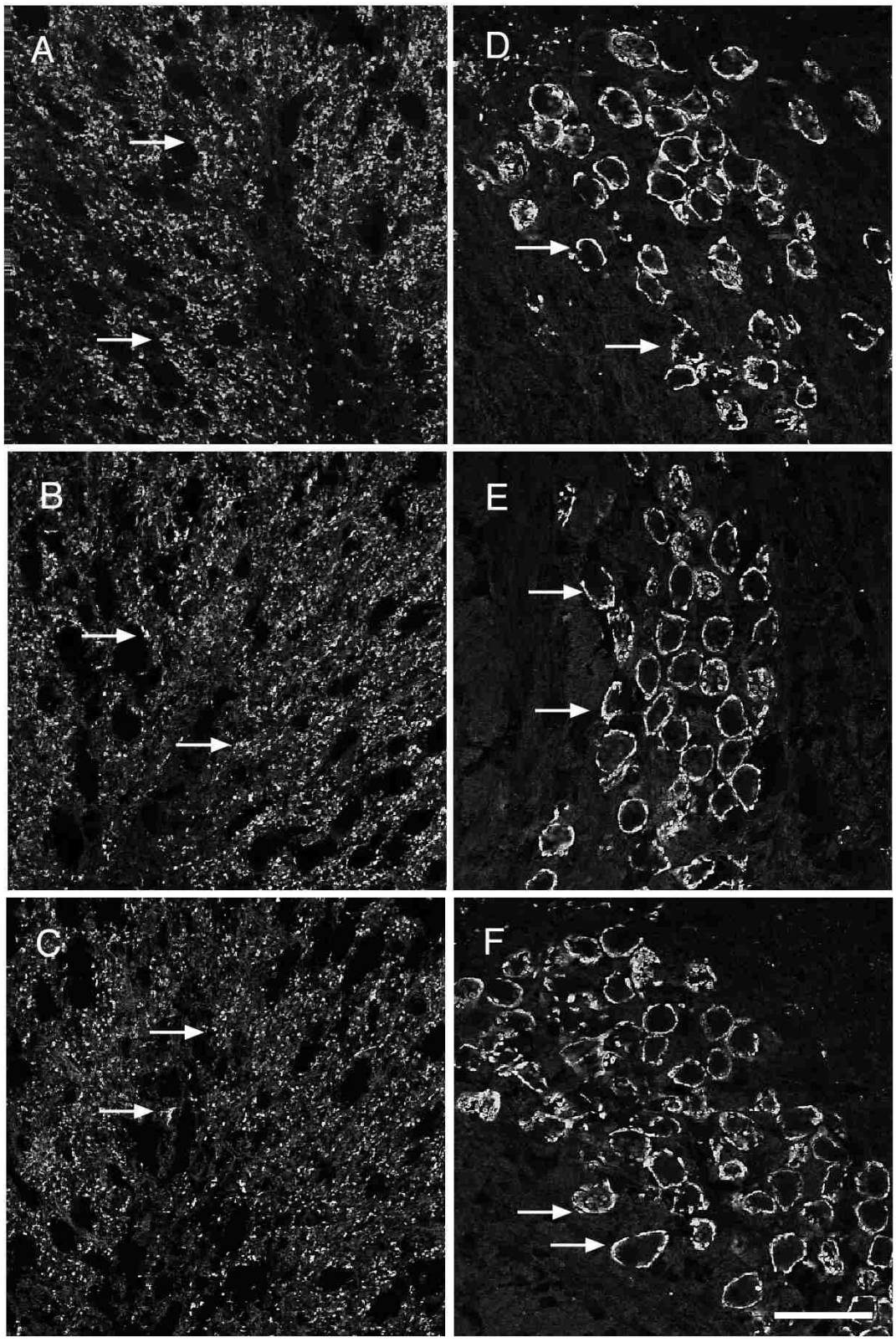

Figure 3. Pb exposure does not affect VGLUT1 expression levels in either LSO or MNTB. A-C) Immunofluorescent staining for VGLUT1 in the LSO in response to No (A), Very Low (B), and Low (C) Pb treatment shows no change in immunoreactivity with Pb exposure (arrows). D-F) VGLUT1 immunostaining in the MNTB in response to No (D), Very Low (E), and Low (F) Pb exposure also demonstrates no change in expression levels (arrows). (n=5 per group). Bar = 50 μm for panels A-F.

Figure 4. Pb treatment does not affect VAChT or VGAT expression levels in the SOC.

