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THE EFFCT OF LOW-LEVEL LEAD ON SEROTONIN EXPRESSION IN THE DEVELOPING MOUSE SUPERIOR OLIVARY COMPLEX AND SOMATOSENSORY CORTEX

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The effect of low-level Pb exposure on serotonin expression in the developing mouse superior olivary complex and somatosensory cortex

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Abstract

Low-level Pb exposure is a risk factor for neurobehavioral and cognitive deficits in both humans and animals. These neurological dysfunctions have been associated with deficits in auditory temporal processing. The developing central nervous system is particularly susceptible to Pb exposure, and Pb decreases the immunoreactivity of serotonin (5-HT) and VMAT2 in the lateral superior olive (LSO). During early developmental, "non-serotonergic" sensory neurons, including LSO neurons and thalamocortical neurons of the somatosensory system, transiently take up 5-HT from the extracellular environment through the transient expression of the serotonin reuptake transporter SERT. Maintenance of appropriate 5-HT levels is important for development of these sensory systems. The current study was undertaken to define the effect of developmental Pb exposure on the transient uptake of 5-HT in LSO and thalamocortical neurons and is the first of its kind. CBA mice were exposed to 0 mM (control), 0.01 mM (very low), and 0.1mM (low) Pb acetate from gestation through postnatal day 4 (P4), P8, or P21. Brainstem sections were immunostained for 5-HT, SERT, TPH, MAOA, VMAT2, SYP and GAP-43. Total brainstem levels of 5-HT and its metabolite 5-hydroxindole-3-acetic acid (5HIAA), were measured by HPLC. We found that Pb extends the normal developmental uptake of 5-HT by LSO neurons through prolonged expression of SERT. Total brainstem levels of 5-HT remain largely unchanged. Pb also decreases VMAT2, SYP, and GAP-43 immunostaining within the P8 LSO, indicating that modulation of SERT by Pb leads to impaired maturation of synapses in the LSO. These effects persist into adulthood. The major afferent target of the LSO, the Inferior Colliculus (IC), also shows altered immunoreactivity for 5-HT and VMAT2 following developmental Pb exposure. Finally, Pb decreases the immunoreactivity of 5-HT at the thalamocortical axon terminals (TCAs) in the region of the somatosensory cortical barrel field. In the forebrain, Pb appears to decrease 5-HT levels in general. This differs from its effect in the brainstem where Pb appears to specifically target central auditory nuclei.

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General Introduction

Lead toxicity

Lead (Pb) has long been recognized as a significant environmental pollutant with neurotoxic effects causing behavioral and cognitive deficits in humans (Finkelstein et al., 1998; Canfield et al., 2003; Lanphear et al., 2005) and animals (Vazquez and Pena de Ortiz, 2004; Burger and Gochfeld, 2005). Major environmental sources of Pb include Pb-based paints, food/drink cans, gasoline, and drinking water (Toscano and Guilarte, 2005). Pb-based paint, used between 1884 and 1978, is one of the major sources of Pb exposure in young children and accounts for exposure in 38 million living units in the United States in 2002 (Jacobs et al., 2003). Drinking water can be contaminated by lead pipes and a recent study of the Washington, DC area by the CDC showed that about 18% of households in the DC area have Pb service pipes (Toscano and Guilarte, 2005). Canned food containing Pb solder was the major dietary source of Pb before 1995 (Goyer, 1996). Emmisions from the combustion of leaded fuels was a major source of atmospheric and soil Pb accumulation (Toscano and Guilarte, 2005).

Regulatory initiatives limiting the lead content of paint and gasoline reduced the median blood Pb levels in children from 15 μg/dL in the late 1970s to 1.9 μg/dL in 2002 (Bellinger, 2008). The removal of Pb solder from canned foods have reduced the average dietry intake of Pb in a 2-year-old child from 30 μg/dL in 1982 to about 2 μg/dL in 1991 in the United States (Toscano and Guilarte, 2005). However, Pb still persists in the environment. Pb paint and pipes remain in old residences and off-road vehicles, such as farm tractors, still use leaded gasoline. In addition, Pb is found in toys with leaded paint,

cosmetics, folk medicines, glazed ceramics, bullets, and storage battery casings (Bellinger and Bellinger, 2006; Bellinger, 2008).

The developing central nervous system (CNS) is particularly susceptible to environmental Pb exposure (Landrigan and Todd, 1994; Moreira et al., 2001), thus children are most vulnerable. Currently, the Center for Disease Control (CDC) limit of concern for childhood blood Pb level remains at 10 μg/dL, and this level is thought to be a threshold for childhood cognitive deficits (Landrigan, 2000). However, recent data suggests that detrimental effects of Pb on childhood cognition may be present at blood Pb levels lower than 10 μg/dL (Bellinger, 2008). Children with blood Pb level of 5-10 μg/dL have a 5.0 point total lower IQ score, with 7.8 points lower in reading scores, and 6.9 points lower in math scores, compared to children with blood Pb levels of 1-2 μg/dL (Surkan et al., 2007). Lanphear et al. reported that in children with blood Pb levels lower than 5 μg/dL, every 1 μg/dL increase of blood Pb concentration results in a 0.7 point decrease of mean arithmetic scores, an approximate one point decrease in mean reading scores, a 0.1 point decrease in nonverbal reasoning scores, and a 0.5 point decrease of short-term memory scores (Lanphear et al., 2000). In addition, not only do the studies suggest the existence of adverse effect of Pb below 10 μg/dL, but also that the rate of decline in IQ scores may be greater at blood Pb levels below 10 μg/dL than at levels above 10 μg/dL (Bellinger and Bellinger, 2006). Canfields et al. reported that in the children whose maximal blood Pb levels remain lower than 10 μg/dL, an increase of blood Pb level from 1 to 10 μg/dL results in an IQ decline of 7.4 points, whereas each increase of 10 μg/dL in the lifetime blood Pb level is associated with a 4.6 point decrease in IQ (Canfield et al., 2003).

Pb is also a risk factor for neurobehavioral deficits in children (Bellinger and Bellinger, 2006). For example, a recent study found that children with blood levels greater than 2.0 μg/dL are at a 4.1-fold increased risk for attention deficit hyperactivity disorder (ADHD), and the authors conclude that approximately 290,000 cases of ADHD among U.S. children 4–15 years old can be attributed to environmental Pb exposure (Braun et al., 2006). Aggression, explosive temper, and antisocial behavior related with crime rate have also been shown to be related to childhood Pb exposure (Nevin, 2007).

According to the estimation by the US EPA in 2001, a reduction in a single IQ point results in an added financial burden of $8,346 dollars per Pb exposed child (Toscano and Guilarte, 2005). Grosse et al. reported that the economic benefits of the IQ gain resulting from the substantial reduction in children's blood Pb levels between 1976 and 1999 is $110 to $319 billion dollars for each year's cohort of 2-year-old children (Grosse et al., 2002).

In spite of the extensive documentation of the toxic effects of Pb on cognitive and neurobehavioral functions, the underlying mechanisms by which Pb exerts its effect on the central nervous system, especially during development, has yet to be defined. Several studies suggest that exposure to Pb during gestation and the early postnatal period produces greater deficits in learning performances than Pb exposure in older animals (Jett et al., 1997; Kuhlmann et al., 1997). Thus, there seems to be a critical period of brain development in which neuronal processes are highly vulnerable to the presence of Pb, leading to cognitive and behavioral abnormalities (Toscano and Guilarte, 2005). In general, Pb has been implicated in diverse processes such as mitochondrial dysfunction, oxidative stress, decreased cellular energy metabolism, altered regulation of

gene transcription, and alterations in neurotransmitter systems (Toscano and Guilarte, 2005; Bellinger and Bellinger, 2006).

Lead and auditory temporal processing

Compared to the intensive studies conducted on the effect of developmental Pb exposure on cognitive disorders, little effort has been made to understand the impact of Pb exposure on sensory systems, including auditory and visual processing. For example, auditory processing is vital for speech perception, especially in difficult listening situations when auditory signals can be masked due to background noise. Subtle impairments of auditory processing by Pb exposure could therefore have profound long term effects on learning and memory (Otto and Fox, 1993). In fact, Pb exposure is a risk factor for dyslexia (Glotzer et al., 1995) and ADHD (Braun et al., 2006), and children with either dyslexia or ADHD have deficits in auditory temporal processing, including backward masking (Breier et al., 2003; Facoetti et al., 2003; Putter-Katz et al., 2005; Wright and Conlon, 2009) and amplitude modulation detection (McAnally and Stein, 1997).

It is important to note that Pb appears to affect auditory temporal processing in the central auditory system, rather than producing sensorineural hearing loss at peripheral sites, including the cochlea and spiral ganglion (Otto and Fox, 1993). Children who are exposed to Pb show altered auditory processing and a decreased performance in tests requiring appropriate timed reactions (Finkelstein et al., 1998). An electrophysiological study in asymptomatic Pb-exposed children also shows increased latencies in the auditory brainstem response (ABR) in waves III and V and the interpeaks I-III and I-V (Holdstein

et al., 1986). Impaired central auditory processing by Pb exposure is also found in animal models. Our lab has demonstrated that Pb exposure in mice during development results in increased latencies in the ABR as measured in the interpeak interval between peaks I-V as well as impaired temporal processing within the inferior colliculus (IC) (Jones et al., 2008).

The cellular mechanism that underlies Pb-induced auditory temporal processing deficits is largely unknown. In 1999, Gray and Holian developed an avian model, in which Pb exposure during development results in deficits in auditory temporal processing, including backward masking (Gray et al., 1999). Using this avian model, our lab demonstrated that Pb exposure produces a significant decrease in the amount of medium weight neurofilament (NFM) protein as well as decreases in the NFM phosphorylation in the axons connecting auditory nuclei in the brainstem (Lurie et al., 2006). Changes in the neurofilament protein were also observed in a mouse model. In mice exposed to Pb during development, Pb results in increased phosphorylation of both the medium and high weight neurofilament within the auditory brainstem nuclei (Jones et al., 2008). Neuritic beading in the axons within auditory region of the brainstem also increases with Pb exposure, suggesting that Pb could affect axonal transport, and possibly impairing synaptic transmission of neural signals in respond to sound (Jones et al., 2008). This in turn, might affect auditory temporal processing.

Serotonin and auditory temporal processing

While the physiological basis of auditory temporal processing has yet to be fully elucidated, serotonin (5-HT), as a neuromodulator, has been shown to modify responses

to sensory stimuli, rather than to elicit neural firing directly. Especially in the IC, a midbrain auditory nucleus, 5-HT has been shown to preferentially facilitate or depress the response of some IC neurons to certain sounds, ranging from simple tone bursts to complex-species specific vocalizations, thus altering both the magnitude and latency of neural responses (Hurley and Pollak, 1999; Hurley et al., 2002; Hurley and Pollak, 2005a, b). For example, ionophoretic application of 5-HT in the IC of Mexican free-tailed bats results in altered first-spike latencies and spike counts, depending on the type of neuron within the IC (Hurley and Pollak, 2005a). In this study, 5-HT has been shown to create a unique spatiotemporal pattern of activity among neurons within the IC in response to specific vocalizations (Hurley and Pollak, 2005a). 5-HT also has been shown to play a neuromodulatory role in the in the dorsal and posteroventral cochlear nucleus, in that application of 5-HT to cochlear neurons inhibit acoustically evoked neuronal firing, indicating that 5-HT could be used to control background noise (Cransac et al., 1998). Thus, 5-HT plays a key role in modulating auditory temporal processing.

Because previous studies have demonstrated that Pb induces auditory temporal processing deficits, it is possible that Pb alters the serotonergic system within central auditory nuclei, thereby affecting auditory temporal processing. In support of this hypothesis, our lab has found that developmental Pb exposure results in decreased levels of 5-HT and norepinephrine (NE), and decreased expression of VMAT2 in brainstem auditory nuclei, especially in the lateral superior olive (LSO) (Fortune and Lurie, 2009).

Serotonergic innervation in the central auditory system

Serotonin (5-HT) and its receptors are present in the auditory brainstem and midbrain nuclei, including the superior olivary complex (SOC), cochlear nucleus (CN) and the inferior colliculus (IC) (Thompson et al., 1994; Behrens et al., 2002; Hurley et al., 2002; Thompson and Hurley, 2004; Hurley et al., 2008). While most of the serotonergic neurons innervating the IC and CN originate in the dorsal and, to a lesser extent, the median raphe nuclei (Klepper and Herbert, 1991; Thompson et al., 1991; Thompson et al., 1994), the origin of the 5-HT fibers in LSO has not been fully defined. It is likely that there is more than one source of serotonergic afferents to the SOC (Harvey et al., 1993), and dorsal and median raphe nuclei are not the major source of the serotonin found in the SOC (Thompson and Thompson, 2001).

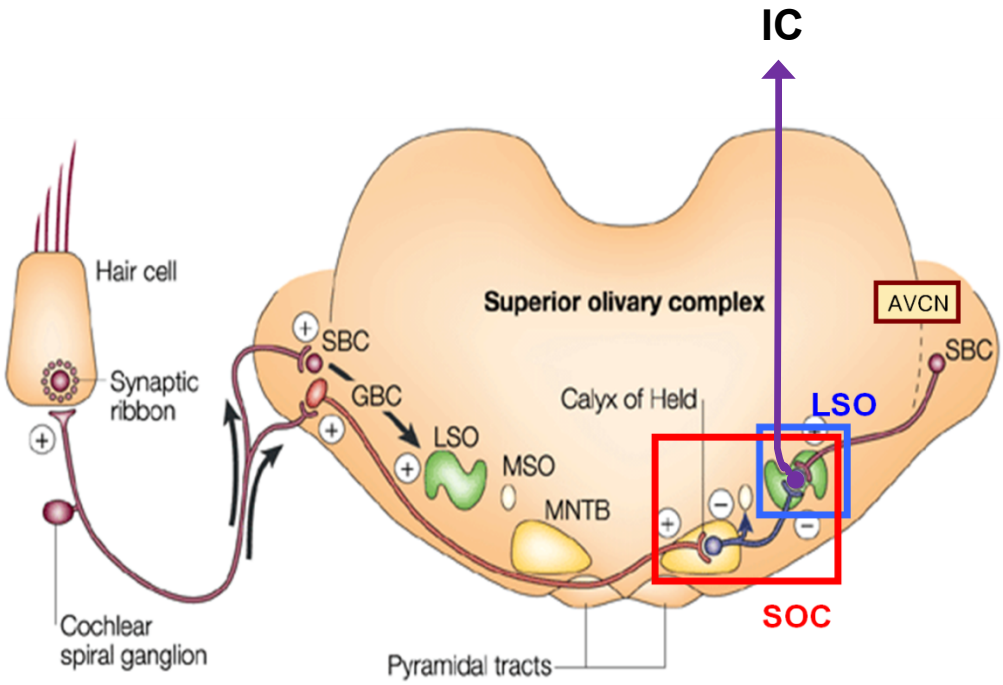
Various serotonin receptors are present in the SOC, CN, and IC (Harvey et al., 1993; Thompson et al., 1994; Thompson et al., 1995). Immunohistochemical studies reveal that 5-HT1A and 5-HT2B receptors are localized in the CN (Thompson et al., 1994; Tadros et al., 2007). In the IC, seven main families of 5-HT receptor have been detected using techniques including radioligand binding, immunohistochemistry, and in situ hybridization (e.g., 5-HT1: (Thompson et al., 1994; Peruzzi and Dut, 2004); 5-HT2: (Cornea-Hebert et al., 1999); 5-HT3: (Morales et al., 1998); 5-HT4: (Vilaro et al., 2005); 5-HT7: (To et al., 1995; Heidmann et al., 1998). Several reports have suggested that members of the 5-HT1 receptor family are especially strongly represented, with a radioligand binding study indicating enrichment of the 5-HT1A receptor in the IC (Thompson et al., 1994). An immunohistochemical study further suggested that 5-HT1A and 1B receptors are present on many IC neurons (Peruzzi and Dut, 2004). Iontophoretic

application of the 5-HT1A receptor agonist, 8-OH-DPAT, results in either increased or altered spike counts, as well as changes in the spike temporal pattern as measured by first spike latency in the IC (Hurley and Pollak, 2005a; Hurley, 2006). In the LSO, 5-HT1 and 5-HT2 receptors are involved in the modulation of LSO synapses by inducing evoked excitatory postsynaptic currents (EPSCs) and depressing inhibitory postsynaptic currents (IPSCs) (Fitzgerald and Sanes, 1999).

Serotonin in the developing central auditory system

Serotonin (5-HT) has also been implicated in the development of two auditory nuclei, the anteroventral cochlear nucleus (AVCN), which is a part of the cochlear nuclear complex, and the lateral superior olive (LSO), which is a part of the superior olivary complex (SOC) (Cases et al., 1998). The AVCN provides a major afferent input to the ipsilateral LSO, and the LSO, in turn, provides afferents to the inferior colliculus (IC) (Figure 1, (Thompson and Schofield, 2000; von Gersdorff and Borst, 2002).

In MAOA knockout mice with abnormally high levels of 5-HT, almost all the cell bodies in LSO contain 5-HT immunostaining starting from embryonic day 18 (E18), and reaching maximal expression at birth (P0) to postnatal day 7 (P7). This 5-HT immunoreactivity then disappears by P10 (Cases et al., 1998). 5-HT immunoreactive LSO neurons are also present in wild type mice examined at P1 and P8 (Thompson, 2006). LSO ascending neurons use glycine and glutamate as a neurotransmitter and thus are not considered being intrinsic serotonergic neurons (Thompson and Schofield, 2000).



Modified from (Gersdorff and Borst, 2002)

Figure 1. Diagram of the auditory brainstem. Auditory signals arriving at the cochlea are transmitted to the ipsilateral anterior ventral cochlear nucleus (AVCN). The AVCN then provides a major afferent input to the ipsilateral lateral superior olive (LSO), and the LSO, in turn, provides afferents to the inferior colliculus (IC). The LSO is a part of the superior olivary complex (SOC) that is composed of the medial nucleus of the trapezoid body (MNTB) and the medial superior olive (MSO) in addition to the LSO.

Developing LSO neurons do not have the capability of synthesizing 5-HT, as evidenced by a lack of immunoreactivity for the 5-HT synthesizing enzymes, tryptophan hydroxylase (TPH) (Thompson, 2008) and L-amino acid decarboxylase (AADC) (Cases et al., 1998). Instead, it is thought that these LSO neurons take up extracellular 5-HT through the 5-HT transporter (SERT) (Thompson and Thompson, 2009b). In MAOA k/o mice, 5-HT and 5-HTT staining is co-localized in LSO neurons at P0 and P5-6, and all staining for both proteins disappears by P15 (Thompson and Thompson, 2009b).

Transient expression of 5-HT during peri and postnatal development in LSO neurons is proposed to have two functional applications. First, 5-HT may be involved in the maturation of synapses within the LSO. During postnatal development, the transient expression of 5-HT precedes the auditory inputs from cochlear nucleus and is concomitant to the maturation of synapses and dendrites in the LSO. For example, the mature pattern of collateral branching of the afferents from the cochlear nucleus is established in the LSO during the period of E18 to P5 in rats (Kandler and Friauf, 1993; Friauf et al., 1999). In rats, the refinement and pruning of dendrites of LSO neurons begins at P4 and is most dramatic after P14, which is shortly after the onset of hearing (around P12; (Rietzel and Friauf, 1998). Furthermore, the expression of growth associate protein (GAP-43), a marker for axonal elongation and maturation of presynaptic endings, is strongly expressed starting from P1 in rats. By P8, GAP-43 staining becomes restricted to the neuropil (Horvath et al., 1997) indicating more mature synapses. In agreement with the GAP-43 data, 5-HT application activates prolonged bursts of spontaneous inhibitory postsynaptic currents (IPSCs) in young animals but this effect is not observed after P8 within slice preparations of gerbil LSO neurons (Fitzgerald and

Sanes, 1999). Amplifying inhibitory transmission of the LSO in the first postnatal week prevents the normal developmental refinement of LSO dendrites and MNTB terminal arborizations (Sanes and Chokshi, 1992; Sanes et al., 1992; Sanes and Hafidi, 1996). Thus, 5-HT in the early postnatal period may play a developmental role by increasing IPSCs in the LSO.

5-HT may also be involved in the development of the axonal projection from the LSO to its target area, which includes the lateral lemniscus (LL) and the central nucleus of the IC (CNIC) (Oliver, 2000). Transient uptake of 5-HT in non-serotonergic neurons has been shown to be necessary for the precise arrangement and refinement of afferent sensory connections in the thalamocortical and retinothalamic projection neurons (Gaspar et al., 2003; Luo et al., 2003). Alterations in brain 5-HT levels, either increases or decreases, during development disrupt the normal development of the tonographic sensory map in the somatosensory cortex, superior colliculus, and the dorsal lateral geniculate body (Gaspar et al., 2003). Within the auditory system, Thompson and Thompson have recently reported that 5-HT immunoreactive LSO neurons in MAOA k/o mice do project to the CNIC (Thompson and Thompson, 2009a), further supporting the role of 5-HT in the proper topographic projection and terminal arborization of LSO neurons within the IC. In the rat IC, developmental processes that include axon collateralization and synapse formation occur predominately from birth to P11 (Kandler and Friauf, 1993) and a marker for mature synapses, chondroitin sulfate proteoglycan, has been observed in the IC beginning at P8 (Friauf, 2000). Thus, the transient expression of 5-HT in the LSO seems to play a developmental role in the IC by affecting

the collateral branching of axons originating from the LSO as well as the formation of functional synapses.

Lead and serotonin

Environmental lead (Pb) adversely affects a variety of neurotransmitter systems, and the developing nervous system seems to be particularly susceptible to Pb exposure (Antonio et al., 2002; Leret et al., 2002; Devi et al., 2005). Neurotransmitters, such as 5-HT, are used as developmental signals, thus alterations in neurotransmitter systems during development can affect the construction and plasticity of brain circuits (Gaspar et al., 2003). Among the neurotransmitter systems, serotonergic neurons are one of the earliest neurons to be generated in the brain and have been involved in a variety of developmental processes, including neurogenesis, programmed cell death, cell migration, dendrite and axonal development, synaptogenesis and synaptic plasticity (Luo et al., 2003).