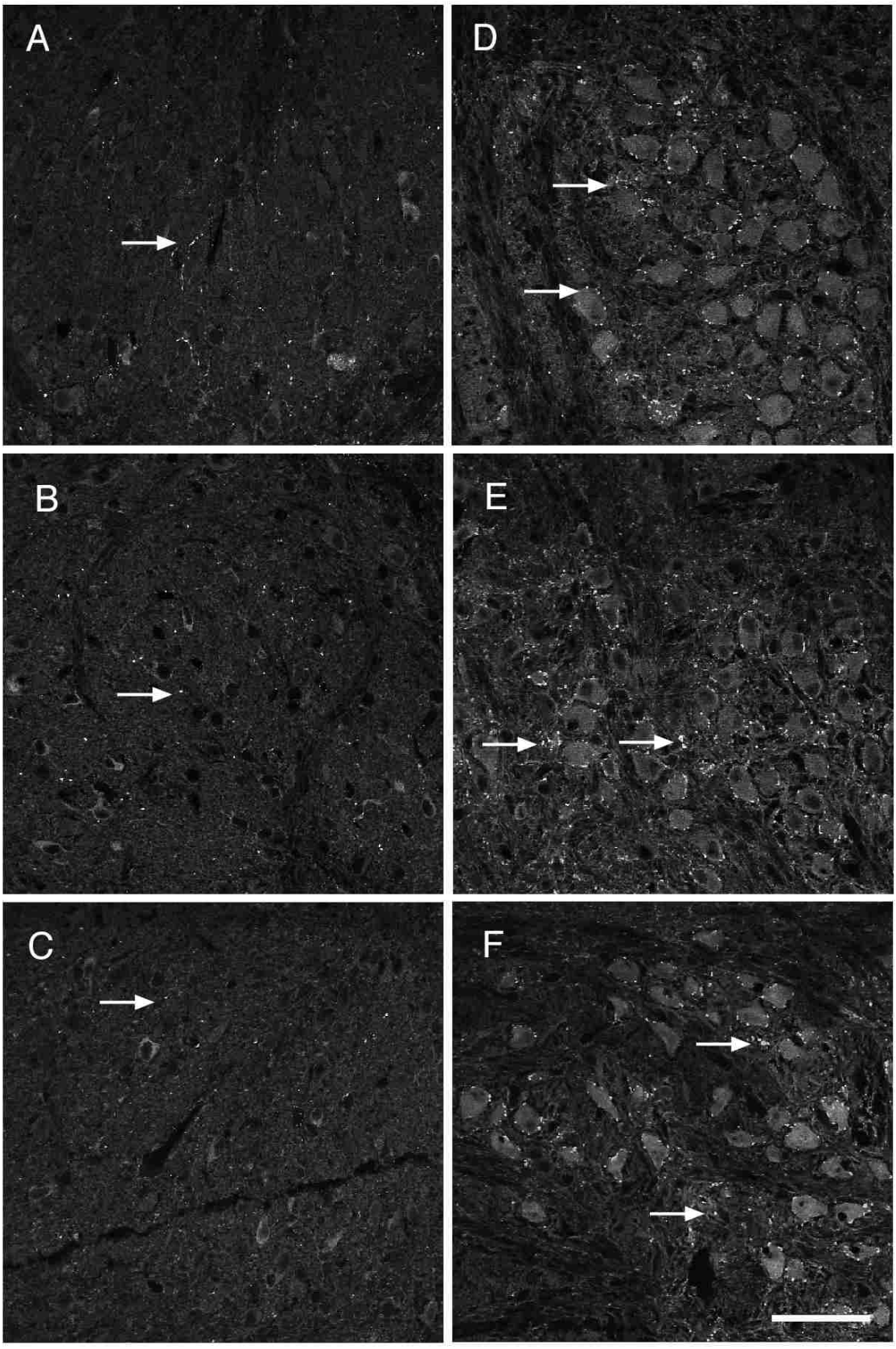

Figure 4. Pb treatment does not affect VAChT or VGAT expression levels in the SOC. A-C) VACHT immunostaining in the LSO does not change in response to No (A), Very Low (B), and Low (C) Pb exposure (arrows). D-F) Similarly, VGAT immunostaining in the MNTB does not differ among the No (D), Very Low (E), and Low (F) Pb groups (arrows). (n=5 per group). Bar = 50 μm for panels A-F.

Figure 5. Quantification of immunostaining confirms that Pb exposure does not affect VGLUT1 (A and B), VGAT (C), or VACHT (D) expression levels in the SOC.

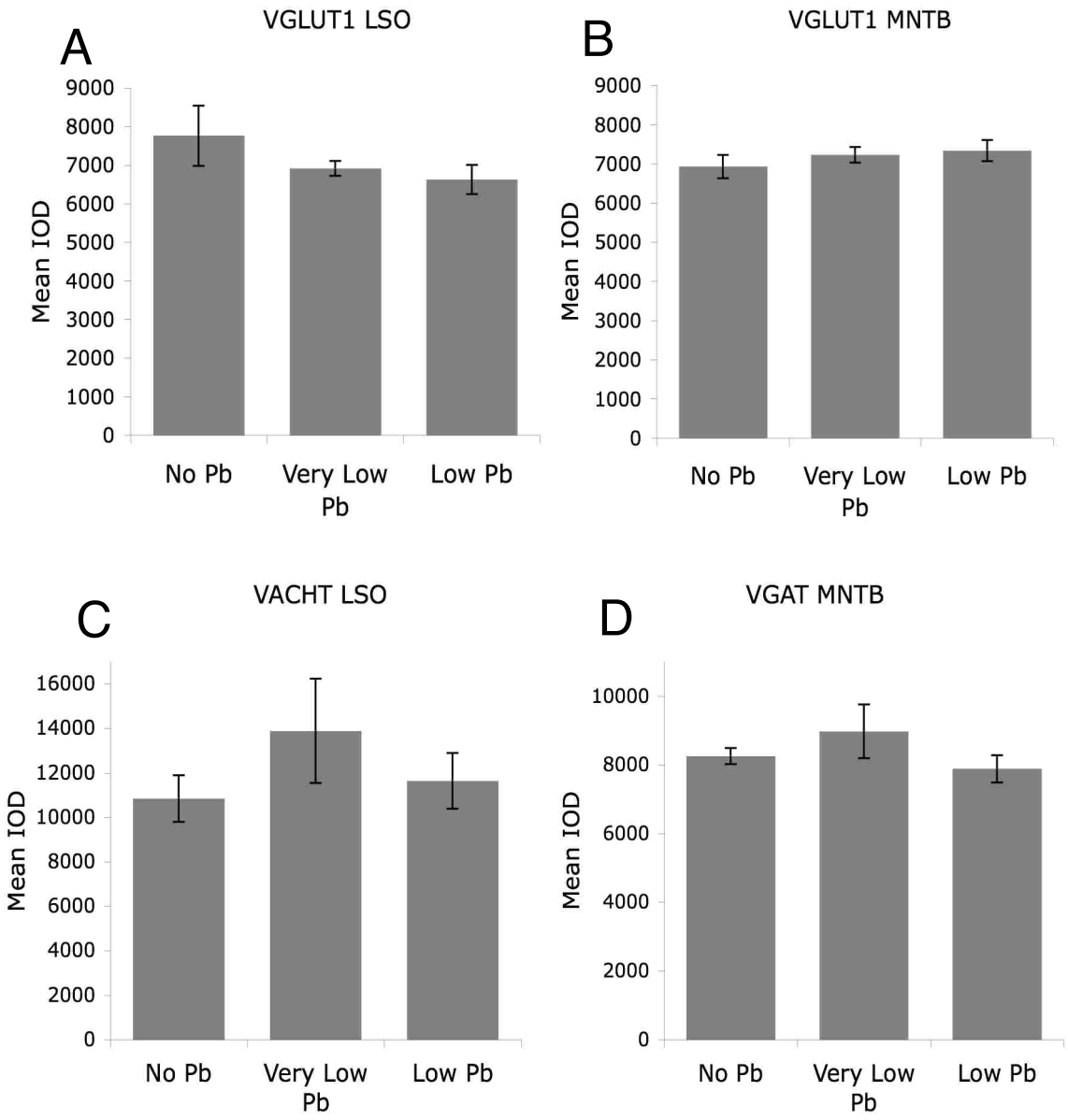

Figure 5. Quantification of immunostaining confirms that Pb exposure does not affect VGLUT1 (A and B), VGAT (C), or VACHT (D) expression levels in the SOC. Graphs illustrate mean + the standard error of the mean (SEM). (n=5 per group). One-way Anova with Tukey's all pairs comparison.

Figure 6 illustrates TH immunostaining in the LSO for all Pb treatment groups. Pb does not cause a decrease in TH immunostaining at either the very low or low dose of Pb (Figure 6), suggesting that dopamine expression does not change with Pb exposure. In contrast, immunoreactivity for both serotonin and DβH is significantly decreased with Pb (Figures 7 and 8). Serotonin expression is decreased approximately 29% compared to controls in the very low Pb group while DβH expression is decreased approximately 30% in both the very low and the low Pb groups (Figures 7 and 8).

In order to determine whether the decreased expression of VMAT 2, serotonin, and DβH correlated with a loss of synapses in LSO, brainstem sections were immunolabeled with antibodies to the synaptic vesicle protein, synaptophysin. Pb exposure results in a significant decrease in synaptophysin labeling within LSO (but not MNTB) that is similar in magnitude (39%) to the decreased expression of VMAT 2, serotonin and DβH (Figure 9 and10). This is particularly significant because in the mouse, LSO but not MNTB receives serotonergic input and this pattern of innervation differs from that in other mammals (Thompson and Hurley 2004). We also found little DβH staining in MNTB. The fact that synaptophysin labeling decreases in LSO but not MNTB suggests the Pb-induced loss of DβH and serotonin immunoreactivity within LSO is correlated with loss of synapses.

Figure 6. TH expression levels in the LSO are not altered with Pb treatment.

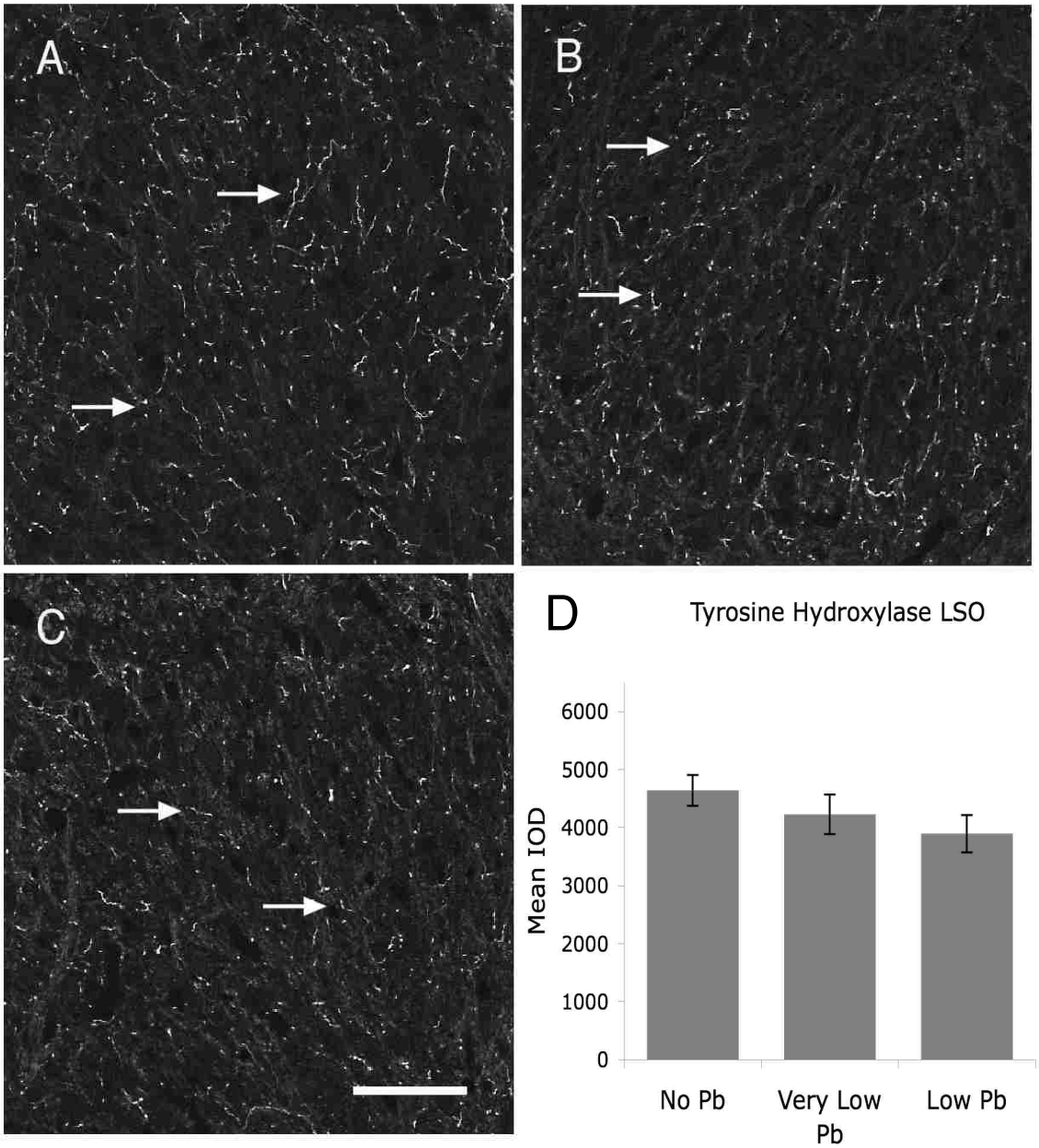

Figure 6. TH expression levels in the LSO are not altered with Pb treatment. A-C) Immunofluorescent staining for TH in the LSO in response to No (A), Very Low (B), and Low (C) Pb treatment shows no change in immunoreactivity (arrows). Quantification of TH immunostaining in the LSO confirms that Pb has not affect on TH immunostaining (D). Graphs illustrate mean + the standard error of the mean (SEM). (n=5 per group). One-way Anova with Tukey's all pairs comparison. Bar = 50 μm for panels A-C.

Figure 7. Pb treatment decreases 5-HT expression in the LSO.