Indeed, developmental Pb exposure has been shown to alter brain levels of 5-HT and a major metabolite of 5-HT, 5-hydroxytryopatamine (5-HIAA), in various brain regions (Cupo and Donaldson, 1988; Antonio et al., 1996; Antonio et al., 2002; Devi et al., 2005). Mice exposed to 5mM and 25mM Pb-acetate from P1 through P21 show an increase in 5-HT with 0.2% (5mM) of Pb exposure and a decrease with 1% (25mM) of Pb exposure in all brain regions examined, including cerebral cortex, hippocampus, cerebellum, and medulla (Devi et al., 2005). Developmental Pb exposure also alters MAO activity in the mouse and rat brain (Devi et al., 2005). When rats are exposed to Pb-Ac (300 mg/L) from gestation through P5, both 5-HT and 5-HIAA levels decrease,

and increases in 5-HT turnover (5-HIAA/5-HT) are observed in the entire brain (Antonio et al., 1996). When the same concentration of Pb-Ac (300 mg/L) is used to expose rats for a longer period (from gestation through P12), Pb exposure decreases 5-HT and 5-HIAA in the hippocampus, and decreases 5-HT without affecting 5-HIAA levels in the hypothalamus and cerebellum (Antonio et al., 2002). Similarly, Pb exposure from gestation through P28 results in decreases of 5-HT and 5-HIAA levels in the brainstem (Widmer et al., 1991). Pb also increases 5-HT turnover with decreased 5-HT levels and increased 5-HIAA levels in the developing chick brain (Cupo and Donaldson, 1988). Thus, it is not surprising that we have found that very low level Pb exposure (10μM and 100μM) during development (from gestation through P21) decreases immunoreactivity for both 5-HT and the vesicular monoamine transporter (VMAT2) in the lateral superior olive of mice (Fortune and Lurie, 2009).

Serotonin and development of somatosensory barrel fields

In addition to central auditory neurons (including neurons in LSO and AVCN) several other sensory neurons also transiently express serotonin during development. These neurons include the thalamocortical neurons that project to the layer IV of somatosensory cortex where they form a distinct barrel-field pattern (Cases et al., 1998). In the mouse, each of the barrels corresponds to the afferent thalamic axonal projection from one whisker to the somatosensory cortex (Gaspar et al., 2003). This organization emerges over the first postnatal week by axon collateral remodeling (Rebsam et al., 2002).

The transient uptake of 5-HT by thalamocortical neurons occurs through the transient expression of SERT and VMAT2 on the same neurons. SERT is required for the uptake of 5-HT that is released or leaked out of neighbouring serotonergic processes originating from the raphe, and VMAT2 is required for the vesicular storage of 5-HT (Lebrand et al., 1996; Lebrand et al., 1998). Similar to the LSO neurons, these thalamic neurons do not synthesize 5-HT, as evidenced by a lack of TPH and AADC (Cases et al., 1998). Vitalis et al, 2002 has demonstrated that MAOA is not expressed in the thalamocortical neurons, suggesting that the 5-HT taken up by thalamocortical neurons is not degraded and could be immediately stored into synaptic vesicles by VMAT2 (Vitalis et al., 2002).

Transient uptake of 5-HT by thalamocortical neurons is proposed to have two functional applications. One hypothesis is that 5-HT could be used as a borrowed neurotransmitter, as these neurons express both SERT and VMAT2 (Vitalis et al., 2002). However, there is no direct evidence to date showing the actual release of 5-HT from these neurons.

Transient uptake of 5-HT is also a way to clear 5-HT away from the extracellular space during a critical period of somatosensory cortical barrel field development (Gaspar et al., 2003). In fact, evidence suggests that precise regulation of 5-HT levels during development is very important for the proper formation of cortical barrel fields. For example, in MAO k/o mice, where brain 5-HT levels are 6-9 fold higher than wild type mice during the first postnatal weeks, barrels are not formed, as the clustering of both the thalamocortical axons and the cortical granular neurons around thalamocortical axon endings are disrupted (Cases et al., 1996; Lebrand et al., 1996; Vitalis et al., 1998; Upton

et al., 1999). Similar observations are found in SERT k/o mice, in which 5-HT continues to be released but cannot be removed from the synaptic cleft (Persico et al., 2001). An excessive amount of 5-HT in the extracellular space is proposed to activate the 5-HT1B receptor, which is also transiently expressed presynaptically on thalamocortical axon terminals (Mooney et al., 1994; Laurent et al., 2002). In double knock-out mice of MAOA/5HT1B receptor or SERT/5HT1B receptor, barrels are formed normally, despite elevated brain 5-HT levels (Salichon et al., 2001).

In contrast to the complete loss of barrel fields induced by excessive amounts of 5-HT, depletion of 5-HT by pharmacological intervention results in barrel fields that are either smaller in size or are developmentally delayed (Turlejski et al., 1997; Persico et al., 2001). Indeed, depletion of 5-HT results in an overall delay of brain development, as evidenced by reduced brain and body weights (Persico et al., 2001). Thus, the subtle effect of 5-HT depletion on barrel field formation may be the result of a 5-HT induced delay in general brain development, rather than a direct effect of 5-HT on cortical barrel field formation itself (Persico et al., 2001; Gaspar et al., 2003; Luo et al., 2003).

Taken together, the transient uptake of 5-HT by non-serotonergic thalamocortical neurons and precise regulation of 5-HT levels in this system are important for the formation of somatosensory cortical barrel fields. Interestingly, Pb exposure has been shown to disrupt the development of the somatosensory barrel field cortex in rats (Wilson et al., 2000). In rats exposed to Pb from P1 through P10, the total area of the barrel field in primary somatosensory cortex decreases with increasing doses of Pb (Wilson et al., 2000). Pb exposure, especially during early development, has a persistent detrimental effect on cognitive and behavioral decline in humans and animals (Needleman et al.,

1990; Bellinger and Bellinger, 2006; Bellinger, 2008). Thus, the Pb induced deficits in the development of the barrel field cortex in rodents may be indicative of the cognitive and behavioral impairments induced by Pb exposure during human development.

General Hypothesis

The overall hypothesis for the current study is that Pb alters the normal transient uptake of serotonin (5-HT) by sensory neurons, including central auditory and thalamocortical neurons, leading to impaired axonal/dendritic arborization and synapse formation in these systems, and these changes persist in adult animal. The current studies are the first to examine the effect of Pb on the transient expression of 5-HT in central auditory and thalamocortical neurons during development.

The following Specific Aims are designed to test the hypothesis that Pb alters the normal transient uptake of 5-HT by sensory neurons, leading to permanent changes in axonal/dendritic arborization and synapse formation.

Specific Aims

Specific Aim I: Determine whether Pb alters the normal transient uptake of 5-HT by LSO neurons during the first postnatal week.

- *Does Pb alter the normal transient uptake of 5-HT by LSO neurons through modulating SERT expression?*
- *Is the metabolism of 5-HT (Synthesis, degradation, re-uptake of 5-HT) changed with Pb exposure?*
- *What are the outcomes of the Pb-induced alterations in the transient uptake of 5-HT by the LSO neurons?*
    - *Is Axonal/dendritic arborization and synaptogenesis modified in the Pb-exposed LSO*

Specific Aim II: Characterize whether developmental Pb exposure has a permanent effect on the serotonergic innervation in the LSO and IC of adult mice.

- *Define the mechanism underlying the Pb-induced decreases in 5-HT positive processes in the adult LSO.*
    - *Acute (synthesis, degradation, or re-uptake of 5-HT) vs. Developmental effect*
- *Does Pb alter 5-HT expression in the adult IC, and if so, what is the mechanism?*
    - *Acute (synthesis, degradation, or re-uptake of 5-HT) vs. Developmental effect*

Specific Aim III: Determine the effect of developmental Pb exposure on the transient uptake of 5-HT by thalamocortical neurons at their axon terminals in early postnatal mice.

CHAPTER I.

LOW LEVELS OF Pb EXPOSURE RESULTS IN DELAYS IN THE NORMAL TRANSIENT UPTAKE OF 5-HT BY LSO NEURONS THROUGH PROLONGED EXPRESSION OF SERT

Abstract

Low-level Pb exposure has been associated with neurobehavioral and cognitive deficits in children, and also has been shown to alter auditory temporal processing in both humans and animals. We have previously reported that developmental Pb exposure decreases immunoreactivity for serotonin (5-HT) and the vesicular monoamine transporter 2 (VMAT2) in the lateral superior olive (LSO) of P21 mouse. During early peri and postnatal development, certain "non-serotonergic" sensory neurons, including LSO neurons, transiently take up 5-HT from the extracellular environment through transient expression of the serotonin reuptake transporter (SERT), and maintaining proper 5-HT levels is important for the development of these sensory systems. The current study was designed to define the effect of developmental Pb exposure on the serotonergic system in the LSO of the early postnatal mouse. Mice were exposed to very low Pb (0.01mM) or low Pb (0.1mM) throughout gestation and postnatal day 4 (P4) and P8. Brainstem sections from control and Pb exposed mice were immunostainined for 5-HT, SERT, tryptophan hydroxylase (TPH), monoamine oxidase A (MAO-A), and VMAT2. Total brainstem levels of 5-HT and its metabolite, 5-hydroxindole-3-acetic acid (5HIAA), were also measured by HPLC analysis. To test whether Pb exposure affects the synapse formation of LSO, brainstem sections were immunostained for synaptophysin (SYP) and

GAP-43. Pb exposure delays and extends the normal developmental uptake of 5-HT by LSO neurons through prolonged expression of SERT. This effect of Pb targets the transient uptake of 5-HT by LSO neurons through altered expression of SERT. Pb does not alter synthesis or degradation of 5-HT within the entire brainstem. Pb decreases VMAT2, SYP, and GAP-43 immunostaining within the LSO, indicating that the extended developmental uptake of 5-HT by LSO neurons is correlated with impaired maturation of synapses in the LSO. Finally, Pb decreases total brainstem levels of DA and the major metabolites of DA, 3,4-dihydroxyphenylacetic acid (DOPAC). This indicates that Pb may also affect the dopaminergic neurotransmitter systems within the developing brainstem.

Introduction

Lead (Pb) is a widespread environmental pollutant with neurotoxic effects causing neurobehavioral and cognitive deficits in humans (Finkelstein et al., 1998; Canfield et al., 2003; Lanphear et al., 2005). The developing central nervous system (CNS) is particularly susceptible to environmental Pb exposure (Landrigan and Todd, 1994; Moreira et al., 2001), suggesting that Pb may alter critical stages of development. Currently, the Centers for Disease Control (CDC) limit of concern for childhood blood Pb level remains at 10 μg/dL, and this level is thought to be a threshold for childhood cognitive deficits (Lanphear et al., 2000). However, recent data suggests that blood Pb levels lower than 10 μg/dL produce cognitive and neurobehavioral disorders such as attention deficit hyperactivity disorder (ADHD), and dyslexia (Glotzer et al., 1995; Lanphear et al., 2000; Bellinger and Bellinger, 2006; Braun et al., 2006; Bellinger, 2008).

Pb exposure is also associated with deficits in central auditory temporal processing (Finkelstein et al., 1998; Lurie et al., 2006; Jones et al., 2008), and children with either dyslexia or ADHD have been shown to have deficits in auditory temporal processing (Breier et al., 2003; Facoetti et al., 2003; Putter-Katz et al., 2005; Wright and Conlon, 2009).

The cellular mechanism that underlies these Pb-induced cognitive and neurobehavioral dysfunctions are largely unknown. We have previously reported that low-level Pb exposure during development decreases serotonin (5-HT) and vesicular monoamine transporter 2 (VMAT2) immunostaining in brainstem auditory nuclei, particularly in the lateral superior olive (LSO) (Fortune and Lurie, 2009). 5-HT has been implicated in the modulation of auditory temporal processing by altering multiple aspects of neural responses to sound, ranging from simple tone bursts to complex-species specific vocalizations (Hurley et al., 2002; Hurley and Pollak, 2005). However, the role of 5-HT during central auditory development has not been fully elucidated.

During development of the brain, certain "non-serotonergic" neurons transiently express 5-HT. These neurons include the principle projection neurons of sensory systems such as the auditory, visual, and somatosensory systems (Gaspar et al., 2003). These neurons cannot synthesize 5-HT, as evidenced by lack of the 5-HT synthesizing enzymes tryptophan hydroxylase (TPH) and L-aromatic acid decarboxylase (AADC). Instead, they accumulate 5-HT through uptake by SERT, a high affinity 5-HT transporter, which is also transiently expressed in these same neurons (Gaspar et al., 2003; Thompson, 2008). In the auditory system, a subset of LSO neurons transiently express 5-HT from postnatal day 1 (P1) to P8 in wild-type mice, and from embryonic day 18 to P10 in

Monoamine Oxidase A (MAOA) knockout mice (Cases et al., 1998; Thompson, 2006). Alterations in 5-HT homeostasis during development have been shown to cause permanent changes to adult behavior and to modify the fine wiring of neural connections in the brain (Gaspar et al., 2003). For example, in thalamocortical neurons, precise regulation of 5-HT levels is important for the formation of somatosensory cortical barrel fields (Gaspar et al., 2003; Luo et al., 2003). Interestingly, Pb exposure has been shown to disrupt the development of the somatosensory barrel field cortex in rats (Wilson et al., 2000). However, the effect of Pb exposure on the transient uptake of 5-HT in auditory neurons has not been explored.

The current study was undertaken to define the interactions between developmental Pb exposure and the serotonergic system in central auditory nuclei in early postnatal mice. Brainstem sections from postnatal control and Pb-exposed mice were immunostained for 5-HT, SERT, TPH, MAO-A, and VMAT2. Total brainstem levels of 5-HT and its metabolite, 5-hydroxindole-3-acetic acid (5HIAA), were measured by HPLC analysis. To test whether Pb exposure affects the development of LSO, especially synaptic maturation, brainstem sections were also immunostained for synaptophysin (SYP) and GAP-43.

We found that immunoreactivity for 5-HT in LSO somata was less in Pb exposed mice at P4 compared to controls. In contrast, LSO neurons in P8 control mice showed very little 5-HT immunoreactivity, but Pb exposed LSO neurons at P8 continued to be immunopositive for 5-HT. These results indicate that Pb appears to delay, then extend, the normal developmental uptake of 5-HT by LSO neurons. In agreement with these findings, immunoreactivity for SERT in LSO somata was decreased in Pb exposed mice

at P4 and increased at P8. These SERT positive somata were co-localized with 5-HT, demonstrating that Pb appears to modulate SERT expression (and consequently 5-HT uptake) in LSO neurons. We also confirmed that LSO neurons are immune-negative for TPH and MAOA. In addition, total 5-HT levels and 5-HT turnover did not change with Pb exposure in the brainstem fraction of P4 mice. Finally, Pb decreases immunoreactivity for VMAT2, SYP, and GAP-43, demonstrating that the Pb-induced disruption of 5-HT accumulation in LSO neurons is correlated with impaired synaptic maturation within the LSO.

Materials and Methods

Chronic lead exposure to CBA/CaJ mice

Breeding pairs of CBA/CaJ mice were obtained from the Jackson Laboratory (Bar Harbor, MA). Mice were maintained in microisolator units and kept 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 guidelines. 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 (very low) or 0.1 mM (low) lead acetate. Offspring were exposed to lead throughout gestation and through the dam's milk until sacrifice on postnatal day 4 (P4), or day 8 (P8). These concentrations of Pb yield blood Pb levels of 8.0 ± 0.4 µg/dL in very low Pb, and 42.3 ± 1.97 µg/dL in low Pb P21 mice (Fortune and Lurie, 2009).

Tissue preparation for Immunohistochemistry

Mice were deeply anesthetized using 2',2',2'-tribromoethanol (TBE) and perfused transcardially with 4% Na-periodate-lysine-paraformaldehyde fixative (PLP, final concentrations 0.01M sodium periodate, 0.075M lysine-HCl, 2.1% paraformaldehyde, 0.037M phosphate). Brains were removed and post-fixed in PLP overnight at 4°C, rinsed 3 times for 10 minutes each in phosphate buffered saline (PBS) and transferred to a 20% sucrose solution in PBS for 2 days at 4°C. Finally, brains were transferred to a 1:1 mixture of a 20 % sucrose solution in PBS and Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) for 1-2 days at 4°C. Brains were embedded into 1.5 cm square embedding cups filled with O.C.T. compound, and then frozen in dry ice and 100% ethanol and stored at -20°C. Ten micron tissue sections were cut on a Thermo Shandon Cryotome Cryostat (Thermo Shandon, Pittsburg, PA) and a one in three series was collected for each brain.

Immunohistochemistry

Sections were thawed to room temperature and rinsed in PBS three times for 10 minutes each. Sections were then permeabilized for 30 minutes in 0.5% Triton X-100 in PBS and blocked for 30 minutes with 4% of the appropriate normal serum (normal goat serum, normal rabbit serum, normal chicken serum, normal sheep serum, normal donkey serum, Vector Laboratories, Burlingame, CA) in PAB (1% sodium azide, 0.5% bovine serum albumin in PBS) and incubated with primary antibody for 1-4 days in a humid chamber at 4°C. The sections were rinsed in PBS three times for 10 minutes each and incubated with the appropriate secondary antibody, Alexa Fluor-488, 568, 594, 633;

1:400, or 488 Avidin-Biotin complex; 1:500 (Invitrogen, Grand Island, NY) in PAB for 1 hour at room temperature in the dark. Sections were then rinsed in PBS followed by distilled water and coverslipped with FluorSaverTM (Calbiochem®, San Diego, CA) and stored at 4°C. The primary antibodies used for immunohistochemitry were as follows: rabbit polyclonal anti-5-HT (1:10,000); goat polyclonal anti-5-HT (1:500); rabbit polyclonal anti-VMAT2 (1:2000), rabbit polyclonal anti-SERT (1:250), sheep polyclonal anti-TPH (1:500), goat polyclonal anti MAOA (1:500); mouse monoclonal anti-Synaptophysin (1:12,000); rabbit polyclonal anti-GAP43 (1:1000).

Antibodies

The rabbit polyclonal against serotonin (5-HT) was raised in rabbit against serotonin coupled to bovine serum albumin with paraformaldehyde (Cat. No. 20080, ImmunoStar Inc., Hudson, WI). No cross-reactivity of serotonin antisera was seen with 5-hydroxytrytophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's specifications). This 5-HT antibody labels our mouse brainstem sections in a staining pattern virtually identical to that of other studies using the same antiserum to label the mouse superior olive and inferior colliculus (Hurley et al., 2002; Thompson, 2006) and has been previously well characterized in our system (Fortune and Lurie, 2009). In addition, preadsorption with the 5-HT/bovine serum albumin (BSA) conjugate protein (20 µg/ml, Cat. No. 20081, ImmunoStar Inc., Hudson, WI) eliminates all immunoreactivity, whereas preadsorption with BSA does not affect immunostaining.

The goat polyclonal against serotonin (5-HT) was raised in goat against serotonin coupled to bovine serum albumin with paraformaldehyde (Cat. No. 20079, ImmunoStar

Inc., Hudson, WI). No cross-reactivity of serotonin antisera was seen with 5-hydroxytrytophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's specifications). This 5-HT antibody labels our mouse brainstem sections in a staining pattern virtually identical to that of other studies using the same antiserum to label the mouse superior olive (Fortune and Lurie, 2009; Thompson and Thompson, 2009b, a). In addition, preadsorption with the 5-HT/bovine serum albumin (BSA) conjugate protein (20 μg/ml, Cat. No. 20081, ImmunoStar Inc., Hudson, WI) eliminates all immunoreactivity, whereas preadsorption with BSA does not affect immunostaining.

The rabbit polyclonal antibody against serotonin transporter (SERT) was raised in rabbit against synthetic peptide sequence corresponding to amino acids (602-622) of rat 5-HT transporter coupled to keyhole limper hemocyanin (Cat. No. 24330, ImmunoStar Inc., Hudson, WI). This SERT antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in mouse superior olive (Thompson and Thompson, 2009a).

The sheep polyclonal antibody against tryptophan hydroxylase (TPH) was raised in sheep against recombinant rabbit tryptophan hydroxylase, isolated as inclusion bodies from E.coli (Cat. No. AB1541, Millopore, Temecula, CA). This TPH antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in mouse superior olive and raphe nuclei (Thompson and Thompson, 2009a).

The goat polyclonal anti MAOA was raised in goat against a peptide mapping near the C-terminus of MAO-A of human origin (Cat. No. sc-18396, Santa Cruz Biotechnology Inc., Santa Cruz, CA). This MAOA antibody labels our mouse brainstem

sections in a staining pattern virtually identical to that observed in the rat locus coeruleus (Arai et al., 1997).

The rabbit polyclonal antibody against vesicular monoamine transporter 2 (VMAT2, C-terminal) was raised in rabbit against synthetic peptide comprising amino acids sequence (496-515) at the cytoplasmic C-terminus of rat VMAT2 (Cat. No. 135402, Synaptic System, Gottingen, Germany). This VMAT2 antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in the rat median eminence (Erickson et al., 1996) and has been previously well characterized in our system (Fortune and Lurie, 2009).

The mouse monoclonal antibody against synaptophysinwas raised against in the vesicular fraction of bovine brain against the SY38 epitope; a pentapeptide repeat structure in the C-terminal cytoplasmic tail of syanptophysin (Cat. No. MAB5258, Millopore, Temecula, CA). This synaptophysin antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in the ferret superior olive (Alvarado et al., 2004), and the rat superior olive (immunostained with a different mouse monoclonal antibody against synaptophysin (Sigma; Caminoset al., 2007)). This antibody has also been characterized in our mouse system (Fortune and Lurie, 2009).

The rabbit polyclonal anti-GAP43 was raised in rabbit against GAP-43 synthetic peptide sequence corresponding to amino acids (216-226) of rat GAP-43 (Cat. Ab7462, Abcam Inc., Cambridge, MA). This GAP-43 antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in the rat optic nerve (Lingor et al., 2007), and the rat superior olive immunostained with a different mouse monoclonal antibody against GAP-43 (Roche; (Horvath et al., 1997)).

Tissue Imaging and analysis

All fluorescent slides were viewed at either a 40x or a 60x objective using either a Bio-Rad Radiance 2000 Confocal microscope or an Olympus FV 1000 Fluoview Confocal microscope. Quantitative analysis for immunostaining was performed as previously described (Fortune and Lurie, 2009). Briefly, images were collected and then converted from color tiff files to black and white 12-bit tiff files, and the integrated optical density (IOD) of the immunostaining was measured using MediaCybernetics Image-Pro software (Bethesda, MD). IOD 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. A threshold of immunostaining in control (No Pb) animals was set for each antibody such that all immunoreactivity met threshold, and was used as a comparison with the Pb treatment groups. Immunostaining within the LSO in control and Pb-exposed mice was then quantified and averaged. To separately measure the IOD of 5-HT immunostaining in the LSO neuronal cell bodies and processes,, a threshold for object size was also set such that either only neuronal cell bodies or processes were selected for IOD measurement. Statistical differences in immunostaining between control and Pb-exposed mice were analyzed using KaleidoGraph software (Synergy Software, Reading, PA) or GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA).

Sample preparation for HPLC analysis

Brains from P4 mice were quickly dissected and the forebrain was removed with a coronal cut separating dorsally the superior and inferior colliculus. After dissection, the

remaining basal cerebral cortex and cerebellum were gently removed from the separated brainstem fraction under the dissecting microscope. The remaining brainstem fraction includes inferior colliculus, ventral and dorsal brainstem. Brainstem fractions were immediately weighed, and quickly frozen in the liquid nitrogen. Brainstem fractions from two mice were then combined, and homogenized in 500 µl of 0.05 M perchloric acid solution (PCA) (Branson Sonifier 150, Branson, Danbury, CT), and stored at -20 ºC until ready to use.