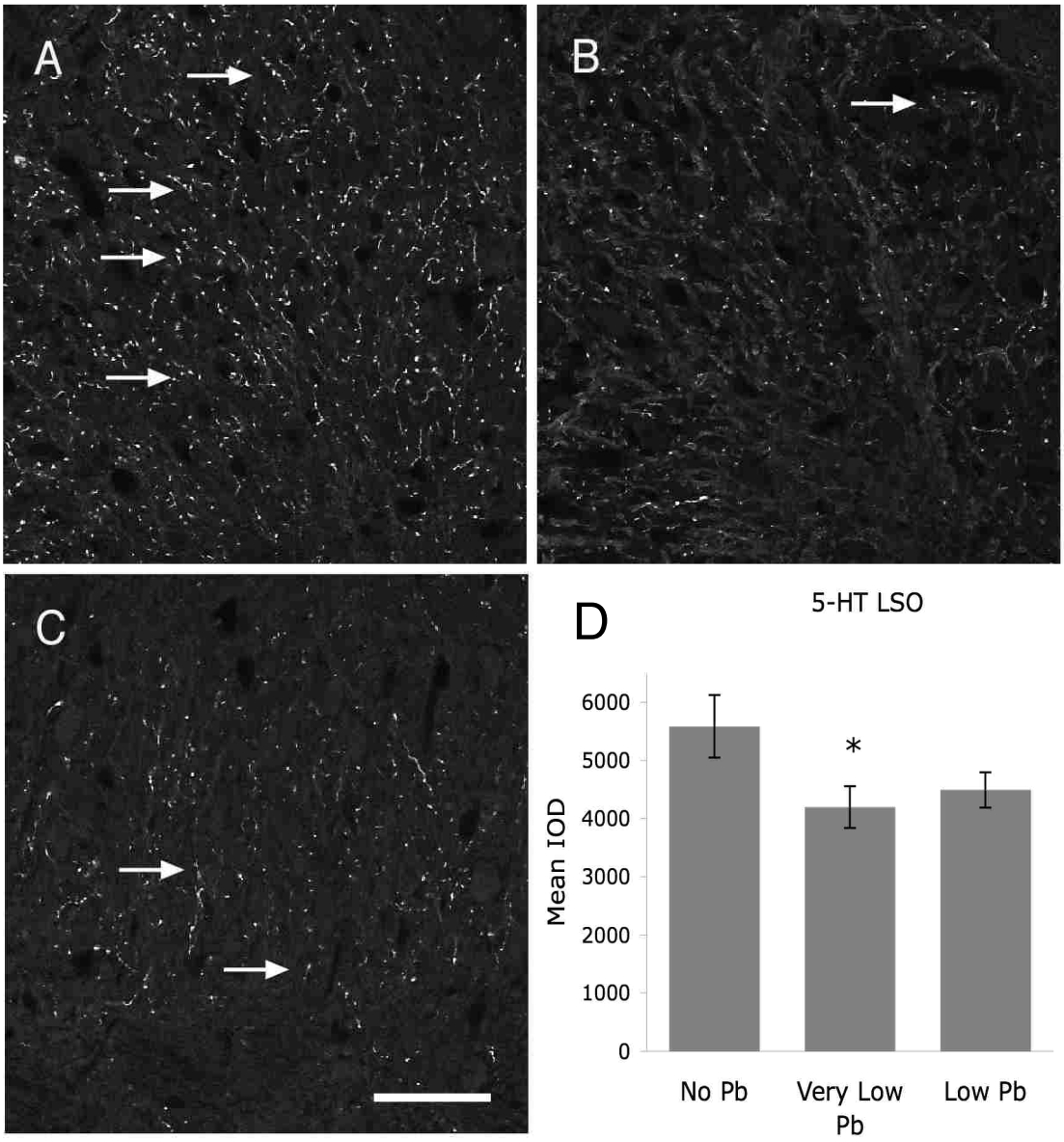

Figure 7. Pb treatment decreases 5-HT expression in the LSO. A-C) Immunoreactivity for 5-HT is decreased with both very low (B) and low (C) Pb compared to no Pb controls (A) (arrows). Quantification of 5-HT immunoreactivity in the LSO confirms that this decrease is statistically significant (D). The graphs illustrate mean + the standard error of the mean (SEM). (n=5 per group) *P < 0.05, One-way Anova with Tukey's all pairs comparison. Bar = 50 μm for panels A-C.

Figure 8. Pb decreases DβH expression in the LSO.

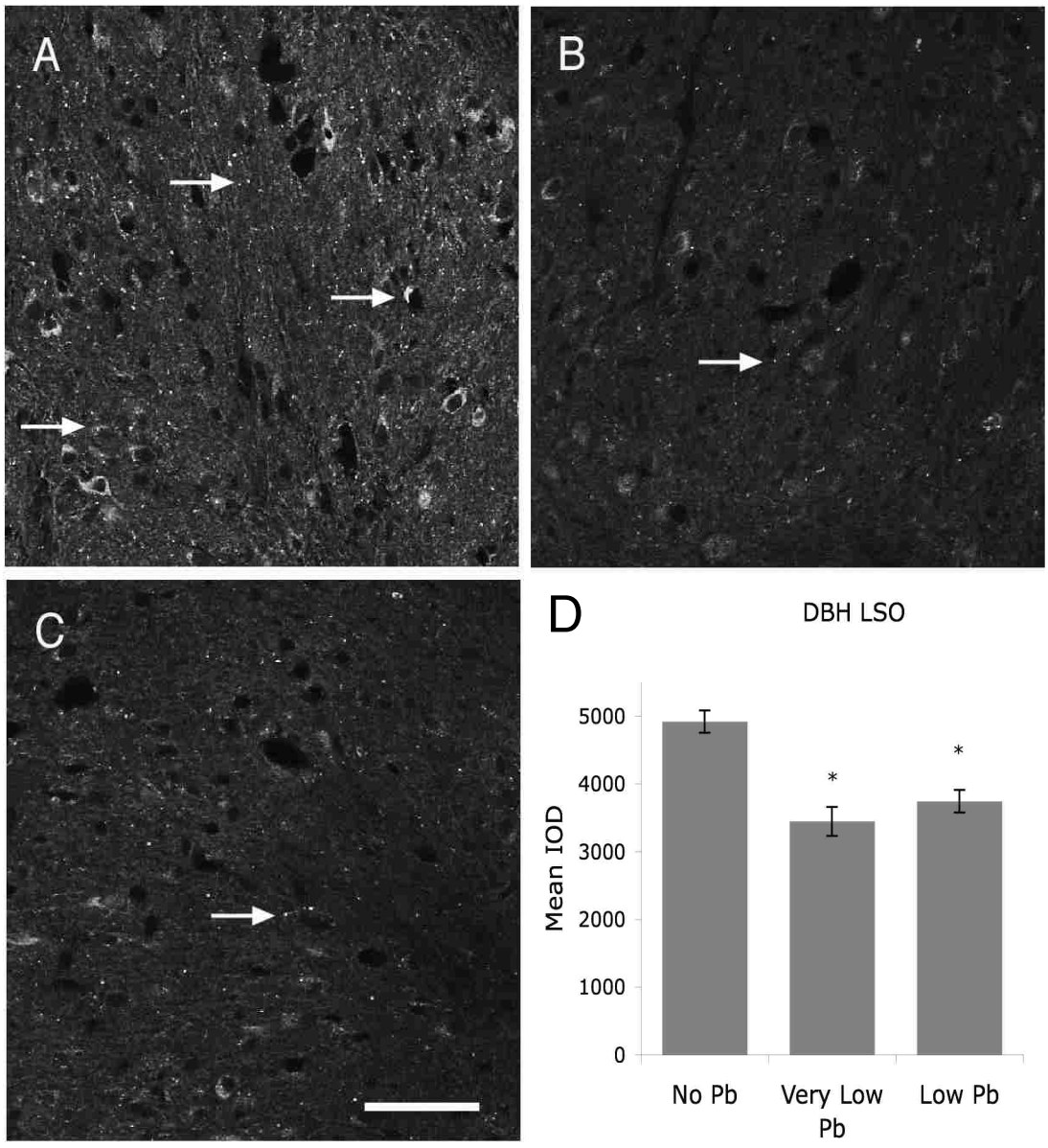

Figure 8. Pb decreases DβH expression in the LSO. A-C) Pb decreases immunoreactivity for DβH in Very Low (B), and Low (C) Pb treatment groups compared to no Pb controls (A) (Arrows). D) Quantification of staining for DβH in the LSO cofirms that this decrease is statistically significant. The graphs illustrate mean + the standard error of the mean (SEM). (n=5 per group) *P < 0.05, One-way Anova with Tukey's all pairs comparison. Bar = 50 μm for panels A-C.

Figure 9. Pb treatment decreases synaptophysin immunoreactivity within the LSO and but has no effect on synaptophysin staining in the MNTB.

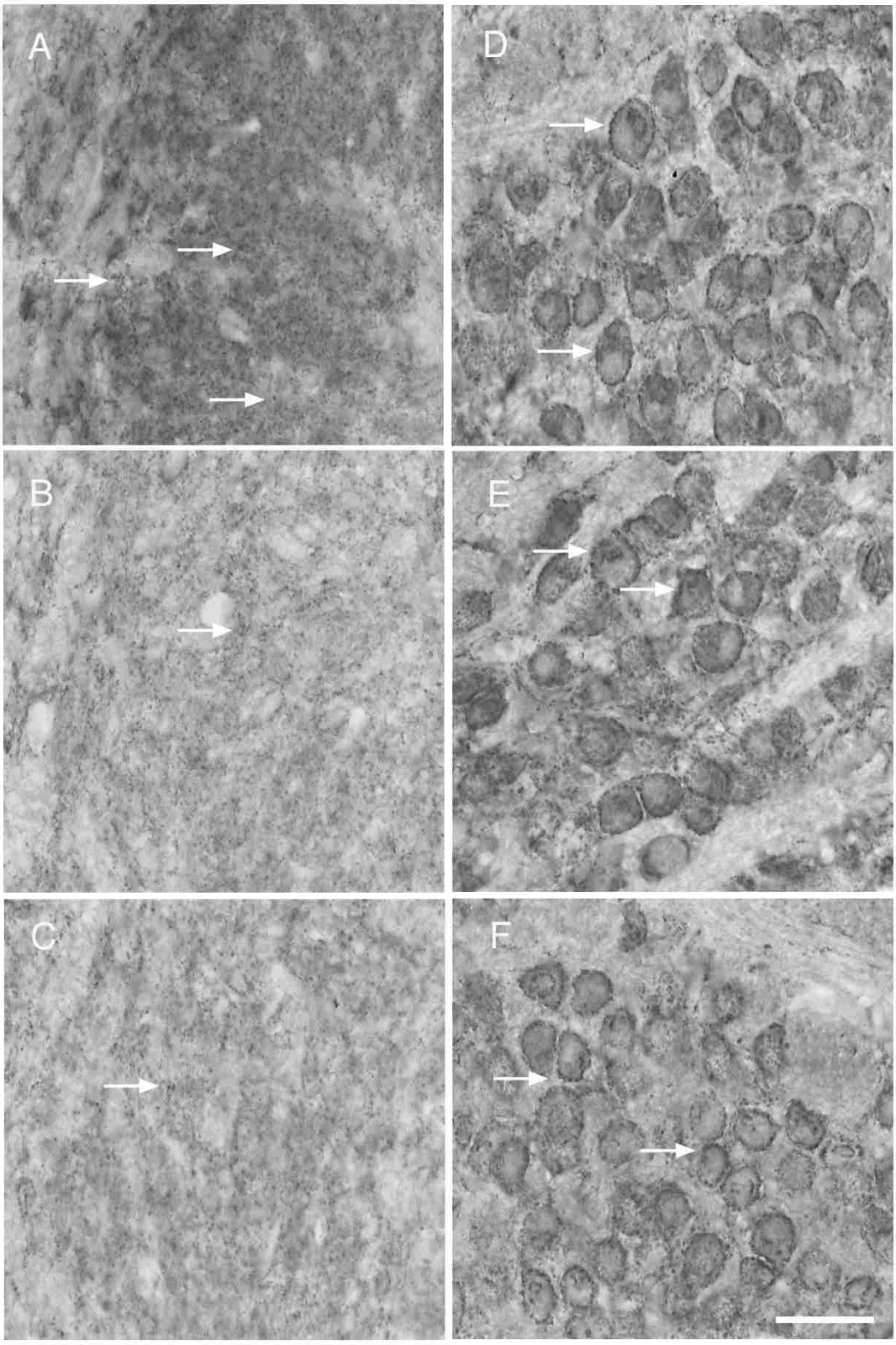

Figure 9. Pb treatment decreases synaptophysin immunoreactivity within the LSO but has no effect on synaptophysin staining in the MNTB. A-C) Very low (B) and low (C) Pb results in decreased immunoreactivity for synaptophysin compared to no Pb controls (A) (arrows). In contrast, MNTB synaptophysin immunostaining remains unchanged from controls (D) with very low (E) and low (F) Pb treatment. Bar = 50 μm for panels A-F.

Figure 10. Pb treatment results in a significant decrease in synaptophysin immunoreactivity in the LSO but not the MNTB.

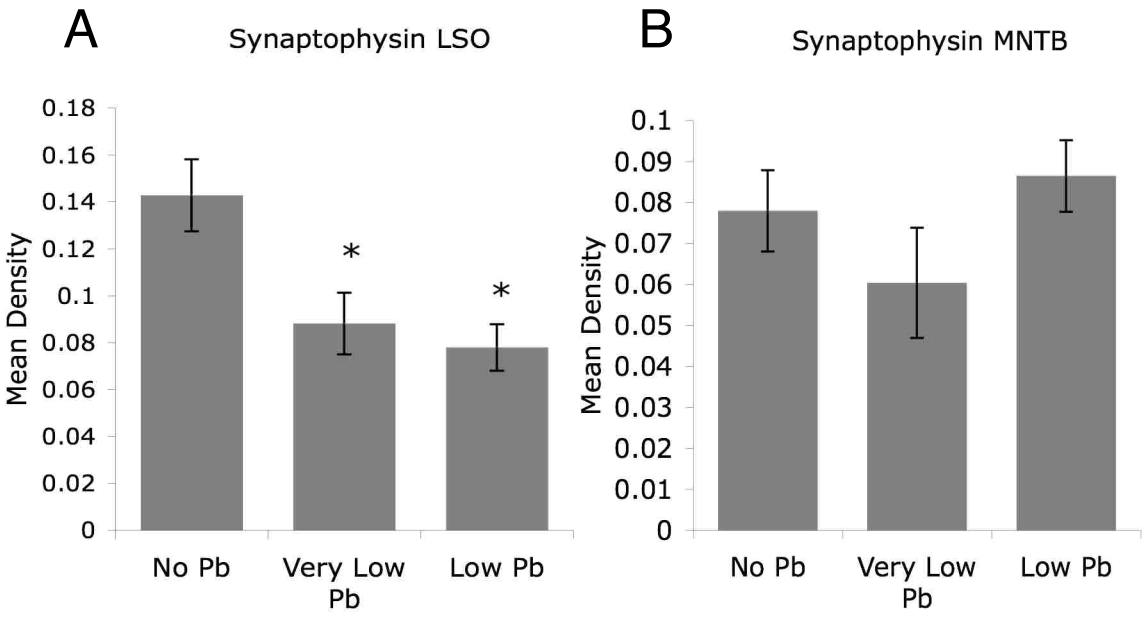

Figure 10. Pb treatment results in a significant decrease in synaptophysin immunoreactivity in the LSO but not the MNTB. A) Quantification of synaptophysin immunostaining in the LSO shows a significant decrease with Pb treatment. B) In contrast, quantification of synaptophysin expression in the MNTB reveals no significant decrease with Pb treatment. Graphs illustrate mean + the standard error of the mean (SEM). (n=4 per group) *P < 0.05 One-way Anova with Tukey's all pairs comparison.

Discussion

The current study demonstrates that developmental exposure to very low levels of Pb results in decreased immunoreactivity for VMAT 2, serotonin, DβH, and synaptophysin within the LSO. This effect appears to be specific for monoaminergic systems because no changes in protein expression level were observed for VGLUT1, VAChT, and VGAT within either LSO or MNTB, or the entire brainstem. In addition, immunostaining for TH did not change with Pb exposure, suggesting that dopamine levels are not altered by Pb. The loss of synpatophysin staining within LSO, but not MNTB, indicates that the decreased expression of VMAT 2, 5-HT, and DβH may be correlated with the loss of monoaminergic synapses. It is significant that the magnitude of the decreased expression for the monoaminergic markers and for synaptophysin is approximately 30%, lending further support to the hypothesis that Pb exposure results in loss of monoaminergic synapses within LSO. Indeed, preliminary studies in our laboratory suggest that Pb interferes with the loading of serotonin into synaptic vesicles. Double-labeling studies with serotonin and synaptophysin reveal that serotonin in the Pb exposed LSO is not localized within synaptic vesicles.