RP-HPLC analysis of 5HT/5HIAA and DA/DOPAC/HVA

5-HT and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) levels were measured using reverse phase high performance liquid chromatography (RP-HPLC) with electrochemical detection. The homogenates were centrifuged at 14,000 x g for 20 min at 4ºC and the supernatants filtered through a Millex® hydrophilic LCR (PTFE) 0.45 um filter (Millipore, Bedford, MA). Sample filtrates were loaded into autosampler vials and were placed into an ESA Model 5 autosampler (ESA, Chelmsford, MA). An OmniSpher 5 C18 chromatographic column (Varian Inc., Lake forest, CA) with a length of 25 cm and an internal diameter of 4.6 mm was used to separate analytes. The mobile phase consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12 M sodium hydroxide, 0.6 mM EDTA and 1.30 mM sodium octyl sulfate, and the pH was adjusted to 3.2 with glacial acetic acid. A constant flow rate of 1ml/min was maintained and the column effluent was analyzed with a Model 5600A ESA CoulArray® electrochemical detector (ESA, Chelmsford, MA). Potentials of the three ESA Model 6210 four channel electrochemical cells, placed in series, were as follows: (channels 1

through 5)-50, 0, 25, 100, 200 mV, (6 through 12) 300 mV. 5-HIAA and 5-HT were monitored at 200 mV. DA and DOPAC were monitored at 100 mV, and HVA was monitored at 300 MV. Peak area ratios were used to calculate 5-HT and 5-HIAA levels from a calibration curve (peak area of analyte/ peak area of DBA).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; $P < 0.05$ was considered significant.

Results

Pb exposure alters the normal uptake of 5-HT by developing LSO neurons

In order to determine whether developmental Pb exposure affects the normal uptake of 5-HT by LSO neurons in early postnatal ages, control and Pb-exposed brainstem sections from postnatal day (P) 4 and P8 were immunostained for 5-HT. Many 5-HT immuno-positive cell bodies were observed in the LSO of control mice at P4 (Figure 2A). Very low Pb exposure significantly decreased 5-HT immunoreactivity in the LSO somata (Figure 2B) compared with controls (Figure 2A). Quantification of the immunostaining showed a 54% decrease in 5-HT staining within LSO cell bodies (Figure 2D). In contrast, low Pb exposure did not change 5-HT immunoreactivity in LSO neurons (Figure 2C, 2D). 5-HT immunoreactivity in the processes within the LSO was not altered in either the very low and low Pb exposed mice (Figure 2E), demonstrating that the effect of Pb exposure is restricted to the uptake of 5-HT by LSO neurons.

By P8, control LSO neurons have lost most of their immunoreactivity for 5-HT with very few LSO cell bodies demonstrating any 5-HT immunostaining (Figure 3A). In contrast, in the Pb-exposed mice, 5-HT positive cell bodies continued to be observed in two out of the five mice in the very low Pb group (Figure 3B) and in five out of five mice in the low Pb group (Figure 3C). Quantification of 5-HT staining in the LSO somata demonstrated the low Pb mice continued to express significant amounts 5-HT immunostaining at P8 (Figure 3D). 5-HT staining intensity in the processes within the LSO was not significantly changed in Pb exposed mice compared to control mice (Figure 3E), once again confirming that the effect of Pb is on the uptake of 5-HT within neuronal cell bodies.

Taken together, these findings demonstrate that Pb appears to delay the normal developmental uptake of 5-HT by LSO neurons. Pb exposed LSO neurons contained less 5-HT at P4 than controls, suggesting a delay in the onset of the ability to take up 5-HT. However, Pb-treated LSO neurons continued to express 5-HT at P8, a time when LSO neurons usually lose the ability to transport 5-HT into the cell body. These results illustrate that Pb temporally shifts the ability of LSO neurons to transiently take up 5-HT. Our results also document that this effect of Pb is specific for the transient uptake of 5-HT by LSO neurons, because Pb did not alter 5-HT immunostaining in processes within LSO. These 5-HT positive processes are thought to represent the serotonergic innervation from the raphe (Thompson, 2006).

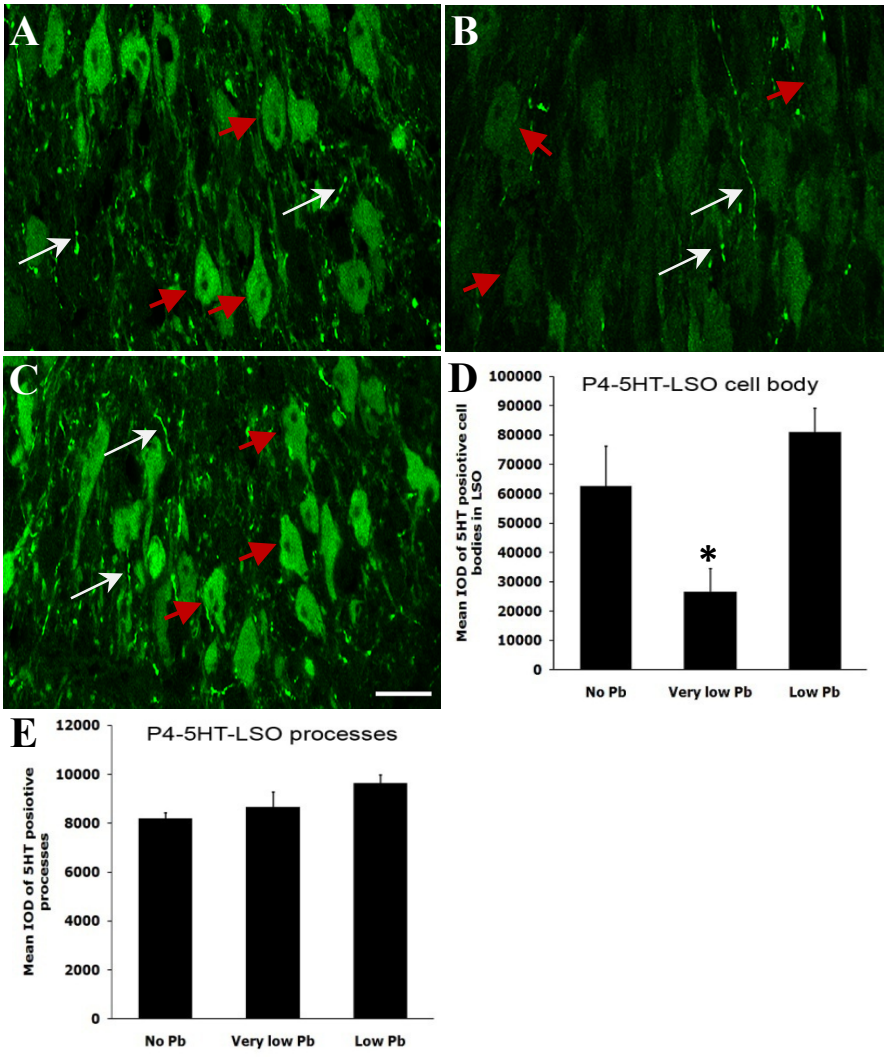


Figure 2. 5-HT immunofluorescence labeling (green) in the LSO somata and processes within the LSO of postnatal day (P) 4 mice. **A-C:** Very low Pb (B) exposure decreases 5-HT expression in the LSO somata (red arrows) compared to control mice (A). Low Pb (C) does not result in significant changes in 5-HT expression. Pb exposure (B, C) does not affect 5-HT expression levels in the processes (white arrows) within the LSO. **D:** Quantification of 5-HT immunostaining in the LSO somata confirms a statistically significant decrease in very low Pb exposed mice. **E:** Pb has no effect on 5-HT immunostaining in LSO processes at P4. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6-8). Scale bar = 15 µm.

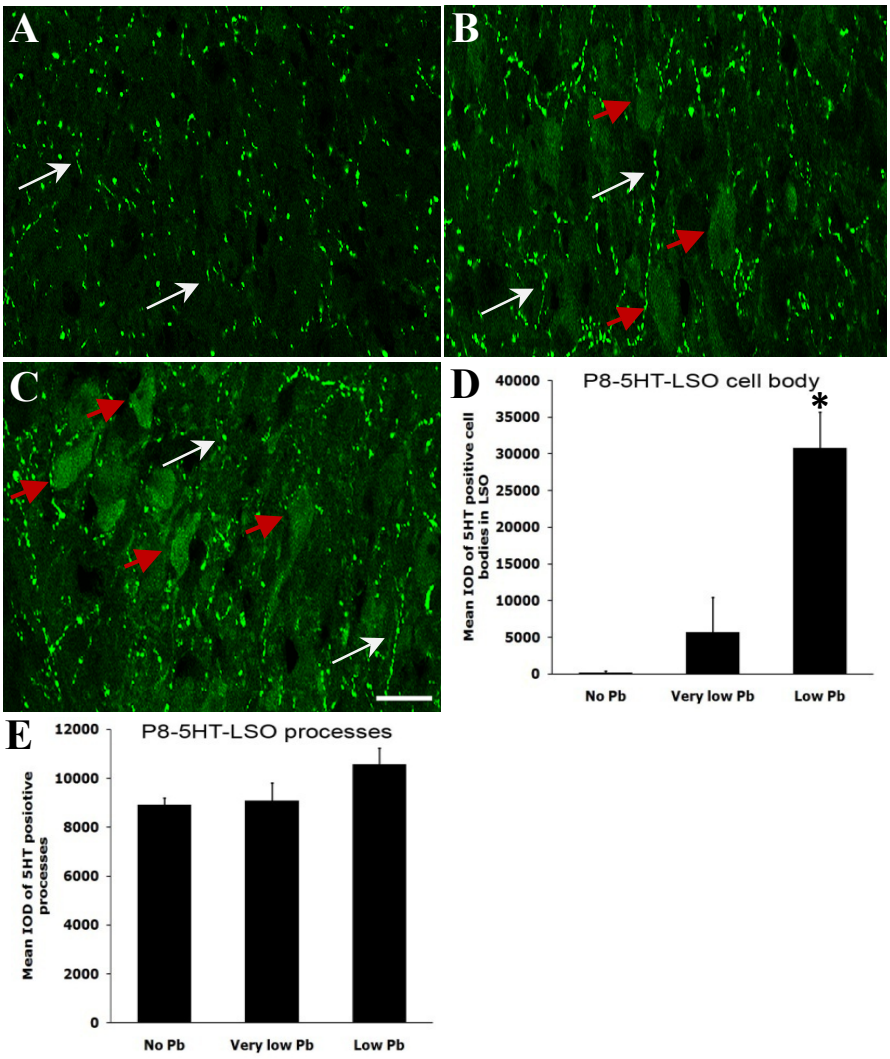


Figure 3. 5-HT immunofluorescence labeling (green) in the LSO somata and processes within the LSO of P8 mice. **A-C:** Immunoreactivity for 5-HT in the LSO somata (red arrows) increases slightly in the very low (B) mice and shows a significant increase with low (C) Pb exposure compared to control mice (A). Pb exposure (B, C) does not significantly affect 5-HT expression levels in the processes (white arrows) within the LSO. **D:** Quantification of 5-HT immunostaining in the LSO somata confirms a statistically significant increase in low Pb mice. **E:** Pb does not have a significant effect on 5-HT immunostaining in LSO processes at P8. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test ($n = 5$). Scale bar = 15 µm.

5-HT uptake into LSO neurons is correlated with SERT expression

The literature has demonstrated that LSO neurons take up 5-HT transiently during development through the transient expression of SERT on LSO neuronal cell bodies. However, these previous studies were performed in MAO/ko mice with abnormally high levels of 5-HT. To confirm that 5-HT positive LSO neurons in wild type mice also express SERT, brainstem sections from control and Pb exposed P4 and P8 mice were double immunostained for 5-HT and SERT.

Somata as well as processes in the LSO were indeed double-labeled for both 5-HT and SERT in P4 and P8 mice (Figure 4). At P4, the Pb-induced decrease in 5-HT staining within LSO neurons (very low dose) was correlated with a loss of SERT immunoreactivity in these same cells (Figure 4A-C). At P8, Pb-exposed LSO neurons continued to express 5-HT, and these neurons also expressed a considerable amount of SERT (Figure 4D-F). Taken together, these results indicate that Pb appears to delay the normal developmental uptake of 5-HT through delayed expression of SERT in the LSO neurons.

It is important to note that the effect of Pb on SERT appears to be specific for SERT expression on LSO somata. Total levels of SERT immunoreactivity within the LSO (which mostly represent SERT immunoreactivity localized in neuronal processes) did not change with Pb exposure (Figure 5). This suggests that Pb specifically targets SERT expression on neuronal cell bodies. Figure 5 also illustrates that the amount of SERT immunoreactivity almost doubled from P4 to P8.

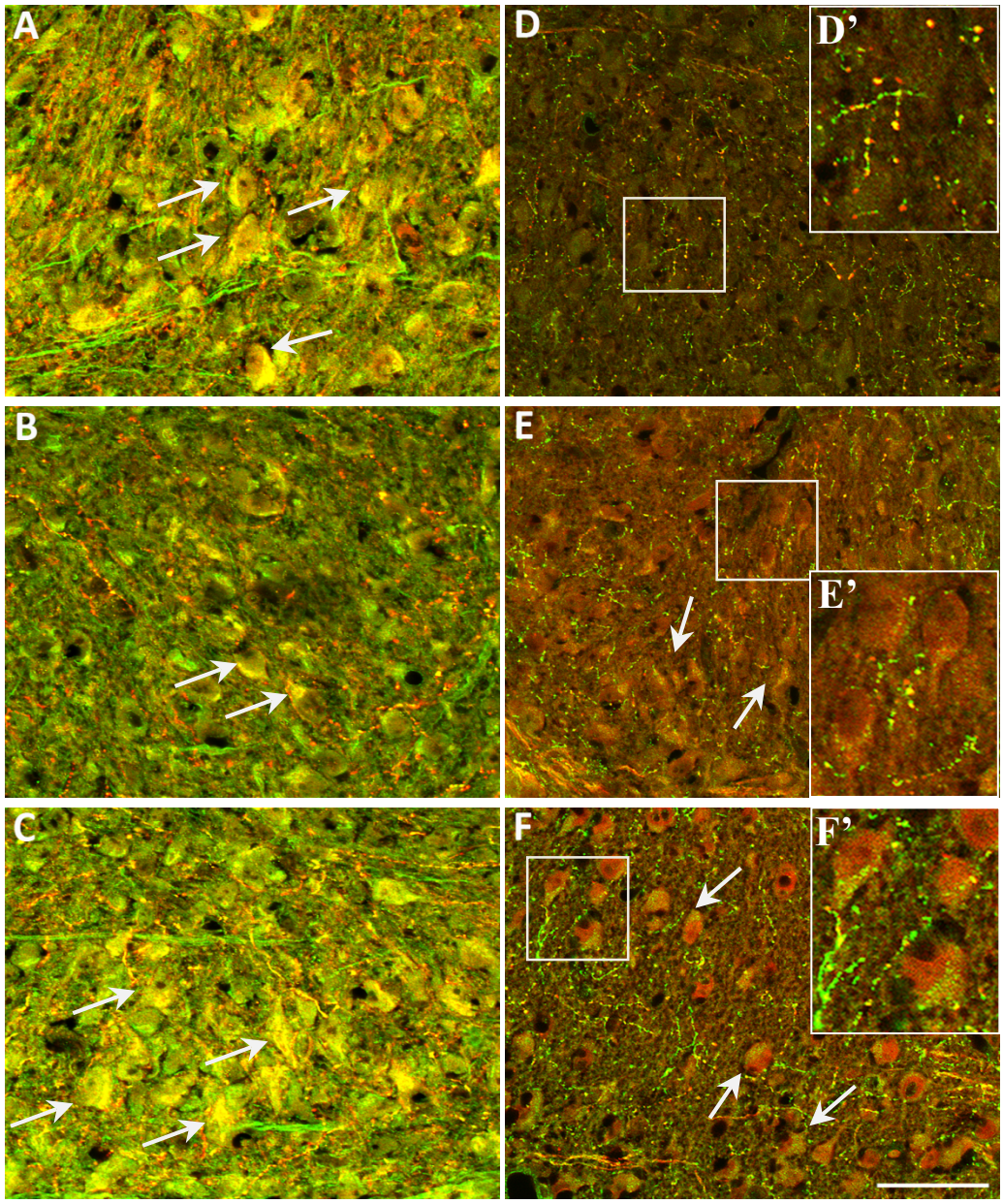

A
D
D'
B
E
E'
C
F
F'

Figure 4. High magnification micrographs of LSO double-labeled for 5-HT (red) and SERT (green) in the LSO somata and processes in P4 and P8 mice. **A-C:** At P4, very low (B) Pb exposure decreases co-localization of 5-HT and SERT (arrows) in the LSO compared to control (A). Low (C) Pb exposure does not affect co-localization of 5-HT and SERT (arrows) and the staining is similar to controls. **D-F:** At P8, very low (E) and low (F) Pb exposure increases co-localization of 5HT and SERT in LSO somata compared to control (arrows, D). The boxed areas in D, E, F are enlarged in D', E', F', to better illustrate the co-localization of 5-HT and SERT in the processes (D') and LSO somata (E', F'). Scale bar = 30 μm in F (applied to A-F).

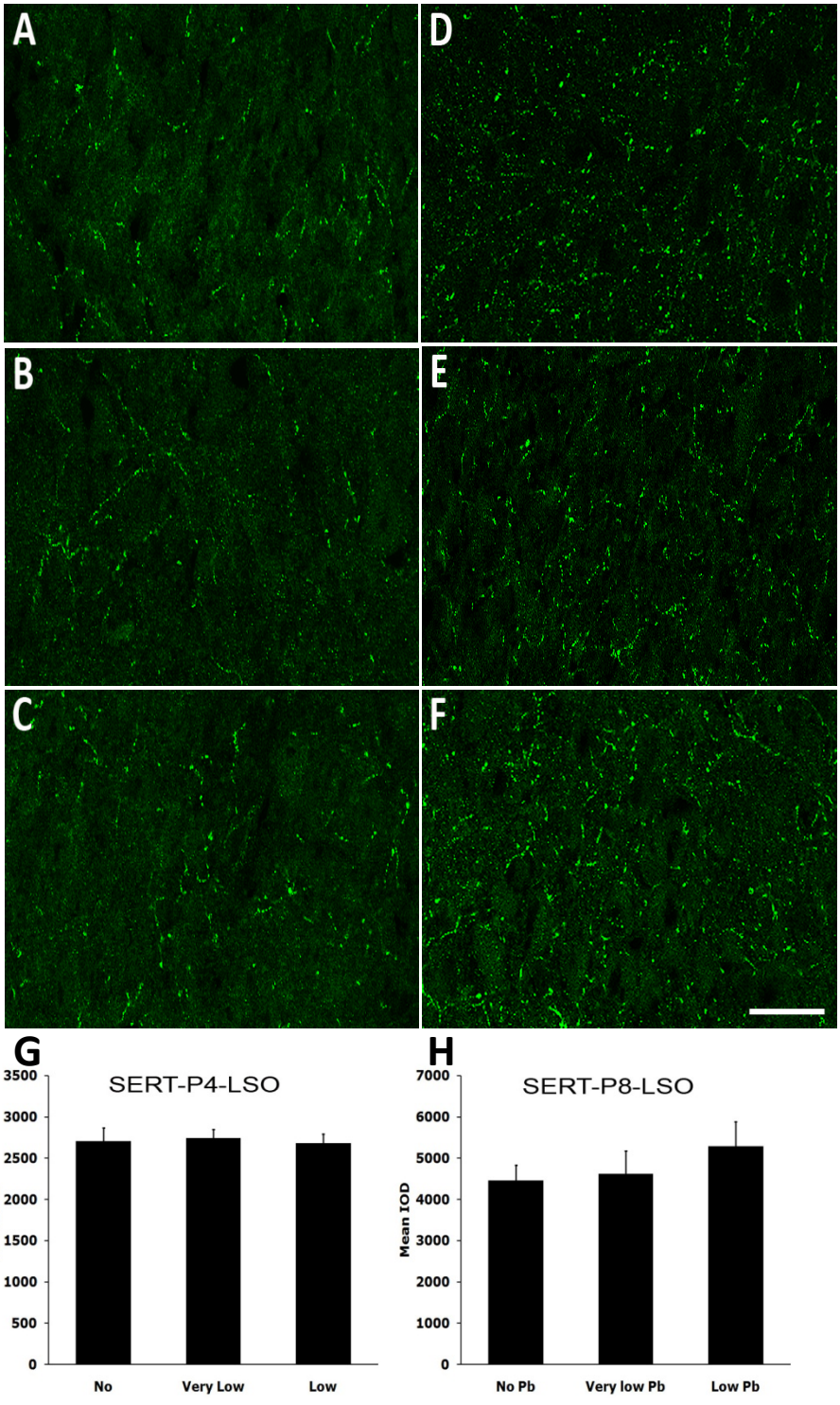

A
D
B
E
C
F
G
SERT-P4-LSO
Mean IOD
3500
3000
2500
2000
1500
1000
500
0
No
Very Low
Low
H
SERT-P8-LSO
Mean IOD
7000
6000
5000
4000
3000
2000
1000
0
No Pb
Very low Pb
Low Pb

Figure 5. Low magnification picture of the majority of LSO immunostained for SERT (green). Pb does not result in significant changes in total SERT expression within LSO at either P4 (A-C) or P8 (D-F). **G;H:** Quantification of SERT immunostaining confirms that Pb does not affect total SERT immunoreactivity in the LSO. However, the amount of SERT immunoreactivity almost doubles from P4 to P8. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 30 μm.

Confirmation that LSO neurons do not contain 5-HT metabolizing enzymes, including TPH and MAOA

To rule out the possibility that Pb induced alterations in 5-HT immunostaining in LSO neurons during early postnatal development is the result of changes in 5-HT synthesis and/or degradation, brainstem sections from control mice were immunostained for the 5-HT synthesizing enzyme, tryptophan hydroxylase (TPH). LSO neurons have been found to be immuno-negative for TPH in P6 MAOA k/o mice (Thompson and Thompson, 2009a). In agreement with these studies, LSO somata were immuno-negative for TPH in our P4 mice (Figure 6A). As expected, neurons in the raphe located in the same brainstem section were strongly immuno-positive for TPH (Figure 6B).

Within intrinsic serotononergic neurons, 5-HT that is taken up from the extracellular space by SERT is rapidly degraded by monoamine oxidase (MAOA) (Vitalis et al., 2002). We tested whether the 5-HT-positive LSO neurons observed at P4 also contain MAOA. MAO activity has been shown to be modulated by Pb (Devi et al., 2005) and therefore could be a potential mechanism by which Pb changes 5-HT levels in LSO neurons during postnatal development. Double-labeling for 5-HT and MAOA in the LSO of P4 control mice demonstrated that 5-HT-positive LSO somata were immuno-negative for MAOA (Figure 6C). In contrast, adjacent raphe neurons were immune-positive for both 5-HT and MAOA (Figure 6D).

These results confirm that the Pb-induced changes in 5-HT immunostaining within LSO neurons are the result of modulation of SERT expression, because LSO neurons were immuno-negative for TPH and MAOA.