The role of the serotonin in LSO

The lateral superior olive forms part of the ascending auditory pathway to the midbrain and is thought to process interaural intensity cues for sound localization (Thompson 2006). LSO neurons project to the inferior colliculus but a subset of neurons

located within the LSO project predominantly ipsilaterally and form synapses onto inner hair cells. The functional role of this efferent system remains unknown but it has been hypothesized to modify or control binaural interactions, reduce the masking effects of background noise, protect the cochlea from noise-induced trauma, and alter the response of the cochlea to sound with changes in attention (Woods and Azeredo 1999);(Darrow, Maison et al. 2007). In the adult mouse, both ascending and descending projections that originate in the LSO receive input from the 5-HT system but LSO ascending neurons use glutamate and glycine as neurotransmitters and are not considered serotonergic (Kelly and Caspary, 2005; Thompson, 2006).

In addition, many 5-HT immunoreactive fibers are found in the inferior colliculus, a target for LSO neurons, and 5-HT has been shown to shift first-spike latencies, neuronal spike count, temporal precision, and the interspike interval of neurons in the inferior colliculus (Hurley and Pollak 2005). Thus, serotonin is considered to refine the representation of acoustic stimuli within the IC. For example, in free-tailed bats, calls become more unambiguous and specific in the presence of serotonin (Hurley and Pollak 1999). In addition, the acoustic startle response is increased with 5-HT depletion (Woods and Azeredo 1999).

Most of the serotonergic fibers found in the IC originate in the dorsal and median raphe nuclei (Klepper and Herbert, 1991) and indeed, the majority of serotonergic neurons within the brain are found in the dorsal and median raphe nuclei, an area that shows high activity levels in awake and alert animals and decreased activity in inattentive or sleeping animals (Hurley and Pollak 2005). While serotonergic neurons innervate

many areas of the brain including the auditory brainstem (Klepper and Herbert, 1991), the origin of the 5-HT fibers in LSO has not been fully defined.

Studies are currently underway in our laboratory to determine whether serotonin expression is decreased in other central auditory areas including the inferior colliculus. Preliminary results indicate that 5-HT immunoreactivity within the IC is reduced with Pb exposure, suggesting that Pb may reduce 5-HT expression within central auditory areas. It will be of importance to determine whether this decrease is restricted to auditory nuclei, or whether there is a decrease in 5-HT expression within raphe neurons themselves.

Noradrenergic Neurons and the auditory system

Both noradrenalin (NA) and 5-HT have been shown to modify auditory neural activity. NA applied directly to the cochlea results in increased absolute and masked auditory thresholds, indicating that NA is able to alter the ability of the auditory system to detect a signal in a noise background (Pickles, 1976). Noradrenergic nerve endings have been found within all nuclei of the SOC and in the rat, these terminals have been shown to originate in the locus coeruleus (Mulders and Robertson 2001). These NA terminals may influence the function of the lateral olivocochlear neurons, located in the LSO, that project to the inner hair cells within the cochlea. While the function of these cells is still unknown, it is intriguing to hypothesize that the NA input to LSO may play a role in detecting a signal in a noise background, as well as to modulate attention during auditory processing. Application of NA to auditory neurons has been shown suppress spontaneous firing rate to a greater extent than the stimulus evoked discharge in cortex,

suggesting that NA is able to increase the "signal to noise ratio" (reviewed in (Berridge and Waterhouse 2003)). The NE system is thought to enhance cognitive function under "noisy" conditions and the presentation of extraneous auditory stimuli impairs sustained attention in rats with forebrain NE depletion, even though these same rats perform normally under non-distracting conditions (Berridge and Waterhouse 2003).

Our finding that Pb exposure reduces DβH immunostaining within LSO is intriguing and further studies are needed to determine whether this effect is restricted to auditory nuclei or whether DβH immunostaining is decreased in other target regions of the locus coeruleus. It is important to note that dysregulation of the locus coeruleous-NE system is associated with cognitive disorders such as attention deficit/hyperactivity disorder (ADHD), and low level Pb exposure has also been shown to be a risk factor for ADHD (Glotzer, Freedberg et al. 1995; Berridge and Waterhouse 2003{Breier, 2003 #45; Braun, Kahn et al. 2006).

Pb exposure and the Monoaminergic System

Our finding that Pb exposure alters the monoaminergic system is consistent with previous work on the effects of Pb on the CNS. Numerous studies have shown decreased function of monoamine oxidase (MAO) following exposure to Pb as well as reduced NE and 5-HT levels in the brain (Devi, Reddy et al. 2005; Jaya Prasanthi, Hariprasad Reddy et al. 2005). However, the mechanism by which Pb reduces NA and 5-HT remains unknown. The current study demonstrates that Pb exposure reduces the expression of VMAT 2, the transporter that loads monamines into vesicles. The decreased expression of VMAT 2 might result in a short-term decrease in levels of 5-HT

and NE within the cytosol of synaptic endings, exposing them to increased degradation, and a long-term reduction in expression levels. We also found that Pb exposure results in decreased expression of the synaptic vesicle protein, synaptophysin, within LSO but not MNTB. This suggests that Pb is targeting the monoaminergic system because there is synaptic loss within LSO, but not MNTB, a nucleus that does not receive a large monaminergic input. Our preliminary studies indicate that in the Pb-exposed LSO, the remaining 5-HT is not localized to synaptic vesicles, lending support to the hypothesis that Pb interferes with the transport of 5-HT into synaptic vesicles. Alternatively, we cannot at this time rule out the possibility that Pb exposure might delay the development of monoaminergic synapses in LSO and the decreased expression of VMAT 2, 5-HT, and NE could be due to delayed development of these synapses. Future studies will address this issue.

In summary, the present study demonstrates that low level Pb exposure during development results in decreased immunoreactivity for VMAT 2, DβH, 5-HT, and synapotophysin within the murine LSO. Other neurotransmitter systems do not appear to be affected by Pb treatment, as VGLUT1, VGAT, and VAChT immunoreactivity remain unchanged following developmental Pb exposure. The decrease in synaptophysin immunoreactivity within LSO but not MNTB suggests that there is a loss of monoaminergic synapses. However, additional studies are needed to confirm that it is the monoaminergic synapses that are decreased with Pb exposure. Finally, the mechanism by which Pb affects the monoaminergic system remains to be elucidated. Clearly, further studies are needed to define the mechanism by which Pb affects VMAT 2 and the

monoaminergic system and to determine whether the effect of Pb is restricted to auditory nuclei or affects all serotonergic and noradrenergic targets.

Extended Discussion

The current study quantifies decreases in protein expression of VMAT2, DβH, 5-HT and synaptophysin in low-level Pb exposed mice. The cause of these alterations in the LSO is still not known but there are several possible mechanisms. One intriguing mechanism involves Pb's inhibitory actions on CREB and Sp1 that could lead to alterations in the transcription of VMAT2. Inhibition of CREB could also affect DβH and the combination of decreased transcription of VMAT2 and DβH with Pb exposure might be a mechanism by which Pb induces decreased protein expression within the monoaminergic system.

Alterations of CREB and Sp1 by lead

CREB (c-AMP response element binding protein) is a leucine zipper transcription factor responsible for transcribing many neuronal survival genes. In relation to the current study CREB is important for its ability to transcribe VMAT2 and its inhibition with Pb exposure. Pb, through a mechanism described later, causes the inactivation of CREB by dephosphorylating it. CREB phosphorylation occurs at the serine-133 residue and can be the result of many parallel pathways, but the CREB phosphorylation pathway that occurs with Pb's toxicity relies on intracellular Ca^{2+} homeostasis (Toscano,

McGlothan et al. 2003). Ca^{2+} has two major pathways for phosphorylating CREB. Intracellular calcium can bind to Ca^{2+} binding protein calmodulin (CaM), which can then activate CaM kinase I, CaM kinase II, and CaM kinase IV, all of which can phophorylate CREB. Ca^{2+} can also activate the Ras/ERK pathway, which leads to phosphorylation of CREB (Lonze and Ginty 2002; Wang, Fibuch et al. 2007). Pb can substitute for Ca++, and thus is positioned to potentially interfere with the phosphorylation of CREB. In addition, Pb can lower levels of intracellular calcium due to inactivation of NMDA receptors in glutamanergic neurons and result in decreased CREB phosphorylation. A decrease in CREB phosphorylation has been shown to result in lowered gene transcription (Toscano, Hashemzadeh-Gargari et al. 2002; Toscano, McGlothan et al. 2003; Toscano and Guilarte 2005).

CREB transcription has many ties to the current study. Phosphorylation of CREB has been shown to take place in both the LSO and MNTB nuclei of the SOC following unilateral cochlear ablation (Mo, Suneja et al. 2006). VMAT2 has a CRE coding site that is conserved in rat, mouse, and human, for which CREB is a proven transcription factor (Takahashi and Uhl 1997; Gerhard, Neumayer et al. 2001; Lonze and Ginty 2002; Zanner, Gratzl et al. 2002; Prinz, Zanner et al. 2003). Interestingly, VMAT2 has binding sites for both CREB and Sp1 and both activation sites are needed for transcriptional activity, implicating a role for Sp1(Takahashi and Uhl 1997; Watson, Deavall et al. 1999; Gerhard, Neumayer et al. 2001). In addition, VMAT2 transcription occurs with increased levels of intracellular calcium, supporting the hypothesis that Ca++ activation of CREB plays a role in VMAT transcription (Watson, Deavall et al. 1999). It should be noted that

DβH also has CRE coding sequences that could modulate protein expression levels (Lonze and Ginty 2002).

The effects of Pb on the Zn finger protein, Sp1, could also play a role in VMAT2 transcription (Takahashi and Uhl 1997; Gerhard, Neumayer et al. 2001). Pb directly competes with Zn in Sp1 binding and has a greater affinity for the Zn-binding site than Zn does (Basha, Wei et al. 2003). At very low levels, Pb increases Sp1 binding to DNA, but with chronic Pb treatment, Sp1 binding has been shown to significantly decrease by postnatal day 20 in rat hippocampus resulting in inhibited transcription (Basha, Wei et al. 2003). This may be one mechanism by which VMAT2 expression is decreased in our studies. In summary Pb^{2+} can produce transcriptional alterations by interfering with the transcriptional factors CREB and Sp1 in an inhibitory manner and this could possibly affect the transcription of both VMAT 2 and DβH. We saw no changes in VGLUT1, VGAT, and VACHT expression and it is important to not that CREB has not been implicated in the transcription of these proteins. Only VGAT has binding sites for Sp1 (Ebihara, Obata et al. 2003) but if the effect of Pb on CREB is playing a significant role in the auditory brainstem, then VMAT2 is the only protein we might expect to show any significant change with Pb treatment.

Lead and tyrosine hydroxylase

Further investigation into the changes on the monoaminergic system showed no decrease in tyrosine hydroxylase protein level. Tyrosine hydroxylase converts tyrosine to L-Dopa, a precursor for dopamine, and is also the rate-limiting step in dopamine synthesis (Pan, Berman et al. 2006; Kaushik, Gorin et al. 2007). TH immunostaining is

commonly used as a marker for dopamine and is also expressed in noradrenergic fibers. Interestingly, TH is also regulated by CREB in a similar method to VMAT2 and DβH (Sabban, Hebert et al. 2004; Shah, Nankova et al. 2006). Likewise TH possesses a similar neuron specific silencing factors, repressor element 1 (RE1), to DβH making it dificult to determine the reason for the differential regulation in the current study (Kim, Yang et al. 1998; Kim, Yang et al. 2006). Noradrenergic fibers are not only dependent on TH but it is also the rate-limiting step. Interestingly, another study has shown decreases in TH activity in rat brain following chronic Pb treatment but whole brain homogenate was used and the exposure differed from the current study (Jadhav 1997). It should be noted that the current study did not look for TH activity but TH protein expression, this expression was not changed by Pb treatment in noradrenergic fibers of the LSO. However, we cannot rule out the possibility that Pb affected TH activity.

Lead and the serotonergic system

Pb has also been shown to cause changes in serotonin within the brain A study by Antonino et al. demonstrated 5-HT decreases in the hippocampus, hypothalamus, cerebellum, and striatum following Pb exposure through dams milk during gestation in male rats (Antonio and Leret 2000). In contrast, Leret et al. 2002 found an increase in 5-HT in the mediobasal hypothalamus and rostral neostiatum and an increase in the ratio products of 5-HIAA to 5-HT in the dorsal hippocampus and mediobasal hypothalamus using a similar experimental protocol to Antonino et al, 2000. Another study by Kala et al. demonstrated that Pb exposed rats had 5-HT decreases in the nucleus accumbens, frontal cortex, and brainstem but showed no changes in striatum, hypothalamus and

hippocampus over a 90 day period (Kala and Jadhav 1995). Further, a study by Szczerbak showed no changes in 5-HT levels in striatum or prefrontal cortex in 12-week-old rats (Szczerbak, Nowak et al. 2007). Interestingly, Jaya Prrasanthi et al. found several brain regions that had increased 5-HT levels in a 0.2% Pb treatment group and decreased levels in a 1% Pb treatment group (Jaya Prasanthi, Hariprasad Reddy et al. 2005). It is clear that 5-HT can be modulated by Pb but the direction of change is dependent on species, age, and treatment regime.

The results found in the current study mostly correlate with several previous studies by showing decreases expression for proteins of the monoaminergic system including VMAT 2, noradrenalin and serotonin. The dephosphorylation of CREB by Pb result in decreased expression of VMAT 2. Decreased expression of VMAT 2 might result in a short-term increase in levels of 5-HT and NE within the cytosol of synaptic endings, exposing them to increased degradation, and a long-term reduction in expression levels. A long-term reduction in expression levels would significantly alter auditory neurotransmission in the brain.