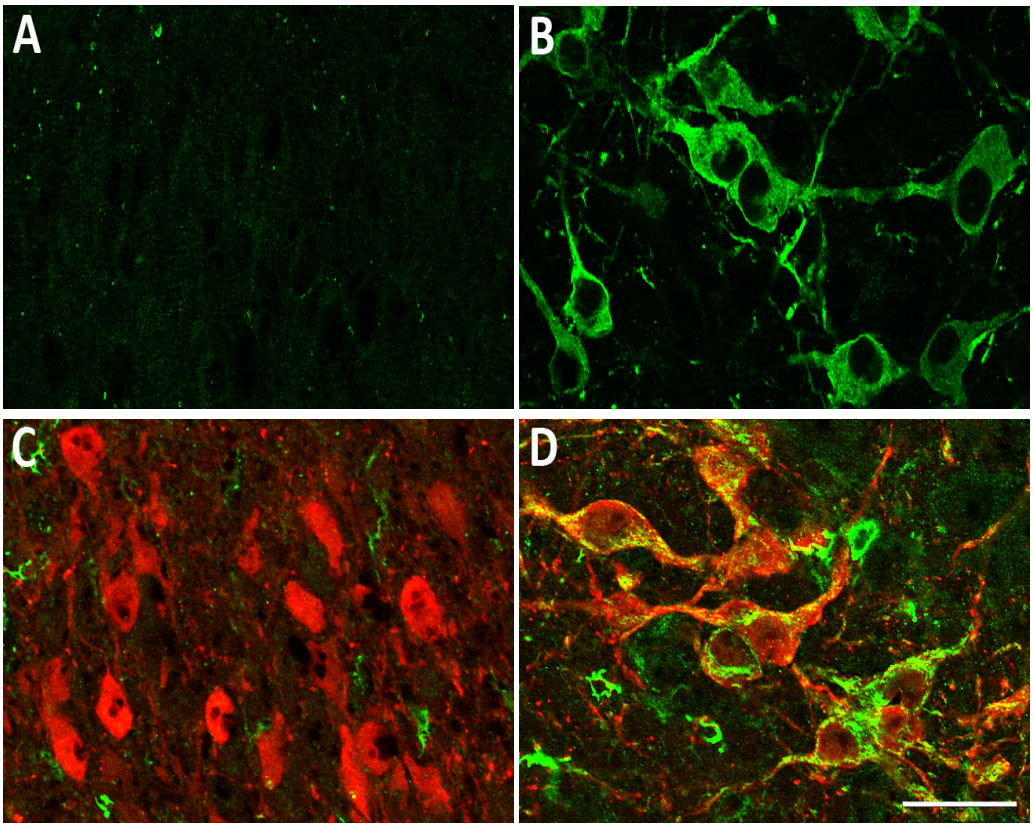


Figure 6. LSO neurons of P4 mice do not express tryptophan hydroxylase (TPH) or monoamine oxidase-A (MAOA). **A, B:** The LSO (A) does not contain any TPH (green) immuno-positive somata unlike neurons in the raphe pallidus (B) where there are many immunopositive neurons. **C, D:** Double immunolabeling of 5-HT (red) and MAOA (green) shows that the 5-HT positive neurons in the LSO (C) do not contain any MAOA immuno-positive somata, unlike neurons in the raphe pallidus where there is obvious double label (yellow) within many neurons (D). Scale bar = 30 μm.

Pb exposure does not affect total 5-HT and 5HIAA levels in the brainstem

Although LSO neuronal cell bodies were immuno-negative for both TPH and MAOA, the possibility remains that Pb affects the synthesis or degradation of 5-HT within the entire brainstem. Therefore levels of 5-HT and a major metabolite of 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), were measured in crude brainstem fractions (which contains all of the serotontergic raphe nuclei) of P4 mice by HPLC analysis. Pb exposure did not result in significant changes in brainstem levels of 5-HT and 5HIAA compared to controls (Figure 7A, 7B). 5-HT turnover (5-HIAA/5-HT) was also not changed, suggesting that Pb does not affect degradation of 5-HT within the brainstem (Figure 7C). These findings demonstrate that the Pb-induced changes in the transient accumulation of 5-HT by LSO neurons are not the result of changes in total brainstem 5-HT levels. This adds further support to our hypothesis that Pb alters the transient uptake of 5-HT by LSO neurons through changes in SERT expression.

Pb exposure decreases immunoreactivity for the vesicular monoamine transporter 2 (VMAT2) within the LSO

The monoamines including dopamine**, s**erotonin, and norepinephrine are transported into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). In addition to the intrinsic monoamine neurons, certain non-serotonergic neurons, such as sensory thalamic neurons that transiently take up 5-HT, have also been shown to express VMAT2 transiently in early postnatal ages, suggesting that the 5-HT taken up by these neurons could be stored in, and released by, synaptic vesicles (Lebrand et al., 1998). It is

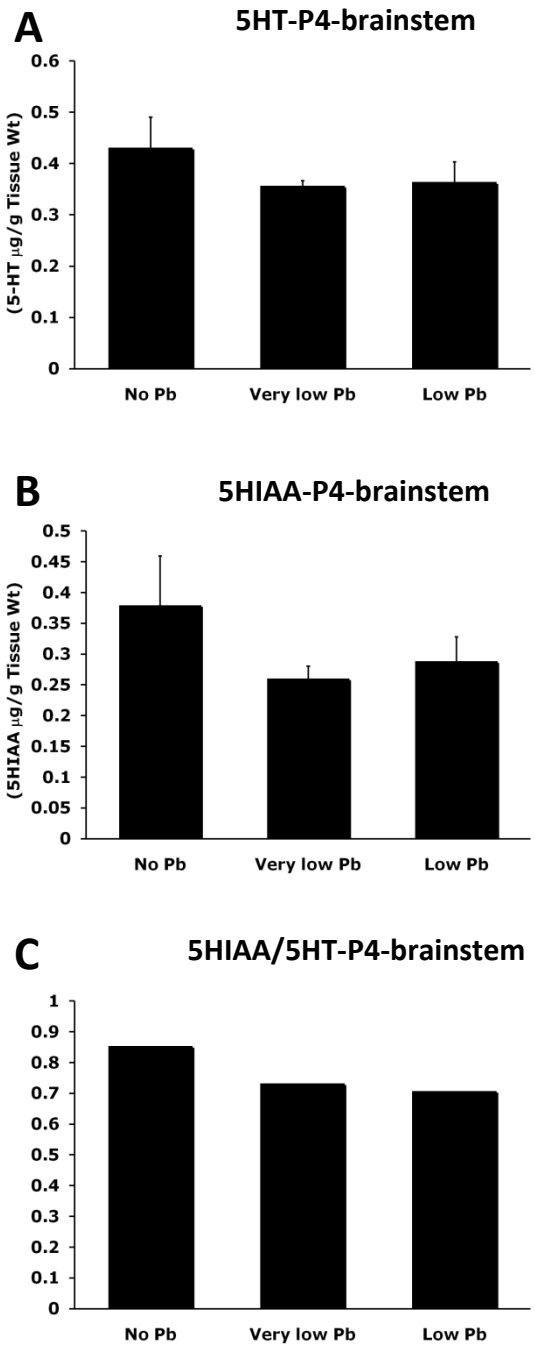


Figure 7. Pb exposure does not result in significant changes in the total brainstem levels of 5-HT and 5HIAA in P4 mice. **A, B:** Total brainstem levels of 5-HT (A) and its metabolite 5HIAA (B) do not significantly change with Pb exposure in P4 mice. **C:** The 5-HT turnover ratio (5HIAA/5-HT) does not change with Pb exposure in P4 mice, indicating Pb does not change 5-HT degradation. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 5).

not known whether the 5-HT immunoreactive LSO neurons also transiently express VMAT2.

To determine whether Pb affects VMAT2 expression in the LSO, control and Pb-exposed brainstem sections from P4 and P8 mice were immunostained for VMAT2. P4 mice showed very little VMAT2 immunostaining (data not shown). VMAT2 immuno-positive LSO somata were observed in control and Pb-exposed mice at P8 (Figure 8). Figure 8 also illustrates that the majority of VMAT2 immunolabel is localized in processes. Very low levels of Pb exposure significantly decreased VMAT2 immunoreactivity in the LSO (Figure 8B, D). In contrast, low Pb exposure did not affect VMAT2 immunoreactivity (Figure 8C). Quantification of VMAT2 staining confirmed a significant decrease of VMAT2 immunostaining in the very low Pb exposed mice compared to control mice (Figure 8D).

These findings demonstrate that Pb decreases VMAT2 immunoreactivity in LSO neurons, and also suggests that Pb decreases the density of monoaminergic synapses. Because we have shown in the current study that Pb does not appear to change the serotonergic innervation in LSO, one possibility is that Pb affects synaptogenesis of other monoaminergic neurons.

Pb exposure decreases total DA and DOPAC levels in the brainstem

To further investigate whether Pb also affects other monoaminergic neurons such as dopaminergic neurons, levels of dopamine (DA) and major metabolites of DA, including 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured in crude brainstem fractions of P4 mice by HPLC analysis. Both the very low

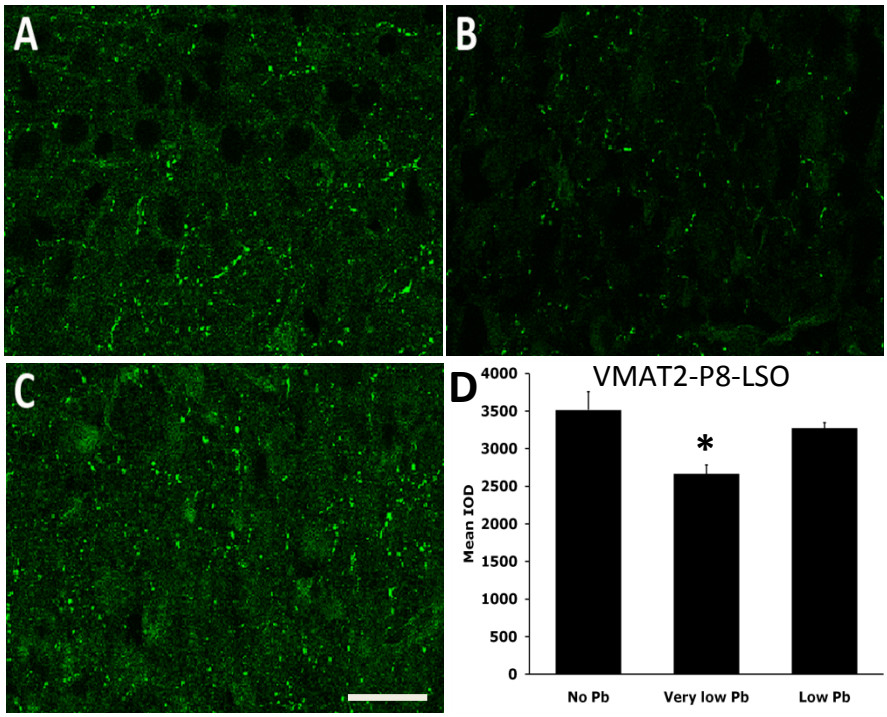


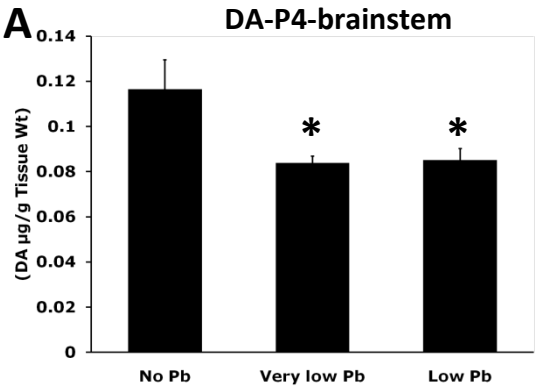
Figure 8. Pb exposure decreases immunofluorescence labeling (green) for the vesicular monoamine transporter 2 (VMAT2) in the LSO of P8 mice. **A-C:** Immunoreactivity for VMAT2 in the LSO decreases with very low Pb exposure (B) compared to no Pb control (A). Low Pb exposure (C) does not result in changes in the total VMAT2 expression. **D:** Quantification of VMAT2 immunostaining in the LSO confirms that this decrease is statistically significant in very low Pb exposed mice. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test ($n = 5$). Scale bar = 30 μm.

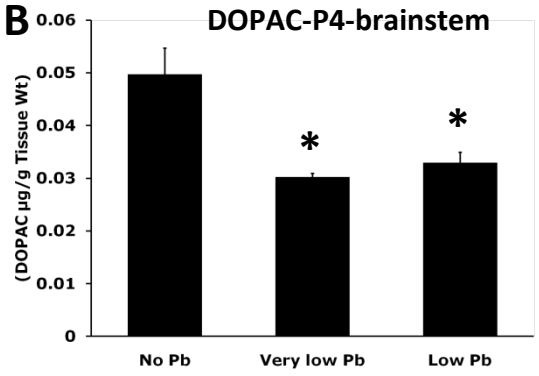
and low Pb significantly decreased brainstem levels of DA and DOPAC compared to controls (Figure 9A, B). In contrast, HVA levels in brainstem fractions did not change with Pb (Figure 9C). DA turnover ((DOPAC+HVA)/DA) was also not affected by Pb, suggesting that Pb does not affect degradation of DA in the brainstem (Figure 9D).

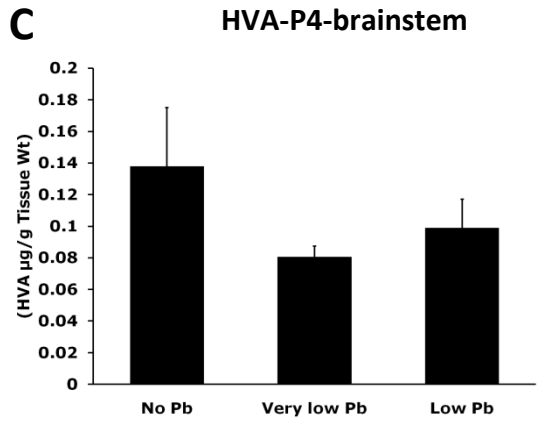
The Pb-induced decreases in total brainstem levels of DA are correlated with decreased VMAT2 expression, and further support the hypothesis that Pb may affect synaptogenesis of non-serotonergic monoamine neurons.

The Pb-induced delay in the normal developmental uptake of 5-HT by LSO neurons is correlated with decreased immunoreactivity for synaptophysin

Alterations in 5-HT homeostasis during development have been associated with disrupted synapse formation, thus modifying the fine wiring of neural connections in the brain (Gaspar et al., 2003). We also found that Pb exposure decreases VMAT2 expression in the LSO, suggesting that Pb impairs synaptic maturation within the LSO. To further examine whether the Pb-induced delay in the normal developmental uptake of 5-HT by LSO neurons is correlated with a loss of synapses, control and Pb-exposed brainstem sections from P8 mice were immunostained for synaptophysin (SYP), a marker of presynaptic terminals, and thus a good indicator of synaptic density. Very low Pb exposure resulted in a significant decrease in SYP labeling within LSO (Figure 10B, D) compared to control (Figure 10A). Low Pb exposure showed a slight decrease, but this change was not statistically significant (Figure 10C, D). Both VMAT2 and SYP decreased with Pb exposure, suggesting that the loss of synapses within LSO is primarily due to decreases in monoaminergic synapses. These findings indicate that Pb exposure


A
DA-P4-brainstem
(DA µg/g Tissue Wt)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
No Pb
Very low Pb
Low Pb


B
DOPAC-P4-brainstem
(DOPAC µg/g Tissue Wt)
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
No Pb
Very low Pb
Low Pb


C
HVA-P4-brainstem
(HVA µg/g Tissue Wt)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
No Pb
Very low Pb
Low Pb

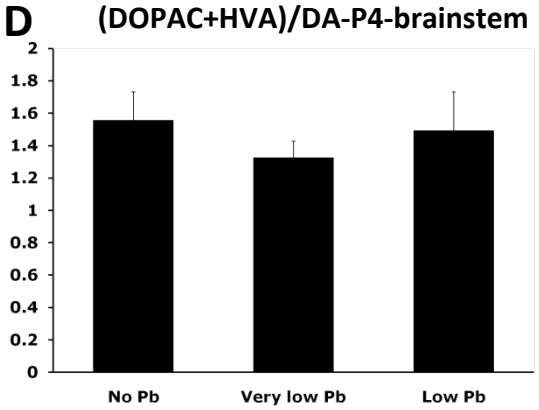

D
(DOPAC+HVA)/DA-P4-brainstem
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
No Pb
Very low Pb
Low Pb

Figure 9. Pb exposure decreases total brainstem levels of DA and DOPAC in P4 mice. **A, B:** Total brainstem levels of DA (A) and DOPAC (B) significantly decreases with Pb exposure in P4 mice. **C:** HVA levels in the brainstem do not change with Pb exposure. D**:** The DA turnover ratio ((DOPAC+HVA)/DA) does not change with Pb. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test ($n = 5$).

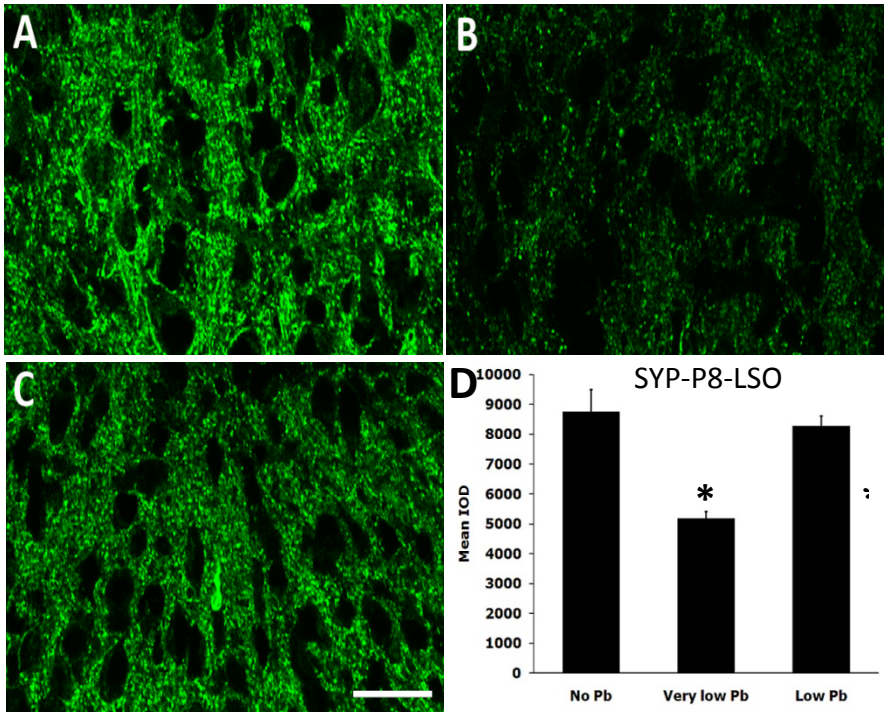


Figure 10. Pb exposure decreases synaptophysin (SYP) immunofluorescence labeling (green) in the LSO of P8 mice. **A-C:** Immunoreactivity for SYP decreases with very low Pb exposure (B) compared to control (A). Low Pb exposure (C) does not result in changes in the SYP expression. **D:** Quantification of SYP immunostaining in the LSO confirms that decrease is statistically significant in very low Pb exposed mice. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test ($n = 5$). Scale bar = 30 μm.

indeed alters the synaptic density within the LSO, and modulation of 5-HT uptake by LSO neurons may be one mechanism by which Pb affects synaptic maturation within the LSO.

The Pb-induced delay in the normal developmental uptake of 5-HT by LSO neurons is correlated with decreased immunoreactivity for GAP-43

Growth associate protein (GAP-43) is a marker for axonal elongation and maturation of presynaptic endings, and has been shown to be strongly expressed in the LSO during postnatal development. In P1 rats, GAP-43 immunostaining shows a diffuse distribution pattern, which is thought to indicate active axonal elongation. By P8, GAP-43 staining becomes restricted to the neuropil, likely reflecting the formation of more mature synapses (Horvath et al., 1997). In order to test whether the Pb-induced decreases in SYP and VMAT2 expressions are correlated with a decreased expression of GAP-43, control and Pb-exposed brainstem sections from P4 and P8 mice were immunostained for GAP-43. Figure 11A illustrates that there are high levels of GAP-43 immunostaining within the LSO of P4 control mice that is characterized by a diffuse distribution pattern. Low Pb exposure resulted in a significant decrease of GAP-43 expression in the LSO (Figure 11D). Very low Pb exposure showed two different patterns of GAP-43 staining, one group of animals demonstrated decreases in GAP-43 expression (Figure 11C, n=3), while the other group showed increases in GAP-43 immunoreactivity (Figure 11B, n=2). This differential response to very low Pb exposure likely indicates that P4 is a transition point for Pb-induced changes in GAP-43 expression within the LSO.

By P8, the control LSO showed a more granular pattern of GAP-43 mmunostaining (Figure 12A). Pb exposure resulted in decreased GAP-43 immunolabeling in the LSO (Figure 12B, C). Quantification demonstrated that the staining intensity of GAP-43 in the low Pb group was significantly decreased compared to control mice (Figure 12D) while very low Pb resulted in a decreasing trend of GAP-43 staining. The changes in GAP-43 immunostaining further support the hypothesis that Pb exposure disrupts the normal development of synapses within the LSO.

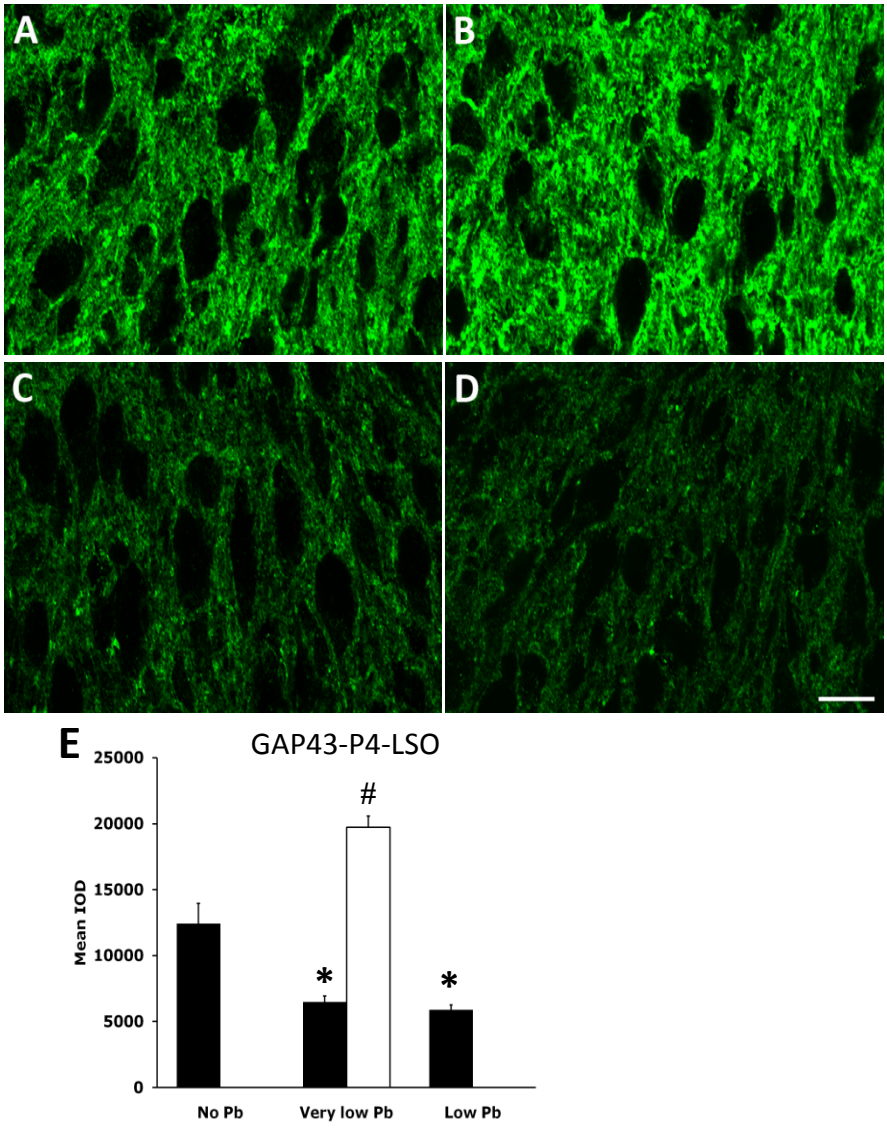


Figure 11. Pb exposure alters GAP-43 immunofluorescence labeling in the LSO of P4 mice. **A -D:** Immunoreactivity for GAP-43 in the LSO decreases with low Pb exposure (D) compared to control mice (A). Very low Pb exposure shows two different patterns of GAP-43 expression, one group shows a decrease GAP-43 labeling (C, n=3) and the other group shows an increase in GAP-43 expression (B, n=2). **E:** Quantification of GAP-43 immunostaining in the LSO confirms the statistical significance of the effect of Pb. Graphs represent mean ± SEM. *, # $p<0.05$; ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 15 μm.

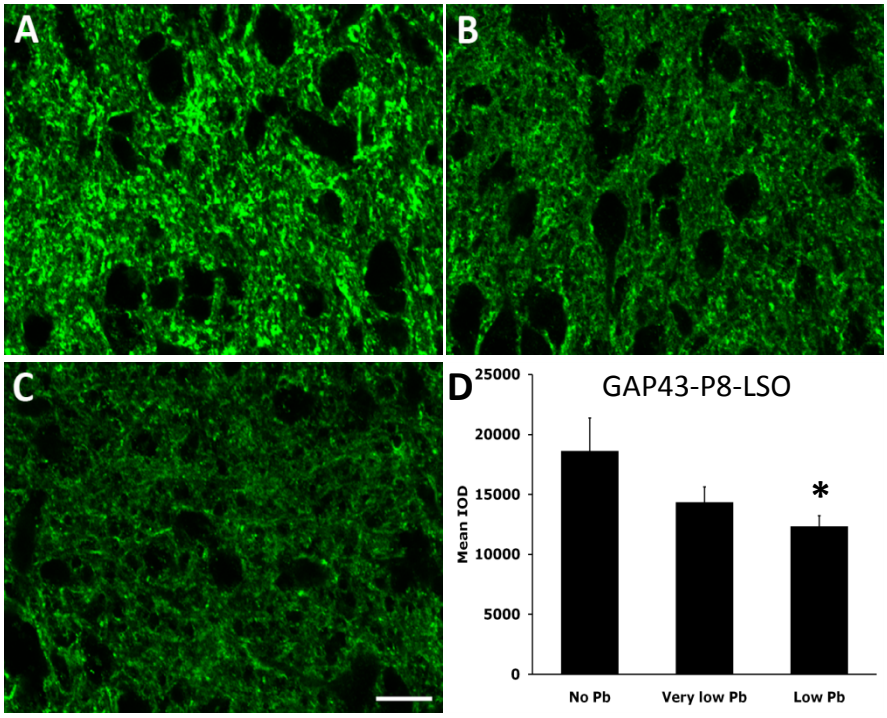


Figure 12. Pb exposure decreases GAP-43 immunofluorescence (green) labeling in the LSO of P8 mice. **A-C:** Immunoreactivity for GAP-43 in the LSO decreases with both very low (B) and low (C) Pb exposure compared to control (A). **D:** Quantification of GAP-43 immunostaining in the LSO confirms this decrease is statistically significant in low Pb exposed mice. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 15 µm.

Discussion

The current study demonstrates that Pb exposure delays the normal developmental uptake of 5-HT by LSO neurons in early postnatal mice (P4 and P8). This effect of Pb is specific for transient uptake because Pb does not alter 5-HT immunostaining in the serotonergic processes that are thought to originate from the raphe. Results from double immunofluorescence staining of 5-HT and SERT indicate that Pb prolongs the expression of SERT in the LSO neurons. Pb did not affect synthesis or degradation of 5-HT in the entire brainstem, further supporting the hypothesis that Pb alters the transient uptake of 5-HT by LSO neurons through changes in SERT expression. The loss of VMAT2 and SYP immunostaining within the LSO indicates that delayed developmental uptake of 5-HT by LSO neurons could be responsible for the decreased synaptic density in the LSO. The changes in GAP-43 immunostaining further support the hypothesis that Pb disrupts axonal arborization and synaptic maturation in the LSO, and also suggest that GAP-43 may be a target molecule for Pb. Finally, total brainstem levels of DA and DOPAC decreased with Pb exposure suggesting that Pb may affect the dopaminergic system as well.

The presence of SERT in LSO somata of a non-genetically modified mouse

LSO neurons receive glutamatergic excitatory input from the ipsilateral anteroventral cochlear nucleus (AVCN) and glycinergic inhibitory input from the contralateral medial nucleus of the trapezoid body (MNTB) (von Gersdorff and Borst, 2002). LSO ascending neurons project to the ipsilateral and contralateral inferior colliculus (IC) and use glycine and glutamate as a neurotransmitter and are not

considered to be intrinsic serotonergic neurons (Thompson and Schofield, 2000). During development, these "non-serotonergic" LSO neurons become transiently immuno-positive for5-HT. In MAOA knockout mouse where brain 5-HT levels are increased up to 6-9 fold compared to wild type, a subset of LSO neuronal somata transiently contain 5-HT immunostaining starting from embryonic day 18 (E18), reaching maximal levels at birth (P0) to postnatal day 7 (P7). This serotonin immunoreactivity then disappears by P10 (Cases et al., 1998). 5-HT immunoreactive LSO somata are also present in wild type mice at P1 and then mostly disappear by P8 (Thompson, 2006).

Unlike intrinsic serotonergic neurons, the 5-HT containing LSO neurons do not have the capacity for synthesizing 5-HT, as evidenced by the lack of tryptophan hydroxylase (TPH), a rate-limiting enzyme in 5-HT synthesis (Thompson and Thompson, 2008, and current study). Instead, Thompson and Thompson 2008 proposed that LSO neurons take up extracellular 5-HT through SERT, a high affinity 5-HT transporter. In MAOA k/o mice, 5-HT and SERT staining is co-localized in LSO somata at P0 and P5-6, and all staining in the LSO somata for both proteins disappears by P15 (Thompson and Thompson, 2009a). In the current study, we also found that 5-HT and SERT are co-localized in the LSO somata of P4 and P8 wild type mice. However, the present study is the first to demonstrate the existence of SERT protein in 5-HT immuno-positive LSO somata in a non-genetically modified mouse, demonstrating that transient expression of 5-HT and SERT is a normal developmental process and is not due to the abnormally high levels of 5-HT in the MAOA k/o mouse.

Pb and SERT expression

The mechanism by which LSO somata transiently express SERT is not clear. The active (functional) SERT is located mainly in axon terminals and along the axons of serotonergic neurons (Zhou et al., 1998). In thalamocortical neurons that transiently accumulate 5-HT through SERT, SERT proteins are transiently expressed in the axons and axon terminals, and are responsible for taking up 5-HT from the extracellular space. This 5-HT is then retrogradely transported to the thalamic neuronal cell bodies in the ventroposterior thalamus (Lebrand et al., 1996; Lebrand et al., 1998). In our model, SERT is expressed directly in LSO neurons themselves and it should be noted that the effect of Pb appears to be specific for the transient expression of SERT in the LSO neurons. Pb does not affect SERT expression in LSO processes.

Factors regulating SERT expression in non-serotonergic neurons are poorly understood. In serotonergic neurons, the transcription factor Pet1 directly activates the transcription of genes that encode TPH, ADAC (L-amino acid decarboxylase), and SERT (Hendricks et al., 1999). However, this gene is not found in neurons transiently expressing SERT (Pfaar et al., 2002).

Interestingly, thyroid hormones have been shown to regulate the transient expression of SERT in thalamocortical neurons (Auso et al., 2001). Auso et al., reported that transient expression of the SERT gene disappears by P11 in normal mice, whereas its expression persists until P15 in hypothyroid rats (Auso et al., 2001). Furthermore, this effect of hypothyroidism is specific for the transient expression SERT, because there is no general delay in brain maturation and SERT is not changed in raphe neurons. Prolonged expression of SERT in hypothyroid rats also leads to reduced axon terminal

arborization and synaptogenesis within the cortical barrel fields, resulting in a smaller barrel area. We found that Pb exposure is also correlated with decreased synaptic density. Interestingly, Pb has been shown to reduce free thyroxine (FT4) levels in Pb exposed adolescents whose average blood Pb level is 7.3 ± 2.92 μg/dL (Dundar et al., 2006). In addition, Pb exposure reduces ^{131}I uptake in animals and impairs the release of thyroid-stimulating hormone (TSH) in children (Slingerland, 1955; Huseman et al., 1987). Therefore, a Pb-induced impairment in thyroid function is one potential mechanism by which Pb might delay the transient expression of 5-HT and SERT in non-serotonergic LSO neurons. Future studies will address this issue.

Possible mechanism of Pb induced impaired synapse formation within the LSO

Precise regulation of transient uptake of 5-HT by "non-serotonergic" sensory neurons has been shown to be critical for the proper formation of highly topographically organized sensory maps (Gaspar et al., 2003). Studies suggest that this transient uptake of 5-HT by non-serotonergic sensory neurons is necessary to clear 5-HT away from the extracellular space, thereby maintaining proper extracellular concentrations of 5-HT during a critical period of development. It is well known that 5-HT functions as a neurotrophic factor during brain development prior to the time when it plays a role as a neurotransmitter (Luo et al., 2003). Depletion of 5-HT levels during development has a long lasting effect on synaptogenesis and brain maturation and maintaining proper 5-HT levels during the critical period of brain development is crucial (Lou et al., 2003; Gaspar et al., 2002). For example, embryonic or neonatal depletion of 5-HT has been shown to decrease synaptic density, reduce both spine density and complexity of cortical pyramidal

neurons and hippocampal dentate granule cells (Mazer et al., 1997; Vitalis et al., 2007), and alter the neural morphology of somatosensory cortical barrel fields (Bennett-Clarke et al., 1994).

We found in the current study that Pb exposure delays the normal uptake of 5-HT by LSO neurons. Thus, an intriguing hypothesis is that Pb could shift the extracellular concentrations of 5-HT during a critical window of neuronal development, thereby resulting in disrupted axonal arborization and synaptogenesis in the LSO. In support of this, we found that Pb decreases SYP immunostaining within the LSO of P8 mice.

5-HT, GAP-43, and synaptogenesis

Despite the growing evidence demonstrating the detrimental effect of 5-HT depletion on synaptogenesis and brain maturation, the factors that mediate these outcomes, especially in the non-serotonergic sensory neurons, remain largely unknown. One possible candidate is GAP-43. GAP-43 is expressed early in development and plays a role in axonal outgrowth and synaptic plasticity in response to extracellular signals (Luo et al., 2003). GAP-43 has shown to be upregulated during the period of barrel field formation, and is then subsequently downregulated (Maier et al., 1999). In GAP-43 k/o mice, thalamocortical axons fail to innervate the cortex, thus barrels are not formed, and forebrain levels of 5-HT and 5HIAA are decreased (Donovan et al., 2002). These studies suggest that GAP-43 may be a factor that mediates the 5-HT induced regulation of sensory neuron development. Within the rat LSO, GAP-43 is upregulated from P1 to P8, and then is subsequently downregulated until P21 (Horvath et al., 1997). Our results demonstrate that Pb exposure also decreases GAP-43 expression in the developing LSO,

perhaps through the Pb-induced changes in extracellular 5-HT levels. Thus, modulation of GAP-43 by changes in 5-HT levels is one potential mechanism by which Pb might impair the normal development of synapses within the LSO.

Finally, in addition to its action as a trophic factor, 5-HT could modulate spontaneous activity in the LSO, thereby affecting LSO development. Within slice preparations of gerbil LSO neurons, 5-HT application activates prolonged bursts of spontaneous inhibitory postsynaptic currents (IPSCs) in young animals but this effect is not observed after P8 (Fitzgerald and Sanes, 1999). Amplifying inhibitory transmission of the LSO in the first postnatal week prevents the normal developmental refinement of LSO dendrites and MNTB terminal arborizations (Sanes and Chokshi, 1992; Sanes et al., 1992; Sanes and Hafidi, 1996). Thus, an intriguing hypothesis is that Pb induces increased concentrations of extracelluar 5-HT in the early postnatal period, resulting in increased frequency of IPSCs that then inhibit proper axonal arborization and synapse formation.

Pb and dopaminergic system

Our finding that Pb exposure decreases the expression of VMAT2 within the LSO of P8 mice and reduces DA and DOPAC levels in the P4 brainstem suggests that Pb may affect other monoaminergic systems including dopaminergic systems within the LSO. In support of this hypothesis, Pb has been shown to decrease brain levels of DA and the major metabolites of DA, including 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in various brain regions (Antonio et al., 1996; Antonio and Leret, 2000; Devi et al., 2005; Szczerbak et al., 2007). The effect of Pb on DA is dependent both on the dose and the length of time it is administered and it is thought that

Pb administered during development has a long lasting effect on DA metabolism (Szczerbak et al., 2007). Future studies that include double-label immunofluorescence for tyrosine hydroxylase (TH, a marker for dopaminergic neurons) and SYP are needed to determine whether the decreased SYP staining that we observe in the Pb-treated P8 LSO reflects a decrease in synapses of dopaminergic neurons.

Summary and Conclusion

The current study demonstrates that Pb exposure delays the normal developmental uptake of 5-HT by LSO neurons during a critical period of development, through the delayed expression of SERT in LSO neurons. SERT appears to be a target for Pb, because Pb did not affect either synthesis or degradation of 5-HT in the entire brainstem . Thus, neurons such as LSO neurons that transiently take up 5-HT during development may be specific cellular targets of Pb. The delayed uptake of 5-HT by LSO neurons was also correlated with the decreased expression of SYP, VMAT2 and GAP-43 within the LSO, suggesting that the delayed uptake of 5-HT by LSO neurons may be one mechanism by which Pb impairs synaptic maturation within the LSO. These synaptic changes persistinto adulthood (Fortune and Lurie, 2009) and studies are in progress to elucidate the permanent effect of Pb on the developing LSO.

CHAPTER II.

LOW-LEVEL Pb EXPOSURE DURING DEVELOPMENT PERMANENTLY ALTERS SEROTONERGIC INNERVATION IN THE LSO AND IC OF ADULT MICE

Abstract

During early postnatal development, certain "non-serotonergic" sensory neurons, including LSO neurons, transiently take up 5-HT from the extracellular environment, and disruption of this transient uptake has been shown to cause permanent changes in adult behavior and result in modifications to the fine wiring of neural connections in the brain. We have previously shown that developmental Pb exposure results in a delay in the uptake of 5-HT by early postnatal LSO neurons, and that this delay is correlated with altered synaptic maturation (chapter I). Because developmental Pb exposure decreases 5-HT and VMAT2 expression in the adult LSO (Fortune and Lurie, 2009), the developmental effects of Pb seem to persist into adulthood. The current study was undertaken to determine whether developmental Pb exposure has an acute effect on the 5-HT system in the adult central auditory system of the brainstem or whether the decrease in VMAT2 and 5-HT observed in the adult is largely due to changes in process arborization and synaptogenesis during development. Mice were exposed to very low Pb (0.01mM) or low Pb (0.1mM) throughout gestation and until postnatal day 21 (P21). Brainstem levels of 5-HT and its metabolite, 5-hydroxindole-3-acetic acid (5HIAA), were measured by HPLC analysis and VMAT activity was measured in isolated synaptic vesicles. Brainstem sections from control and Pb exposed mice were immunostained for

SERT and GAP-43. Pb increased brainstem levels of 5-HT but did not change 5-HT degradation, even though 5-HT immunostaining within the LSO decreased. Pb had no effect on SERT immunoreactivity within the LSO or on VMAT activity. These findings demonstrate that the Pb induced decreases in 5-HT expression within the P21 LSO do not appear to be an acute effect of Pb on the metabolism of 5-HT. Similar trends were observed in the IC, where Pb caused an increase in 5-HT expression within CINC, but no change in total IC 5-HT levels or metabolism, or in SERT immunostaining. Finally, GAP-43 immunoreactivity (a marker of synaptic development) was modified in the adult Pb-exposed LSO, adding support to the hypothesis that developmental Pb exposure modulates process arborization and synaptogenesis during the early postnatal period, and these changes persist into adulthood.

Introduction

Lead (Pb) continues to be a significant environmental toxin. Despite its elimination from household paint, leaded pipes, and food cans, Pb is still found in leaded paint and pipes in old residences, leaded gasoline used by off-road vehicles, toys with leaded paint, cosmetics, folk medicines, glazed ceramics, bullets, and storage battery casings (Bellinger and Bellinger, 2006; Bellinger, 2008). The developing central nervous system (CNS) has long been recognized as a primary target for environmental Pb exposure, thus children are most vulnerable (Landrigan and Todd, 1994; Moreira et al., 2001). In addition, the Centers for Disease Control (CDC) reported that Pb exposure during the first 6 years of life can produce permanent neurological damage (CDC, 1991), indicating that the effects of developmental Pb exposure persist into adulthood.

Moreover, several studies in animals suggest that exposure to Pb during gestation and the early postnatal period produces greater deficits in learning performances than Pb exposure in older animals (Jett et al., 1997; Kuhlmann et al., 1997). Thus, there seems to be a critical period of brain development in which neuronal processes are highly vulnerable to the presence of Pb (Toscano and Guilarte, 2005).

Currently, the CDC limit of concern for childhood blood Pb level remains at 10 μg/dL, however, recent data suggests that there is no safe level of Pb. Blood Pb levels lower than 10 μg/dL can produce neurobehavioral-cognitive deficits in children, including IQ decline, and attention deficit hyperactivity disorder (ADHD) (Bellinger and Bellinger, 2006; Braun et al., 2006; Bellinger, 2008). Pb exposure is also associated with deficits in central auditory temporal processing (Finkelstein et al., 1998; Lurie et al., 2006; Jones et al., 2008), and, interestingly, children with ADHD have been shown to have deficits in auditory temporal processing (Breier et al., 2003; Facoetti et al., 2003; Putter-Katz et al., 2005; Wright and Conlon, 2009). Compared to the intensive studies conducted on the effect of developmental Pb exposure on cognitive disorders, the cellular mechanism underlying Pb-induced sensory system dysfunction, including dysfunction of the central auditory system, is largely unknown.

We have previously reported that low-level Pb exposure during development decreases the expression of serotonin (5-HT) and the vesicular monoamine transporter 2 (VMAT2) in brainstem auditory nuclei, particularly in the lateral superior olive (LSO), suggesting that the serotonergic system is a target of Pb (Fortune and Lurie, 2009). During development, certain "non-serotonergic" neurons, including the principle projection neurons of the auditory, visual, and somatosensory system transiently take up

5-HT through temporary expression of the serotonin re-uptake transporter, SERT (Gaspar et al., 2003). Disruption of this transient uptake has been shown to cause permanent changes in adult behavior and to result in modifications to the fine wiring of neural connections in the brain (Gaspar et al., 2003). In the auditory system, a subset of LSO neurons transiently expresses 5-HT during the peri and postnatal period (Cases et al., 1998; Thompson, 2006). We have previously shown that developmental Pb exposure results in a delay in the uptake of 5-HT by postnatal LSO neurons, and that this delay delay is correlated with altered synaptic maturation (chapter I). Because we also observed decreases in 5-HT and VMAT2 immunoreactivity in the adult LSO following developmental Pb exposure (Fortune and Lurie, 2009), it may be that Pb results in the mis-wiring of neuronal connections that persist into adulthood. Alternatively, the Pb induced changes in 5-HT and VMAT2 immunoreactivity within the adult LSO could be a result of an acute effect of Pb on 5-HT metabolism. In fact, Pb has been shown to alter brain 5-HT levels following acute exposure in the adult animal (Xu et al., 2005).

The current study was undertaken to determine whether developmental Pb exposure has an acute effect on the 5-HT system in the adult central auditory system of the brainstem or whether the decrease in VMAT2 and 5-HT observed in the adult is largely due to changes in process arborization and synaptogenesis during development as we have previously suggested (chapter I). HPLC was utilized to measure whether Pb affects 5-HT metabolism in the adult and VMAT activity was measured in isolated synaptic vesicles to determine whether Pb acutely modulates the loading of 5-HT into presynaptic vesicles. In addition, brainstem sections from P21 control and Pb-exposed mice were immunostained for SERT to establish whether the transporter expression is

changed by Pb in the adult. Finally, the serotonergic system within the central nucleus of Inferior Colliculus (IC) was examined to ascertain whether Pb disrupts 5-HT within a major target of the LSO.

We found that Pb exposure increased brainstem levels of 5-HT, even though 5-HT immunostaining within the LSO decreases. Thus, the effect of Pb on the LSO is specific to that nucleus and is not a general response of the brain to Pb. Pb had no effect on SERT immunoreactivity within the LSO or on VMAT activity. Taken together, these findings demonstrate that the Pb induced decreases in 5-HT immunostaining within the P21 LSO are not the result of altered synthesis, degradation or re-uptake of 5-HT. We found similar trends in the IC, although in this case Pb caused an increase in 5-HT immunoreactivity within the central nucleus, but no change in total 5-HT levels within the IC or in SERT immunostaining. Again these studies indicate that the Pb-induced increases in 5-HT immunoreactivity within the IC are not the result of altered synthesis, degradation, or re-uptake of 5-HT. Finally, GAP-43 immunoreactivity (a marker of synaptic development) was modified in the adult Pb-exposed LSO, adding support to the hypothesis that developmental Pb exposure modulates process arborization and synaptogenesis during the early postnatal period, and these changes persist into adulthood.