The serotonergic neurotransmitter system significantly modifies auditory signaling in the brain. The serotonergic system also innervates most of the ascending auditory pathway and is able to modulate auditory signaling (Hurley and Pollak 1999). Serotonergic innervation of the SOC originates in the dorsal raphe nuclei; an area that has its highest levels of activity in awake and alert animals and lowest levels of activity in unfocused or sleeping animals (Hurley and Pollak 1999). It has been previously shown that alterations in the serotonergic inputs from the raphe nuclei to the inferior colliculi can alter complex species-specific vocalizations (Hurley, Thompson et al. 2002;

Thompson and Hurley 2004; Hall and Hurley 2007). Serotonin has been shown to modulate neuronal spike count, first-spike latency, temporal precision, and the interspike interval, all of which may alter temporal processing (Hurley and Pollak 2005). In the cochlear nucleus, 5-HT application has been shown to inhibit spontaneously active neurons (Ebert and Ostwald 1992). 5-HT levels as measured by HPLC, increased as the intentsity of white noise increased in the cochlear nucleus (Cransac, Cottet-Emard et al. 1998). Several studies have investigated the signal altering effects of serotonin in auditory transmission. Latency, one of the most important modulatory effects, can vary in effect with stimulation characteristics. For example, in some echolocating bats the wide range of latencies of neurons in the inferior colliculus is responsible for calculating target range and location of sound (Hurley and Pollak 1999). More specifically serotonin refines the representation of acoustic stimuli. In free-tailed bats, calls become more unambiguous and specific in the presence of serotonin (Hurley and Pollak 1999). Further, the acoustic startle response is increased with 5-HT depletion. Pb alters many of the same auditory temporal characteristics as serotonin, providing a link between serotonin and the behavioral deficits caused by Pb (Woods and Azeredo 1999).

Lead and the noradrenergic system

The noradrenergic fibers of the LSO showed a large decrease in DβH staining in both the very low and low treatment groups in the current study. Previous studies have found both increases and decreases in DβH following Pb exposure. The Antonino group found decreases in noradrenalin in the cerebellum, striatum and hypothalamus, but found no change in the hippocampus following Pb exposure through dams milk during gestation

in male rats (Antonio and Leret 2000). A later study by Devi et al. showed decreases in noradrenalin levels in the cerebral cortex, hippocampus and cerebellum of rats exposed to 1% Pb but found no changes at 0.2% Pb in drinking water at postnatal day 21(Devi, Reddy et al. 2005). Similarly, Prasanthi et al. found several brain regions with increases in noradrenalin levels in 0.2% Pb treatment group and decreases in the 1% Pb treatment group. Another study showed no changes in noradrenalin levels in striatum or prefrontal cortex in 12-week-old rats exposed to low levels of Pb through dams milk (Szczerbak, Nowak et al. 2007). The problem once again remains the lack of a consistent model in these Pb exposure studies.

The noradrenergic system is aligned with serotonin in terms of neuronal modulation. In general, stimulation of noradrenergic fibers has a depressive effect on auditory neurons. This resuls in lowered levels of spontaneous firing, while maintaining the neuronal response to specific synaptic input. The end result is an increased signal to noise ratio (Berridge and Waterhouse 2003). Further, noradrenalin has been shown to enhance auditory temporal contrast by using phasic alteration of neuronal activity to selectively increase tone stimuli and inhibit tonic auditory response components (Kossl and Vater 1989). In some instances noradrenergic neurons enhanced the efficacy of both excitatory and inhibitory inputs. One study showed systemic and local injections of noradrenalin to the cochlear nucleus increased frequency selectivity of neuronal responses, demonstrating the ability of noradrenalin to extract a signal out of noise background (Cransac, Cottet-Emard et al. 1998).

DβH is the protein that converts dopamine to noradrenalin inside noradrenergic synaptic vesicles. Primarily, the effect of a decrease in DβH would be less readily

releasable noradrenalin and DβH is commonly used as a marker for noradrenalin. Decreased VMAT2 would also reduce the amount of dopamine that reaches the interior of synaptic vesicles resulting in less dopamine that can be converted to noradrenalin. Another factor is Cu^{2+}, a divalent cation similar to Pb, that has the ability to inhibit the v-ATPase activity, lowering the proton concentration gradient that is needed to drive VMAT2 co-transport (Wimalasena, Wiese et al. 2007). Pb could possibly mimic Cu^{2+} and also inhibit v-ATPase activity, which would mean less noradrenalin in the synaptic vesicle pool. Less noradrenalin would mean more spontaneous firing, less noradrenalin for phasic transmission, and a lowered signal to noise ratio of auditory nerves. Postsynapticaly, Pb has also been reported decrease β-adrenergic receptor density (Kala and Jadhav 1995; Tsao, Yu et al. 2000).

Noradrenergic fibers have two characteristic releasing patterns, tonic and phasic. Tonic activity consists of sustained, low-frequency and regular discharge patterns that change most notably with sleep wake cycles. Phasic activity, in contrast, consists of short latency, brief burst action potentials followed by prolonged suppression of discharge activity and are characteristic of overt attending to a novel stimulus within a particular environmental location (Berridge and Waterhouse 2003). Further phasic activity is weaker in times of lowered tonic discharge levels and lower levels of vigilance (Berridge and Waterhouse 2003). Previous studies have shown noradrenalin's enhancing action is restricted to phasic neuronal activity at the onset of tone stimuli (Kossl and Vater 1989). A phasic response could easily be hindered by depletion of readily releasable noradrenalin . While the role of noradrenalin in the LSO has not been defined, it is possible that Pb could alter the noradrenergic systems phasic responses, resulting in

lowered attention to novel stimuli. This is descriptive of ADHD and might explain in part why Pb is a risk factor for ADHD.

Lead and monoamine oxidase

Another area of interest is monoamine oxidase activity in Pb exposed animals. Several studies using different models have shown decreases in monoamine oxidase activity (Devi, Reddy et al. 2005; Jaya Prasanthi, Hariprasad Reddy et al. 2005). In contrast, Leret et al. shows an increase in the degradation product for both dopamine and 5-HT in several brain regions following Pb exposure (Leret, Garcia-Uceda et al. 2002). This would imply increased function or increased protein expression for monoamine oxidase. The current study did not determine the activity of monoamine oxidase but 5-HT decreases could be caused by increased degradation as a result of decreased vesicular storage. Future studies will investigate Pb-induced changes in the activity of MAO should be investigated in our system.

In summary, the decrease in VMAT2 protein expression levels following Pb exposure could be the result of CREB-induced changes in VMAT 2 transcription. Lowered expression of VMAT2 could in turn alter noradrenalin and serotonin storage in synaptic vesicles. The effects on noradrenalin would be further increased by the decreases in the noradrenalin-synthesizing enzyme, DβH, which would decrease the readily releasable pool of noradrenalin. The decrease in storage of neurotransmitters leaves them open for degradation in the cytosol further reducing expression levels and altering synaptic transmission. The decrease within the monoaminergic system in LSO is correlated with synaptic loss as measured by synaptophysin immunohistochemistry. The

resulting reduction in monoaminergic transmission could cause alterations in auditory temporal processing that include a decreased signal to noise ratio, and could explain many of the behavioral effects of developmental Pb exposure.

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