Materials and Methods

Chronic lead exposure to CBA/CaJ mice

Breeding pairs of CBA/CaJ mice were obtained from the Jackson Laboratory (Bar Harbor, MA). Mice were maintained in microisolator units and kept 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 guidelines. 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 (very low) or 0.1 mM (low) lead acetate. Offspring were exposed to lead throughout gestation and through the dam's milk until sacrifice on postnatal day 21 (P21).

Tissue preparation for Immunohistochemistry

Mice were deeply anesthetized using 2',2',2'-tribromoethanol (TBE) and perfused transcardially with 4% Na-periodate-lysine-paraformaldehyde fixative (PLP, final concentrations 0.01M sodium periodate, 0.075M lysine-HCl, 2.1% paraformaldehyde, 0.037M phosphate). Brains were removed and post-fixed in PLP overnight at 4°C, rinsed 3 times for 10 minutes each in phosphate buffered saline (PBS) and transferred to a 20% sucrose solution in PBS for 2 days at 4°C. Finally, brains were transferred to a 1:1 mixture of a 20 % sucrose solution in PBS and Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) for 1-2 days at 4°C. Brains were embedded into 1.5 cm square embedding cups filled with O.C.T. compound, and then frozen in dry ice and 100% ethanol and stored at -20°C. Ten micron tissue sections were cut on a Thermo Shandon Cryotome Cryostat (Thermo Shandon, Pittsburg, PA) and a one in three series was collected for each brain.

Immunohistochemistry

Sections were thawed to room temperature and rinsed in PBS three times for 10 minutes each. Sections were then permeabilized for 30 minutes in 0.5% Triton X-100 in PBS and blocked for 30 minutes with 4% of the normal goat serum (Vector Laboratories, Burlingame, CA) in PAB (1% sodium azide, 0.5% bovine serum albumin in PBS) and incubated with primary antibody for 1-2 days in a humid chamber at 4°C. The sections were rinsed in PBS three times for 10 minutes each and incubated with the goat Alexa Fluor-488 anti-rabbit secondary antibody (1:400, Invitrogen, Grand Island, NY) in PAB for 1 hour at room temperature in the dark. Sections were then rinsed in PBS followed by distilled water and coverslipped with FluorSaverTM (Calbiochem®, San Diego, CA) and stored at 4°C. The primary antibodies used for immunohistochemistry were as follows: rabbit polyclonal anti-5-HT (1:10,000); rabbit polyclonal anti-VMAT2 (1:2000), rabbit polyclonal anti-SERT (1:250); rabbit polyclonal anti-GAP-43 (1:1000).

Antibodies

The rabbit polyclonal against serotonin (5-HT) was raised in rabbit against serotonin coupled to bovine serum albumin with paraformaldehyde (Cat. No. 20080, ImmunoStar Inc., Hudson, WI). No cross-reactivity of the serotonin antisera was seen with 5-hydroxytrytophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's specifications). This 5-HT antibody labels our mouse brainstem sections in a staining pattern virtually identical to that of other studies using the same antiserum to label the mouse superior olive and inferior colliculus (Hurley et al., 2002; Thompson 2006). This antibody has also been characterized in our mouse system (Fortune and Lurie, 2009). In

addition, preadsorption with the 5-HT/bovine serum albumin (BSA) conjugate protein (20 μg/ml, Cat. No. 20081, ImmunoStar Inc., Hudson, WI) eliminates all immunoreactivity, whereas preadsorption with BSA does not affect immunostaining.

The rabbit polyclonal antibody against the serotonin transporter (SERT) was raised in rabbit against the synthetic peptide sequence corresponding to amino acids (602-622) of the rat 5-HT transporter coupled to keyhole limper hemocyanin (Cat. No. 24330, ImmunoStar Inc., Hudson, WI). This SERT antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in mouse superior olive (Thompson and Thompson, 2008).

The rabbit polyclonal antibody against the vesicular monoamine transporter 2 (VMAT2, C-terminal) was raised in rabbit against the synthetic peptide comprising amino acids sequence (496-515) at the cytoplasmic C-terminus of rat VMAT2 (Cat. No. 135402, Synaptic System, Gottingen, Germany). This VMAT2 antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in the rat median eminence (Weihe et al., 1996) and has been previously characterized in our system (Fortune and Lurie, 2009).

The rabbit polyclonal anti-GAP43 was raised in rabbit against the GAP-43 synthetic peptide sequence corresponding to amino acids (216-226) of rat GAP-43 (Cat. Ab7462, Abcam Inc., Cambridge, MA). This GAP-43 antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in the rat optic nerve (Lingor et al., 2007), and the rat superior olive (immunostained with a different mouse monoclonal antibody against GAP-43 (Roche; Horvath al., 1997)).

Tissue Imaging and analysis

All fluorescent slides were viewed with a 60x objective using either a Bio-Rad Radiance 2000 Confocal microscope or an Olympus FV 1000 Fluoview Confocal microscope. Quantitative analysis for immunostaining was performed as previously described (Fortune and Lurie, 2009). Briefly, images were collected and then converted from color tiff files to black and white 12-bit tiff files, and the integrated optical density (IOD) of the immunostaining was measured using MediaCybernetics Image-Pro software (Bethesda, MD). IOD 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. A threshold of immunostaining in control (No Pb) animals was set for each antibody such that all immunoreactivity met threshold, and was used as a comparison with the Pb treatment groups. Immunostaining within the LSO and IC in control and Pb-exposed mice were then quantified and averaged. Statistical differences in immunostaining between control and Pb-exposed mice were analyzed using KaleidoGraph software (Synergy Software, Reading, PA) or GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA).

Sample preparation for HPLC analysis

Brainstems from P21 mice were dissected using a 1mm mouse brain matrix. First, a 1mm thick section was cut and the inferior colliculus region (IC) was separated from the rest of midbrain. Then, a 2mm thick section was cut and frozen on a razor blade using liquid nitrogen and dissected into two separate regions: the ventral brainstem region (VBS), containing the superior olivary complex (SOC), and the dorsal brainstem region

(DBS), containing no auditory nuclei. In terms of the raphe nuclei, the DBS fraction contains most of the rostral group nuclei, including the dorsal and median raphe, and the VBS fraction contains most of the caudal group of the raphe (including the raphe pallidus, and magnus), and part of the rostral group nuclei, including the median raphe. These fractions were immediately weighed and individual VBS, DBS, and IC fractions from three mice were then combined, and homogenized in 150 µl of 0.05 M perchloric acid solution (PCA) containing 3,4-dihydrobenzylamine (DBA, 31ng/ml) as an internal standard (Branson Sonifier 150, Branson, Danbury, CT), and stored at -20 ºC until ready to use.

RP-HPLC analysis of 5HT/5HIAA

5-HT and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) levels were measured using reverse phase high performance liquid chromatography (RP-HPLC) with electrochemical detection. The homogenates were centrifuged at 14,000 x g for 20 min at 4ºC and the supernatants were filtered through a Millex® hydrophilic LCR (PTFE) 0.45 um filter (Millipore, Bedford, MA). Sample filtrates were loaded into autosampler vials and were placed into an ESA Model 5 autosampler (ESA, Chelmsford, MA). An OmniSpher 5 C18 chromatographic column (Varian Inc., Lake forest, CA) with a length of 25 cm and an internal diameter of 4.6 mm was used to separate analytes. The mobile phase consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12 M sodium hydroxide, 0.6 mM EDTA and 1.30 mM sodium octyl sulfate, and the pH was adjusted to 3.2 with glacial acetic acid. A constant flow rate of 1ml/min was maintained and the column effluent was analyzed with a Model 5600A ESA CoulArray®

electrochemical detector (ESA, Chelmsford, MA). Potentials of the three ESA Model 6210 four channel electrochemical cells, placed in series, were as follows: (channels 1 through 5)-50, 0, 25, 100, 200 mV, (6 through 12) 300 mV. 5-HIAA and 5-HT were monitored at 200 mV. Peak area ratios were used to calculate 5-HT and 5-HIAA levels from a calibration curve (peak area of analyte/ peak area of DBA).

Vesicular Monoamine Transporter (VMAT) Assay

Synaptic vesicles were isolated according to the method of Kish and Ueda (1989), and modified accordingly (Kish and Ueda, 1989; Bartlett et al., 1999). Sprague-Dawley rats (200-220 g) were sacrificed by decapitation. The brains were removed and minced in ice cold buffer containing 0.32 M sucrose, 1.0 mM $NaHCO_3$, 1.0 mM magnesium acetate, and 0.5 mM calcium acetate (pH 7.2). The minced brains were homogenized (motorized Potter-Elvejham, Teflon/glass; Wheaton) and centrifuged for 15 min at 12,000g (4ºC, Sorvall SS-34 rotor, Du Pont, Newton, CT). The resulting pellets were resuspended in an ice-cold lysing solution (6 mM Tris-maleate, pH 8.1) for 45 min and centrifuged at 43,000g for 15 min. Supernatants were then centrifuged for 55 min at 222,000g (Beckman Ti 70 rotor; Beckman Instrumentation, Fullerton, CA). The final pellets were resuspended by homogenization in 0.32 M sucrose, 1.0 mM $NaHCO_3$, and 1.0 mM dithiothreitol (pH 7.2). The final synaptic vesicles were stored at -80 ºC until ready to use.

Vesicular uptake of 5-HT was performed as described previously (Bartlett et al., 1998). Synaptic vesicles were resuspended in a buffer containing 5.0 mM $MgCl_2$, 375 mM sucrose, and 5.0 mM N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]

(HEPES, pH 7.4) and maintained at 4 ºC. Duplicate aliquots (1.0 mg protein/mL) of vesicles were preincubated for 5 min at 30 ºC. The uptake was initiated with the addition of a concentrated stock solution (mixture of 5-HT and Pb-Ac, reserpine, or KNO_3; 20 µL, 30 ºC) to 7.815 µM [^{3}H]-serotonin, which yielded a final assay mixture of 100 nM 5-HT, 2.0 mM ATP, 4.0 mM $MgCl_2$, 4.0 mM KCl, 300 mM sucrose, and 5.0 mM HEPES (pH 7.4). The concentrations of Pb-Ac used in the assay were 0.1µM, 0.5 µM, 1 µM, 5 µM, and 50 µM. The final mixtures (100 µL) were incubated for 1.5 min and terminated by the addition of 3.0 mL of ice cold 150 mM KCl. Termination of uptake was followed by filtration through Millipore Hawp (25 mm diameter; 0.45 µm pore size). The filters were washed twice more with 3.0 mL of the 150 mM KCl. The filters were transferred to 5 mL glass scintillation fluids. Liquid scintillation fluid (3.5 mL; National Diagnostics) was added to the vials. Radioactivity was quantified using a liquid scintillation counter (LSC, Beckman LS6500). Residual radioactivity (filters without ATP) was subtracted to account for non-specific binding. These nonspecific binding was quantified and accounted for by measuring [^{3}H]-serotonin signal in the absence of ATP, and subtracted from all radioactive counts. Protein concentrations of the assays were quantified using the Pierce BCA protein assay kit (bicinchoninic acid; Thermo Scientific, Rockford, IL).

Statistical Analysis

Data from the immunocytochemistry and HPLC analysis are expressed as mean ± SEM. In the VMAT assay, data were expressed as nmol/min/mg protein mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; $P < 0.05$ was considered significant.

Results

Pb exposure increases total 5-HT levels but does not change the degradation of 5-HT in the brainstem of P21 mice

At P21, the murine LSO has been shown to be heavily innervated by serotonergic axon terminals from the raphe (Thompson and Hurley, 2004). Thus, it is possible that Pb may decrease synthesis, or increase degradation of 5-HT in the adult raphe, thereby resulting in decreased 5-HT immunoreactivity in the P21 LSO. To determine whether Pb alters either synthesis or degradation of 5-HT within the entire brainstem (which contains the raphe nuclei) of P21 mice, 5-HT and 5-HIAA were measured in total brainstem fractions by HPLC analysis. The brainstem was dissected into the ventral brainstem (VBS, which is enriched in auditory nuclei, including the LSO) and the dorsal brainstem (DBS, which contains no auditory nuclei, but instead contains most of the dorsal raphe nuclei), as described in the *Methods*. Very low Pb increased 5-HT levels in both the VBS and DBS fractions compared to controls (Figure 13). In contrast, 5HIAA levels and 5-HT turnover (5-HIAA/5-HT) were not affected by Pb exposure, demonstrating that Pb does not affect degradation of 5-HT, and may increase the synthesis of 5-HT in the brainstem (Figure 13).

These findings reveal that the Pb-induced decreases in 5-HT immunoreactivity within the P21 LSO are not the result of either decreased brainstem 5-HT levels or increased degradation of 5-HT. In fact, total 5-HT levels within the brainstem increased with Pb exposure, even though 5-HT immunoreactivity within LSO was decreased. These results add support to the hypothesis that Pb exerts a developmental effect on the 5-HT system within the LSO by modulating serotonergic innervation within the LSO.

Pb exposure does not affect SERT expression in the LSO of P21 mice

In order to determine whether the Pb-induced decrease in 5-HT in the adult LSO is a result of impaired uptake into processes, the expression of SERT was characterized. Brainstem sections from P21 control and Pb-exposed mice were immunostained for SERT. Both very low and low Pb exposure did not change SERT immunoreactivity within the LSO compared to controls (Figure 14). Quantification of SERT immunostaining confirmed that Pb has no effect on SERT expression (Figure 14D), demonstrating that Pb induced decreases in 5-HT immunoreactivity at P21 is not correlated with altered SERT expression.

Pb does not affect VMAT activity

We have previously reported that Pb also decreases VMAT expression in the LSO of P21 mice (Fortune and Lurie, 2009), but it is not known whether Pb also impairs the function of VMAT. Therefore, VMAT activity was measured in isolated synaptic vesicles of adult rats treated with various concentrations of Pb-acetate (Pb-Ac), ranging from 0.1 μM to 50 μM.

These concentrations were chosen so the in-vitro assay mimicked the estimates of brain concentrations of Pb used in our in vivo mouse model. Specifically, our Pb concentrations in vivo yielded blood Pb levels of 8.0 ± 0.4 μg/dL in very low Pb, and 42.3 ± 1.97 μg/dL in low Pb exposed mice (Fortune and Lurie, 2009). Based on these blood Pb levels, we then estimated brain Pb concentrations based on the previous studies of Widzowski and Cory-Slechta (1994) who reported that blood Pb levels of 17 μg/dL

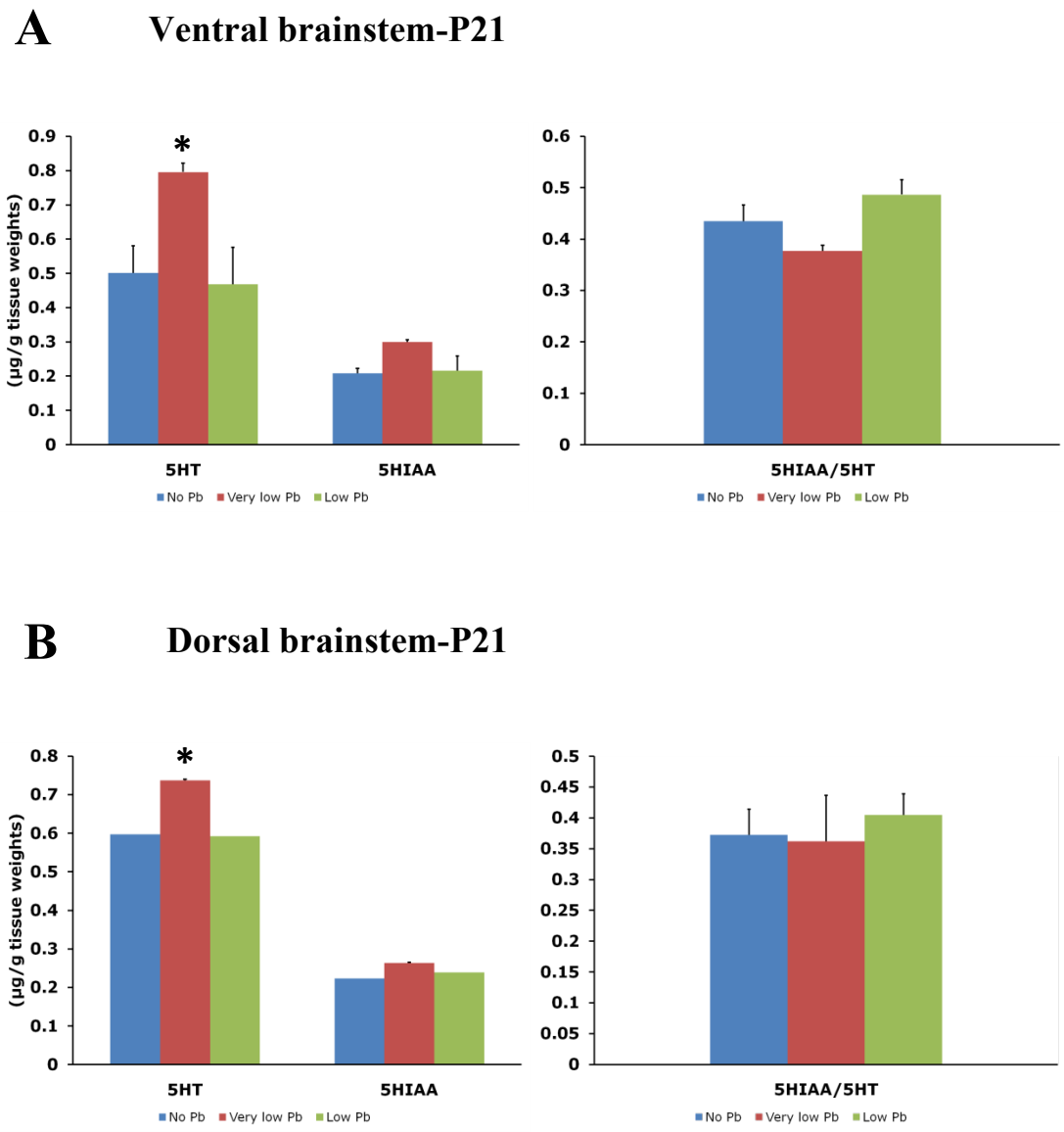


Figure 13. Pb exposure increases brainstem levels of 5-HT in P21 mice as measured by HPLC. Pb exposure increases 5-HT levels both in the VBS (A) and DBS (B) fractions. Pb does not affect 5-HIAA levels or the 5-HT turnover (5HIAA/5-HT) in either fraction (A, B). Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test ($n = 6$).

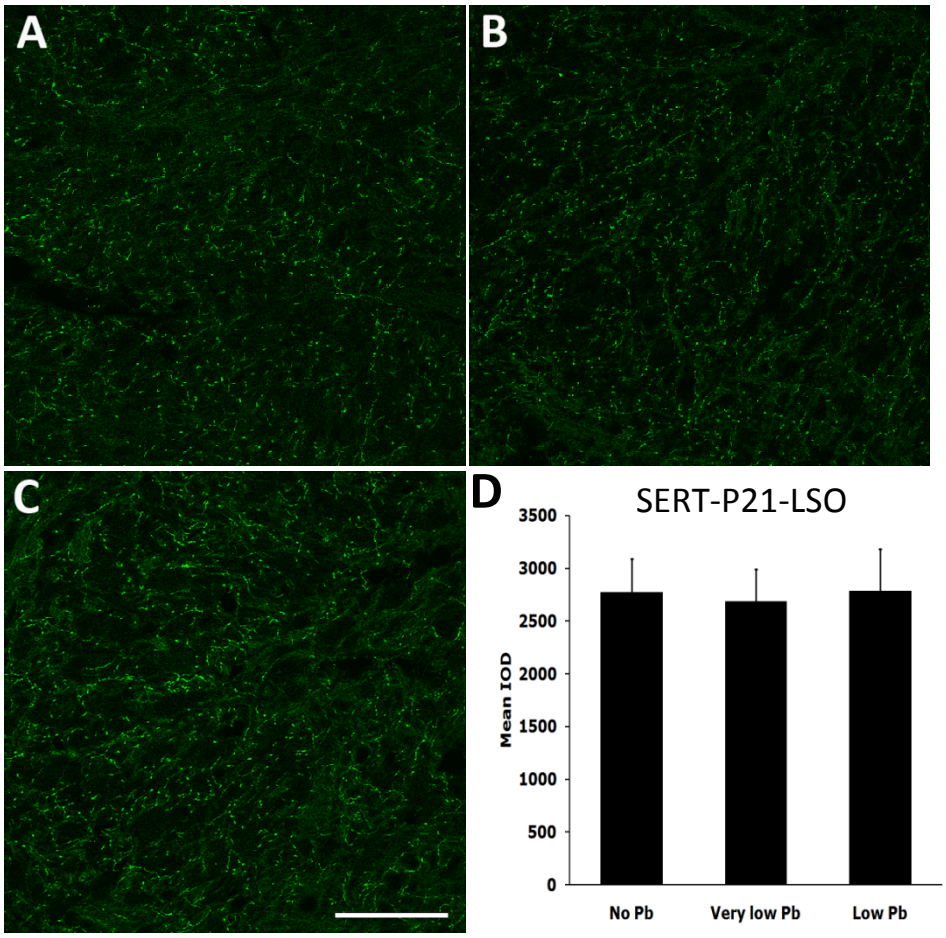


Figure 14. Pb exposure does not affect SERT immunofluorescence (green) labeling within the LSO of P21 mice. **A-C:** Immunoreactivity for SERT does not change with Pb exposure (B, C) compared to the no Pb control (A). **D:** Quantification of SERT immunostaining confirms that Pb does not affect SERT expression levels in the LSO. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 50 μm.

and 38 μg/dL correspond to brainstem Pb levels of 0.1 μg/g wet tissue weight (0.330 μM Pb-Ac) and 0.48 μg/g wet tissue weight (1.582 μM Pb-Ac) (Widzowski and Cory-Slechta, 1994). Table 1 illustrates that Pb did not affect VMAT activity in any of the concentrations used compared to no Pb controls. In contrast, the uptake of 5-HT was almost completely inhibited in synaptic vesicles treated with reserpine, a known VMAT inhibitor, and KNO3, a known VATPase inhibitor (Table 1). These findings demonstrate that Pb does not acutely affect the function of VMAT.

Pb changes the expression pattern of GAP-43 in the LSO of P21 mice

The findings that Pb increases 5-HT within the total brainstem and does not change either SERT expression or VMAT activity, lends support to our hypothesis that Pb exerts a developmental effect on the serotonergic system and produces changes in axonal aborization and synaptogensis that persist into adulthood. We have previously shown that Pb modulates the expression of GAP-43 within the LSO of early postnatal mice (chapter I). GAP-43 is a marker for axonal growth and plasticity (Illing et al., 2000). Within auditory brainstem nuclei, both the intensity of immunoreactivity as well as the staining pattern of GAP-43 is altered following injuries such as cochleotomy and acoustic trauma (Illing et al., 1997; Michler and Illing, 2002).

Unlike other auditory brainstem nuclei where GAP-43 mRNA levels are high during the first postnatal week but gradually decrease and are almost entirely lost in the adult animal, LSO neurons are unique in that they continue to express high levels of GAP-43 mRNA into adulthood. The persistence of GAP-43 indicates the plasticity potential of LSO neurons (Illing et al., 1999).

Table 1. VMAT activity assay

Compound	Percent of Control
0.1 μM Pb-Ac	81 ± 14.5
0.5 μM Pb-Ac	99 ± 4.7
1 μM Pb-Ac	113
5 μM Pb-Ac	103 ± 4.7
50 μM Pb-Ac	130 ± 8.4
0.1 μM Reserpine	6 ± 2.3
50 mM KNO_3	18 ± 4.6

Table 1. Pb does not significantly alter the VMAT-mediated transport of ^{3}H-serotonin into synaptic vesicles isolated from rat brains. Data are presented as % of control (Mean ± SE). Reserpine, an inhibitor of VMAT, is used as a positive control for VMAT inhibition and does inhibit the activity of VMAT in this assay. Similarly, KNO3, an inhibitor of VATPase, also inhibits the activity of VMAT.

We have also found that decreases in SYP are accompanied by decreased expression of GAP-43 in the Pb exposed LSO of early postnatal mice (Figure 10-12 in chapter I). In order to determine whether Pb results in changes in GAP-43 in adult (P21) mice as well, control and Pb-exposed brainstem sections from P21 mice were immunostained for GAP-43. Figure 15A illustrates that the control LSO contains a moderate amount of GAP-43 immunostaining that appears to be localized within distinctly delineated fibers and varicosities, presumably reflecting the presynaptic endings (Horvath et al., 1997). Quantification of total GAP-43 staining in the LSO demonstrated that Pb exposure did not affect total intensity of GAP-43 staining compared to controls (Figure 15D). However, the pattern of staining changed. In the Pb treated mice, there was increased immunostaining within varicosities that likely represent synaptic boutons onto LSO neurons. These varicosities (or boutons) were rather diffusely distributed, and the size of these varicosities was generally larger in the LSO of Pb exposed mice (Figure 15B, C). The modulations in GAP-43 expression together with the known Pb-induced decreases in syaptophysin within LSO (Fortune and Lurie, 2009) further support the hypothesis that developmental Pb exposure produces permanent changes in synapse formation within the adult LSO.

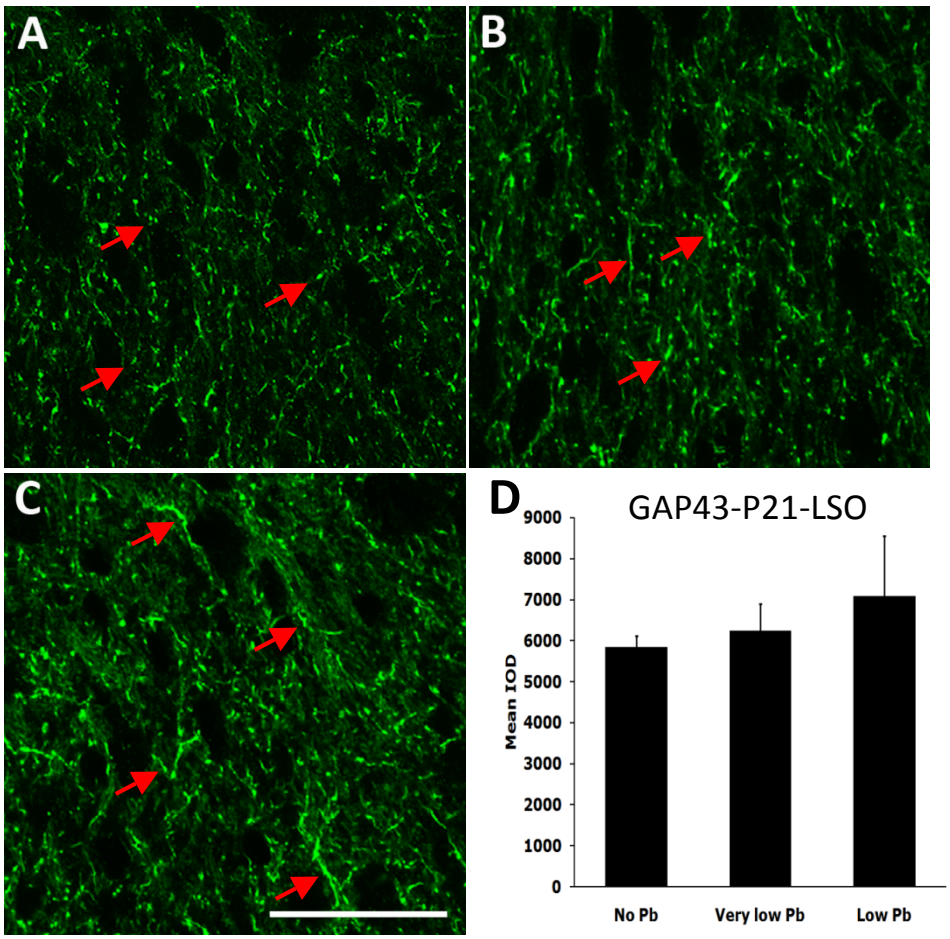


Figure 15. Pb exposure alters the staining pattern of GAP-43 immunolabeling (green) within the LSO of P21 mice. **A-C:** GAP-43 Immunoreactive varicosities in the LSO are rather diffusely distributed, and the size of boutons is generally larger with Pb exposure (arrows in B, C) compared to control (A). **D:** Quantification of SERT immunostaining shows that Pb does not affect total GAP-43 expression levels in the LSO. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 50 µm.

Pb increases 5-HT expression in the IC of P21 mice

LSO neurons provide a major afferent input to the central nucleus of inferior colliculus (CNIC), contributing to the tonographically organized laminar formation within the IC (Thompson and Schofield, 2000). In the rat IC, developmental processes that include axon collateralization and synapse formation occur predominately from birth to P11 (Kandler and Friauf, 1993), the same time period when LSO neurons transiently accumulate 5-HT. Because we have shown that Pb delays the normal developmental uptake of 5-HT by LSO neurons, an intriguing possibility is that Pb could also affect the serotonergic system within the IC.

Therefore, control and Pb exposed brainstem sections from P21 and P4 mice were immunostained for 5-HT. P4 mice showed very little 5-HT immunostaining in the IC, and Pb exposure did not appear to change the levels of immunoreactivity (data not shown). The 5-HT positive processes within the CNIC originate mainly from dorsal and median raphe in adult animal (Klepper and Herbert, 1991; Hurley et al., 2002) and we observed 5-HT immunoreactive processes in the P21 IC of control and Pb exposed mice (Figure 16). Very low levels of Pb significantly increased 5-HT immunoreactivity within the LSO (Figure 16B, D) but low Pb exposure did not affect 5-HT immunoreactivity (Figure 16C). This is very interesting, because Pb decreased 5-HT in the adult LSO, but increased 5-HT in the IC.

Pb does not affect total IC levels of 5-HT and 5HIAA

Levels of 5-HT and 5-HIAA were measured in the IC fractions of P21 mice by HPLC analysis in order to determine whether Pb affects 5-HT synthesis and/or

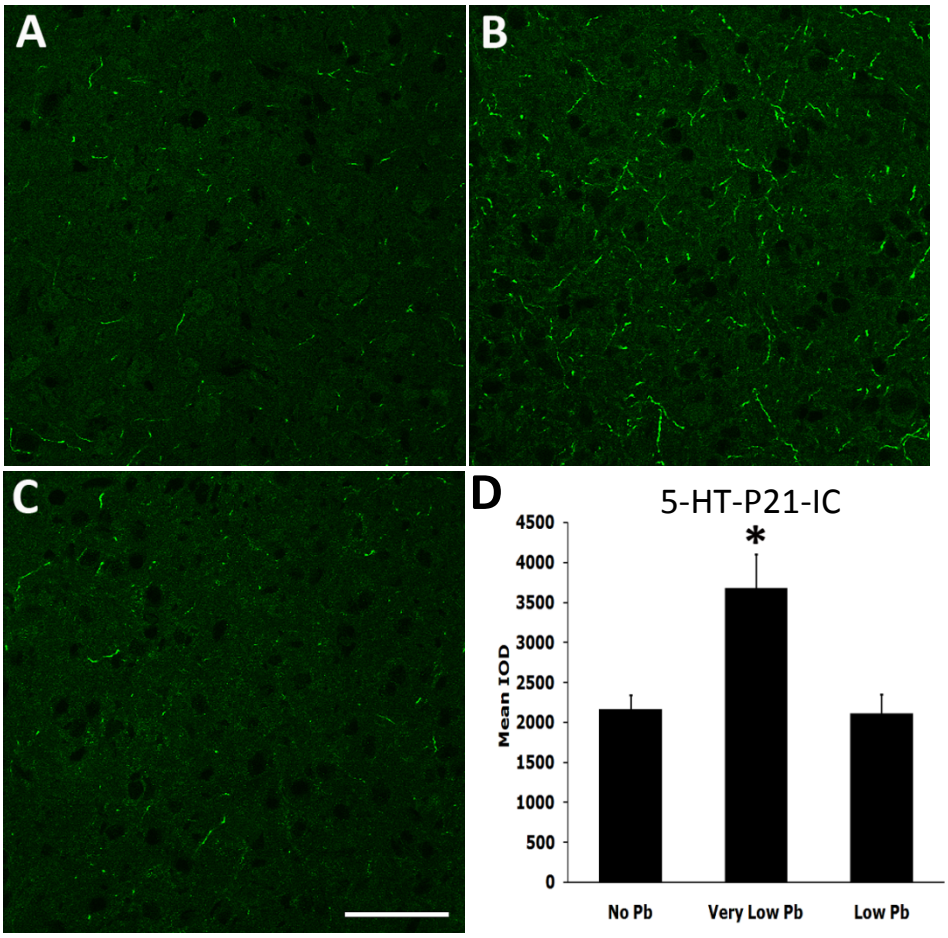


Figure 16. Pb exposure increases 5-HT immunofluorescence (green) labeling in the IC of P21 mice. **A-C:** Immunoreactivity for 5-HT in the IC increases with very low Pb exposure (B) compared to the no Pb control (A). Low Pb exposure (C) does not result in changes in total 5-HT expression. **D:** Quantification of 5-HT immunostaining in the IC confirms a significant increase with very low Pb. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 7). Scale bar = 50 μm.

degradation within the IC. Pb exposure did not affect 5-HT levels in the IC fractions compared to controls (Figure 17A). In addition, 5HIAA levels and 5-HT turnover (5-HIAA/5-HT) were not affected by Pb (Figure 17B, C). Thus, Pb did not acutely affect synthesis or degradation of 5-HT in the adult IC but did increase the number of 5-HT processes within the CNIC. This argues for a developmental effect of Pb on the CNIC, perhaps involving permanent axonal rearrangements of 5-HT fibers.

Pb does not affect SERT expression in the IC of P21 mice

In order to confirm that the increase in 5-HT in the CNIC is not correlated with changes in SERT expression, brainstem sections from P21 control and Pb-exposed mice were immunostained for SERT. Both very low and low Pb did not change SERT immunoreactivity within the IC of P21 mice compared to controls (Figure 18), demonstrating that Pb induced increases in 5-HT expression in the P21 IC were not correlated with any changes in SERT expression. Thus, Pb did not impact 5-HT re-uptake, synthesis or degradation in the P21 IC.

Pb increases VMAT2 expression in the IC of P21 mice

Finally, Pb exposure has been shown to decrease VMAT2 expression in the LSO of P21 mice (Fortune and Lurie, 2009); thus it is possible that Pb also affects VMAT2 expression in the IC. To test whether Pb modulates the expression of VMAT2 within the IC, control and Pb exposed brainstem sections were immunostained for VMAT2. Low Pb significantly increased immunoreactivity for VMAT2 in the CNIC of P21 mice

(Figure 19C, D), compared with controls (19A). In contrast, very low Pb did not result in changes in VMAT2 expression (Figure 19B).

It is of interest to note that Pb increased the number of 5-HT stained processes within the IC following very low level exposure, but there was increased density of VMAT2 (and presumably presynaptic terminals) at a different (low level) exposure. These findings suggest that Pb might have two different effects on axonal maturation at different doses during the period of IC development. At very low levels (0.01 mM), Pb increases axon collateralization or alternatively, interfers with the retraction of serotonergic axonal processes within the IC. In contrast, at higher doses (our low Pb dose, 0.1 mM), Pb may increase the number of axon terminal that contain VMAT2.

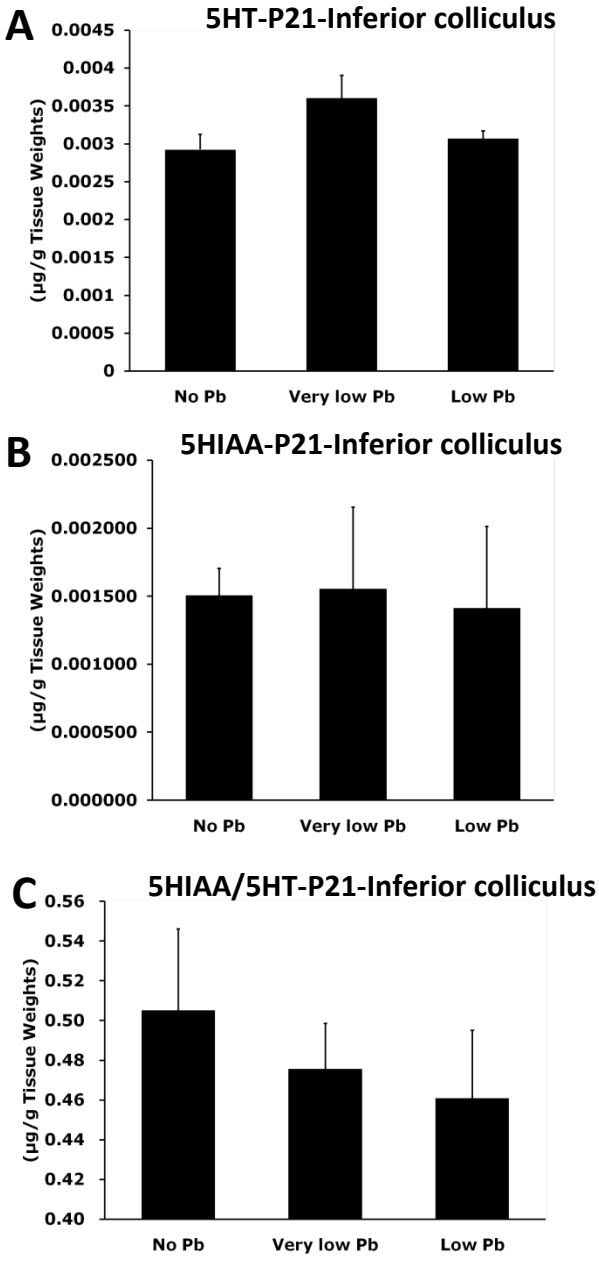


Figure 17. Pb exposure does not change 5-HT levels in the inferior colliculus of P21 mice as measured by HPLC analysis (A, C). In addition, Pb does not change 5-HIAA levels or 5-HT turnover (5HIAA/5-HT) in the IC (B, C). Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 6).

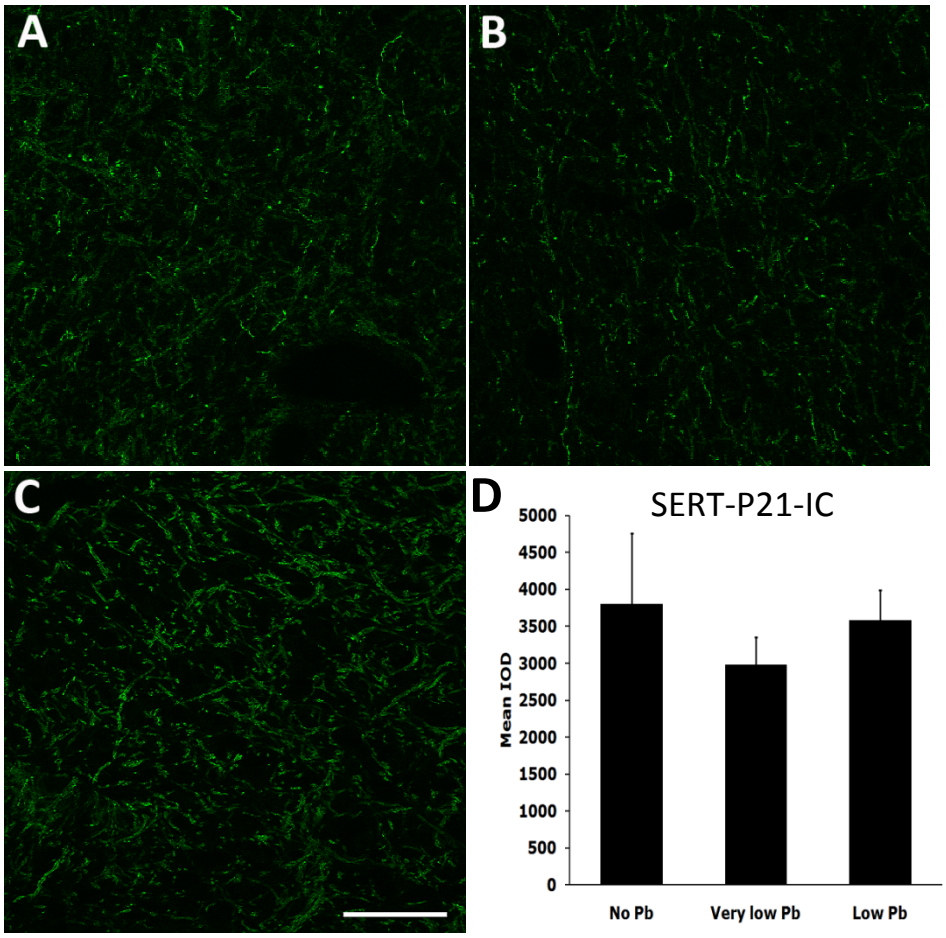


Figure 18. Pb exposure does not alter SERT immunofluorescence (green) labeling within the IC of P21 mice. **A-C:** Immunoreactivity for SERT in the IC does not change with Pb exposure (B, C) compared to the no Pb control (A). **D:** Quantification of the SERT immunostaining confirms that Pb has no effect on SERT labeling in the IC. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 5). Scale bar = 50 μm.

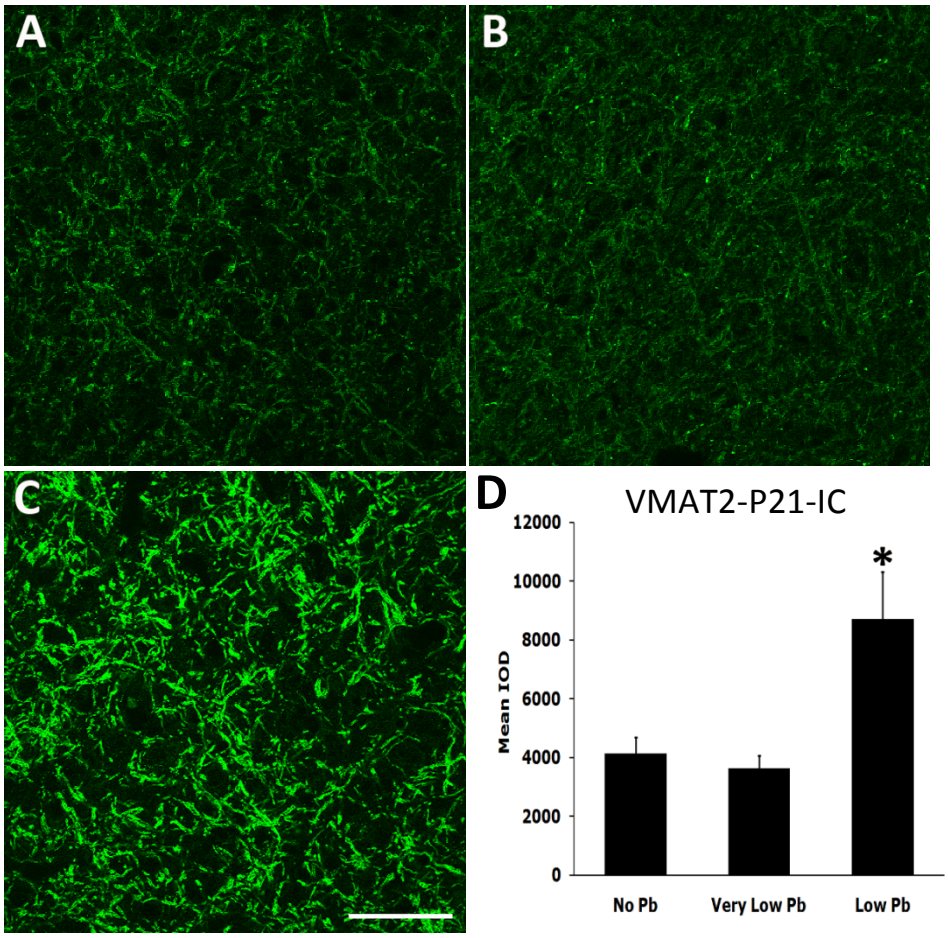


Figure 19. Pb exposure increases vesicular monoamine transporter 2 (VMAT2) immunofluorescence (green) labeling in the IC of P21 mice. **A-C:** Immunoreactivity for VMAT2 in the IC increases with low Pb exposure (C) compared to no Pb control (A). Very low Pb exposure (B) does not result in changes in total VMAT2 expression. **D:** Quantification of VMAT2 immunostaining in the IC confirms that the increase with low Pb is statistically significant. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 7). Scale bar = 50 µm.

Discussion

The current study demonstrates that a Pb-induced decrease in 5-HT immunostaining within the P21 LSO is not the result of an acute effect of Pb on 5-HT metabolism. Changes in the GAP-43 staining pattern in the LSO suggest that decreased 5-HT and VMAT2 immunoreactivity is correlated with altered axonal arborization and synapse development in the LSO. In addition, very low levels of Pb exposure increased 5-HT immunoreactivity in the IC of P21 mice, suggesting that Pb may also affect the development of the IC. Pb did not alter total IC levels of 5-HT and 5HIAA, and had no effect on SERT expression within the IC, suggesting that the Pb induced increase in 5-HT immunoreactive processes that is observed with very low Pb exposure is likely the result of changes in axonal arborization that occurs during development. Interestingly, low levels of Pb exposure increased VMAT2 expression within the IC, suggesting that different doses of Pb might have two different effects on axonal maturation of the IC. To our knowledge, this is the first evidence in vivo that differing doses of Pb affect different aspects of neuronal maturation in the same brain area.

Effect of Pb on 5-HT processes in the LSO and IC

5-HT functions as a regulatory factor for the brain development prior to the time when it plays a role as a neurotransmitter (Luo et al., 2003). Alterations in 5-HT homeostasis during development have been shown to cause permanent changes in the fine wiring of neural connections in the brain and to result in abnormal behaviors that persist into adulthood (Mazer et al., 1997; Vataeva et al., 2007; Vitalis et al., 2007; Vataeva et al., 2008). The most striking evidence of 5-HT induced mis-wiring of brain

connections comes from the somatosensory system, where "non-serotonergic" thalamocortical neurons transiently accumulate 5-HT during early postnatal development. Maintaining normal 5-HT levels in this system is critical for the proper formation of barrels that are the morphological substratum of the somatosensory cortical map, in which each of the barrels corresponds to the afferent thalamic axonal projection from one whisker to the somatosensory cortex (Gaspar et al., 2003). Interestingly, these barrels are absent in the somatosensory cortex of MAOA k/o mice where brain 5-HT levels are increased up to 6-9 fold compared to wild type mice (Cases et al., 1996). In contrast to the complete loss of barrel fields induced by excessive amounts of 5-HT, depletion of 5-HT by pharmacological intervention results in barrel fields that are either smaller in size or are developmentally delayed (Blue et al., 1991; Bennett-Clarke et al., 1994; Turlejski et al., 1997). Importantly, there seems to be a critical period for wiring in the somatosensory cortex, because MAOA inhibition during the first postnatal week only causes a disruption in the barrel field formation (Vitalis et al., 1998).

In the auditory system, LSO neurons also transiently accumulate 5-HT most prominently during the first postnatal week (Cases et al., 1998; Thompson, 2006, 2008). During postnatal development, the transient expression of 5-HT precedes the auditory inputs from cochlear nucleus and is concomitant to the maturation of synapses and dendrites in the LSO. The mature pattern of collateral branching of the afferents from the cochlear nucleus is established in the LSO during the period of E18 to P5 in rats (Kandler and Friauf, 1993; Friauf and Lohmann, 1999) with the refinement and pruning of dendrites of LSO neurons beginning at P4 (Rietzel and Friauf, 1998). From these studies, it appears that maintence of proper extracellular concentrations of 5-HT through uptake

by SERT is necessary for the maturation of LSO synapses. Therefore, continuous removal of 5-HT for an extended period of time through uptake into non-serotonergic LSO neurons (Chapter 1) limits the amount of 5-HT that is available for the appropriate maturation of synapses in the LSO.

LSO neurons provide a major afferent input to the central nucleus of inferior colliculus (CNIC), contributing to the tonographically organized laminar formation within the IC (Schofield, 2006). In the rat IC, developmental processes that include axon collateralization and synapse formation occur predominately from birth to P11 (Kandler and Friauf, 1993), the same time period when LSO neurons transiently accumulate 5-HT. However, IC neurons do not transiently take up 5-HT during development (data not shown) and so the increase in 5-HT positive processes in the IC following Pb exposure could be a secondary effect of Pb on the LSO.

Effect of Pb on synaptogenesis in the LSO and IC

In addition, Pb decreases the total immunostaining of synaptophysin (SYP) and VMAT2 and changes the staining pattern of GAP-43 in the adult LSO indicating that Pb alters synapse maturation within the LSO. SYP is a component of the membrane of presynaptic vesicle, thus immunostaining is thought to label all presynaptic terminals. The current study demonstrates that Pb appears to decrease synaptogenesis in general, including monoaminergic synapses as measured by VMAT2 immunostaining, within the adult LSO. In contrast, Pb resulted in increased VMAT2 staining in the CNIC, but did not change total SYP staining (data not shown). This suggests that Pb specifically affects synaptogenesis of monoaminergic inputs into the IC and has little effect on other synaptic

connections. We have found no significant changes in immunoreactivity for the vesicular glutamate transporter (VGLUT1) in the IC following Pb exposure (data not shown), further supporting the idea that the development of the monoaminergic system is specifically targeted by Pb in central auditory nuclei. The fact that Pb did not affect immunostaining of SYP within the IC also indicates that Pb subtly impairs the precise organization of the topographic map in the IC, instead of causing a general increase or decrease in the total number of synapses. Future studies that include a detailed morphological analysis of single axon arbors immunostained with 5-HT or other monoaminergic markers are necessary to test this possibility.

Summary and Conclusion

The results of the current study reveal that specific changes in 5-HT immunostaining within the LSO and IC are not associated with an acute effect of Pb on 5-HT metabolism, suggesting that Pb may be affecting axonal arborization and synaptic maturation within these central auditory nuclei during development. In addition, we found that Pb affected the pattern of GAP-43 and VMAT2 staining in the adult, further supporting the hypothesis that developmental Pb exposure has lasting effects on synaptic maturation that persist in the adult. The fact that Pb increased the number of 5-HT immunoreactive processes within the IC, but decreased the number of these processes in the LSO further suggests that Pb modulates process arborization differentially in the two nuclei. The mechanism for this remains to be elucidated, but differential effects of Pb on neurite outgrowth have been reported in vitro (Wang et al., 2008). Our results also raise the intriguing possibility that different doses of Pb affect the IC differentially, with very

low doses increasing the number of 5-HT positive processes, and low Pb doses increasing synaptogenesis of monomaminergic neurons. To our knowledge, this is the first evidence in vivo that differing doses of Pb modulate different aspects of neuronal maturation and it will be exciting to further elucidate this mechanism of action of Pb.

CHAPTER III.

LOW LEVELS OF Pb RESULTS IN DECREASED 5-HT EXPRESSION IN THALAMOCORTICAL NEURONS AT THEIR AXON TERMINALS AND DECREASES 5-HT LEVELS IN THE FOREBRAIN OF EARLY POSTNATAL MICE

Abstract

Low-level Pb exposure is a risk factor for cognitive and behavioral dysfunctions including learning disability and ADHD. The cellular mechanism that underlies these Pb-induced dysfunctions, especially during brain development, is incompletely understood. During the early peri and postnatal period of development, certain "non-serotonergic" sensory neurons, including LSO neurons in the auditory system and thalamocortical neurons in the somatosensory system, transiently take up 5-HT from extracellular environment. This is thought to be a way to maintain appropriate levels of extracellular 5-HT that is important for the development of these sensory systems. We have previously reported that low-level Pb exposure results in a delay in the uptake of 5-HT by early postnatal LSO neurons, and that this delay is correlated with altered synaptic maturation (chapter I), suggesting that sensory neurons that take up 5-HT transiently could be a specific target for Pb. The current study was undertaken to determine whether Pb has a similar effect on the transient expression of 5-HT by the thalamocortical neurons of early postnatal mice. Mice were exposed to very low Pb (0.01mM) or low Pb (0.1mM) throughout gestation and until postnatal day 4 (P4), or P8. Forebrain sections from postnatal control and Pb-exposed mice were immunostained for 5-HT and levels of

5-HT and 5HIAA were measured by HPLC analysis. To examine the dorsal raphe, brainstem sections from postnatal control and Pb-exposed mice were immunostained for 5-HT and TPH. We found that Pb decreases immunoreactivity for 5-HT in the thalamocortical axon terminals (TCAs) in the area of somatosensory cortical barrel field. In addition, total forebrain levels of 5-HT and 5HIAA decrease with Pb as does 5-HT immunoreactivity within the DR. Finally, Pb increases TPH expression in the DR, perhaps as a compensatory mechanism. These findings demonstrate that the effect of Pb is not restricted to the TCAs, but appears to have a widespread impact on the forebrain 5-HT system.

Introduction

Lead (Pb) is a highly neurotoxic agent that causes functional and structural abnormalities in the brain (Canfield et al., 2003). The U.S. Environmental Protection Agency (EPA) estimates that 430,000 American children between 1 and 5 years old have blood Pb levels at or above 10 μg/dL (EPA, 2004), and blood levels of 10 μg/dL are thought to be a threshold for childhood cognitive deficits (Landrigan, 2000). The developing central nervous system (CNS) has long been recognized as a primary target for environmental Pb exposure (Landrigan and Todd, 1994; Moreira et al., 2001), therefore Pb could impact critical stages of brain development. Pb has been shown to induce permanent neurological changes when exposure occurs during the first 6 years of life in children (the Centers for Disease Control, CDC, 1991), indicating that this heavy metal has a profound effect on CNS development. Currently, 10 μg/dL is the CDC limit of concern for childhood blood Pb levels (CDC, 1991). However, recent data suggests

that blood Pb levels lower than 10 µg/dL can produce deficits in neurobehavioral-cognitive performances including decline in IQ, and attention deficit hyperactivity disorder (ADHD) (Bellinger and Bellinger, 2006; Braun et al., 2006; Bellinger, 2008).

The cellular mechanism that underlies these Pb-induced cognitive and neurobehavioral dysfunctions, especially during early development, is incompletely understood. We have previously reported that low-level Pb exposure during development decreases the immunoreactivity of serotonin (5-HT) and the vesicular monoamine transporter 2 (VMAT2) in brainstem auditory nuclei, providing a link between Pb exposure and the serotonergic system (Fortune and Lurie, 2009). Indeed, developmental Pb exposure has been shown to alter brain levels of 5-HT and a major metabolite of 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), in various brain regions (Cupo and Donaldson, 1988; Antonio et al., 1996; Antonio and Leret, 2000; Devi et al., 2005). 5-HT functions as a growth regulatory factor in the brain during development, and embryonic or neonatal depletion of 5-HT results in impaired synaptogenesis and abnormal behaviors that persist into adulthood (Mazer et al., 1997; Vataeva et al., 2007; Vitalis et al., 2007; Vataeva et al., 2008). Thus, Pb could have a lasting impact on the brain by modulating 5-HT during critical periods of CNS development.

The rodent somatosensory cortex has been a preferred model of choice to test the effect of 5-HT on brain development, owing to its distinct barrel-like pattern composed of afferent thalamocortical axon terminals (TCAs) and cortical layer IV neurons. Furthermore, these non-serotonergic TCAs transiently take up 5-HT from extracellular sources during the critical period of barrel field formation through SERT, a high affinity 5-HT transporter, which is also transiently expressed in the TCAs (Gaspar et al., 2003).

The cortical barrel fields (BFCx) emerge over the first postnatal week through axon collateral remodeling, and precise regulation of 5-HT levels in thalamocortical neurons is important for the proper formation of the BFCx (Rebsam et al., 2002; Gaspar et al., 2003; Luo et al., 2003). Depletion of 5-HT has been shown to produce a delay in barrel pattern maturation (Blue et al., 1991; Osterheld-Haas and Hornung, 1996) as well as a reduction in barrel field area (Bennett-Clarke et al., 1994; Persico et al., 2000).

Interestingly, in rats exposed to Pb from P1 through P10, the total area of the barrel field in primary somatosensory cortex decreases with increasing doses of Pb (Wilson et al., 2000). This raises the intriguing possibility that the Pb induced deficits in the development of the BFCx in rodents are associated with Pb induced alterations in 5-HT levels in the somatosensory cortex. However, the effect of Pb on the transient uptake of 5-HT by the thalamocortical neurons has not been examined.

The current study was undertaken to determine the effect of developmental Pb exposure on the transient presence of 5-HT within the TCAs in early postnatal mice. Forebrain sections from postnatal control and Pb-exposed mice were immunostained for 5-HT and levels of 5-HT and 5HIAA were measured by HPLC analysis. To test whether Pb exposure affects the synthesis of 5-HT by the dorsal raphe nuclei (DR), a major serotonergic input to the somatosensory cortex, brainstem sections from postnatal control and Pb-exposed mice were immunostained for 5-HT and tryptophan hydroxylase (TPH), a rate limiting enzyme for 5-HT synthesis. We found that Pb exposure decreases immunoreactivity for 5-HT in the TCAs of Pb exposed mice at P4 compared to controls. In addition, total forebrain levels of 5-HT and 5HIAA decrease with Pb exposure as does 5-HT immunoreactivity within the DR. Finally, Pb increases TPH expression in the DR.

These findings demonstrate that a Pb-induced change in 5-HT levels during the early postnatal period is correlated with decreased 5-HT immunostaining in TCAs.

Materials and Methods

Chronic lead exposure to CBA/CaJ mice

Breeding pairs of CBA/CaJ mice were obtained from the Jackson Laboratory (Bar Harbor, MA). Mice were maintained in microisolator units and kept 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 guidelines. 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 (very low) or 0.1 mM (low) lead acetate. Offspring were exposed to lead throughout gestation and through the dam's milk until sacrifice on postnatal day 4 (P4), or day 8 (P8). These concentrations of Pb yield blood Pb levels of 8.0 ± 0.4 μg/dL in very low Pb, and 42.3 ± 1.97 μg/dL in low Pb P21 mice (Fortune and Lurie, 2009).

Tissue preparation for Immunohistochemistry

Mice were deeply anesthetized using 2',2',2'-tribromoethanol (TBE) and perfused transcardially with 4% Na-periodate-lysine-paraformaldehyde fixative (PLP, final concentrations 0.01M sodium periodate, 0.075M lysine-HCl, 2.1% paraformaldehyde, 0.037M phosphate). Brains were removed and post-fixed in PLP overnight at 4°C, rinsed 3 times for 10 minutes each in phosphate buffered saline (PBS) and transferred to a 20%

sucrose solution in PBS for 2 days at 4°C. Finally, brains were transferred to a 1:1 mixture of a 20 % sucrose solution in PBS and Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) for 1-2 days at 4°C. Brains were embedded into 1.5 cm square embedding cups filled with O.C.T. compound, and then frozen in dry ice and 100% ethanol and stored at -20°C. Ten micron tissue sections were cut on a Thermo Shandon Cryotome Cryostat (Thermo Shandon, Pittsburg, PA) and a one in three series was collected for each brain.

Immunohistochemistry

Sections were thawed to room temperature and rinsed in PBS three times for 10 minutes each. Sections were then permeabilized for 30 minutes in 0.5% Triton X-100 in PBS and blocked for 30 minutes with either 4% normal goat or donkey serum (Vector Laboratories, Burlingame, CA) in PAB (1% sodium azide, 0.5% bovine serum albumin in PBS) and incubated with primary antibody for overnight in a humid chamber at 4°C. The sections were rinsed in PBS three times for 10 minutes each and incubated with the appropriate secondary antibody (Alexa Fluor-488 anti-rabbit or Alexa Fluor-488 anti-sheep, Invitrogen, Grand Island, NY) in PAB for one hour at room temperature in the dark. Sections were then rinsed in PBS followed by distilled water and coverslipped with FluorSaverTM (Calbiochem®, San Diego, CA) and stored at 4°C. The primary antibodies used for immunohistochemitry were as follows: rabbit polyclonal anti-5-HT (1:10,000), sheep polyclonal anti-TPH (1:500).

Antibodies

The rabbit polyclonal against serotonin (5-HT) was raised in rabbit against serotonin coupled to bovine serum albumin with paraformaldehyde (Cat. No. 20080, ImmunoStar Inc., Hudson, WI). No cross-reactivity of serotonin antisera was seen with 5-hydroxytrytophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's specifications). This 5-HT antibody labels our mouse forebrain and brainstem sections in a staining pattern virtually identical to that of other studies using the same antiserum to label the mouse raphe nuclei (Thompson 2006), and the mouse somatosensory cortex immunostained with a different rat monoclonal antibody against 5-HT (SeraLab; Lebrand et al., 1996). This antibody has also been characterized in our mouse system (Fortune and Lurie, 2009). In addition, preadsorption with the 5-HT/bovine serum albumin (BSA) conjugate protein (20 μg/ml, Cat. No. 20081, ImmunoStar Inc., Hudson, WI) eliminates all immunoreactivity, whereas preadsorption with BSA does not affect immunostaining.

The sheep polyclonal antibody against tryptophan hydroxylase (TPH) was raised in sheep against recombinant rabbit tryptophan hydroxylase, isolated as inclusion bodies from E.coli (Cat. No. AB1541, Millopore, Temecula, CA). This TPH antibody labels our mouse brainstem sections in a staining pattern virtually identical to that observed in mouse superior olive and raphe nuclei (Thompson and Thompson, 2008).

Tissue Imaging and analysis

All fluorescent slides were viewed at 60x objective using an Olympus FV 1000 Fluoview Confocal microscope. Quantitative analysis for immunostaining was performed as previously described (Fortune and Lurie, 2009). Briefly, images were collected and then converted from color tiff files to black and white 12-bit tiff files, and

the integrated optical density (IOD) of the immunostaining was measured using MediaCybernetics Image-Pro software (Bethesda, MD). IOD 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. A threshold of immunostaining in control (No Pb) animals was set for each antibody such that all immunoreactivity met threshold, and was used as a comparison with the Pb treatment groups. Immunostaining in thalamocortical axon terminals within the area of cortical barrel fields and dorsal raphe nuclei in control and Pb-exposed mice were then quantified and averaged. 5-HT immunostaining has been used to visualize thalamocortical axon terminals within the cortical barrels during the postnatal period (Cases et al., 1996; Lebrand et al., 1996; Rebsam et al., 2002). The 5-HT positive thalammocortical afferents have been shown to reach the cortex early in the embryonic period, and start to form characteristic clusters of cells that constitute barrels at P3. The clusters become more clearly defined by P7, with one thalamocortical axon cluster confined to only one barrel (Rebsam et al., 2002). This 5-HT staining then subsequently decreases and completely disappears by postnatal week two (Cases et al., 1996; Lebrand et al., 1996). Unlike the horizontal section where the distinct columnar structure of barrels is visible, the number and size of barrels varies across the barrel field in the coronal sections that were used in the current study. Therefore, 3-4 random barrels from each of the left and right primary somatosensory cortices from a single coronal section were chosen for analysis, and the IOD from each individual barrel was measured and averaged. We consequently measured intensity of 5-HT immunostaining rather than the total area of staining. A total of 36-40 individual barrels from each mouse were measured. Statistical differences in

immunostaining between control and Pb-exposed mice were analyzed using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA).

Sample preparation for HPLC analysis

Mice from control and Pb exposed groups were decapitated at P4. Brains were quickly dissected and the hindbrain (or brainstem) was removed with a coronal cut separating dorsally the superior and inferior colliculus. After dissection, the remaining basal cerebral cortex was gently removed from the separated hindbrain fraction under the dissecting microscope, and added to the forebrain fraction. Forebrain fractions were immediately weighed, and quickly frozen in the liquid nitrogen. Each forebrain fraction was homogenized in 500 µl of 0.05 M perchloric acid (PCA; containing 30 ng/ml 3,4-dihydrobenzylamine (DBA) as an internal standard) solution (Branson Sonifier 150, Branson, Danbury, CT), and stored at -20 ºC until ready to use.

RP-HPLC analysis of 5HT/5HIAA

5-HT and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) levels were measured using reverse phase high performance liquid chromatography (RP-HPLC) with electrochemical detection. The homogenates were centrifuged at 14,000 x g for 20 min at 4ºC and the supernatants filtered through a Millex® hydrophilic LCR (PTFE) 0.45 um filter (Millipore, Bedford, MA). Sample filtrates were loaded into autosampler vials and were placed into an ESA Model 5 autosampler (ESA, Chelmsford, MA). An OmniSpher 5 C18 chromatographic column (Varian Inc., Lake forest, CA) with a length of 25 cm and an internal diameter of 4.6 mm was used to separate analytes. The mobile phase

consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12 M sodium hydroxide, 0.6 mM EDTA and 1.30 mM sodium octyl sulfate, and the pH was adjusted to 3.2 with glacial acetic acid. A constant flow rate of 1ml/min was maintained and the column effluent was analyzed with a Model 5600A ESA CoulArray® electrochemical detector (ESA, Chelmsford, MA). Potentials of the three ESA Model 6210 four channel electrochemical cells, placed in series, were as follows: (channels 1 through 5)-50, 0, 25, 100, 200 mV, (6 through 12) 300 mV. 5-HIAA and 5-HT were monitored at 200 mV. Peak area ratios were used to calculate 5-HT and 5-HIAA levels from a calibration curve (peak area of analyte/ peak area of DBA).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; $P < 0.05$ was considered significant.

Results

Pb exposure decreases 5-HT immunostaining in developing thalamocortical axon terminals in the BFCx

In order to determine whether developmental Pb exposure affects the normal uptake of 5-HT by thalamocortical axon terminals (TCAs) in early postnatal ages, control and Pb-exposed forebrain sections from postnatal day (P) 4 and P8 were immunostained for 5-HT. In P4 control mice, distinct clusters of 5-HT immuno-positive TCAs were observed in the area of the somatosensory barrel field cortex (BFCx) (Figure 20A). Both

the very low and low Pb exposure significantly decreased 5-HT immunoreactivity in the TCAs (Figure 20B-D) compared with control (Figure 20A). No 5-HT immuno-positive thalamocortical neuronal cell bodies were observed (data not shown) confirming the results of others who have not found 5-HT positive thalamic cell bodies in wild type mice (Cases et al., 1998; Donovan et al., 2002).

By P8, a more defined aggregate of TCAs was observed in the BFCx as delineated by 5-HT immunoreactivity (Figure 21A). Very low levels of Pb resulted in significantly less 5-HT staining in the TCAs (Figure 21B) compared with control (Figure 21A, D). Low levels of Pb, in contrast, produce significantly more 5-HT staining in the TCAs compared to controls (Figure 21C, D). Thus the two different doses of Pb have a differing impact on 5-HT in the TCAs.

Taken together, these findings demonstrate that at very low levels, Pb appears to decrease the normal developmental uptake of 5-HT by the TCAs in the somatosensory cortical barrel field area. It will be of interest to determine whether very low Pb exposed TCAs continue to express 5-HT beyond P10-P14, when TCAs normally lose the ability to take up 5-HT into the cell body.

Pb decreases total 5-HT and 5HIAA levels in the forebrain

To determine whether these observed Pb-induced alterations in 5-HT immunostaining are correlated with changes in either synthesis or degradation of 5-HT within the forebrain, levels of 5-HT and a major metabolite of 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), were measured in crude forebrain fractions of P4 mice by HPLC analysis. Both the very low and low levels of Pb significantly decreased forebrain levels

of 5-HT and 5HIAA compared to controls (Figure 22A, B), suggesting that Pb could be decreasing 5-HT synthesis. 5-HT turnover (5-HIAA/5-HT) was not changed by Pb, demonstrating that Pb does not modulate the degradation of 5-HT in the forebrain (Figure 22C). The HPLC analysis demonstrated that the Pb-induced decreases in the transient accumulation of 5-HT by TCAs are indeed correlated with decreases in forebrain levels of 5-HT. Furthermore, these findings also revealed that the effect of Pb is not restricted to the TCAs, but appears to have a widespread impact on the forebrain 5-HT system.

Pb decreases immunoreactivity for 5-HT in the Dorsal Raphe nuclei

Because Pb decreased 5-HT levels in the forebrain, we examined whether Pb also decreases 5-HT immunoreactivity in the Dorsal Raphe (DR), a major serotonergic input to the cerebral cortex, including the somatosensory cortex (Vitalis and Parnavelas, 2003). Brainstem sections (which contain the DR region) from control and Pb exposed mice were immunostained for 5-HTin P4 and P8 mice. In P4 mice, Pb resulted in significant decreases in 5-HT immunolabeling in the DR (Figure 23B, C). Quantification of 5-HT immunolabeling demonstrated that the staining intensity of 5-HT in very low Pb exposed mice was significantly decreased compared to control mice (Figure 23D). In P8 mice, Pb showed an increasing trend of 5-HT expression in the DR, but these changes were not statistically significant (Figure 24).

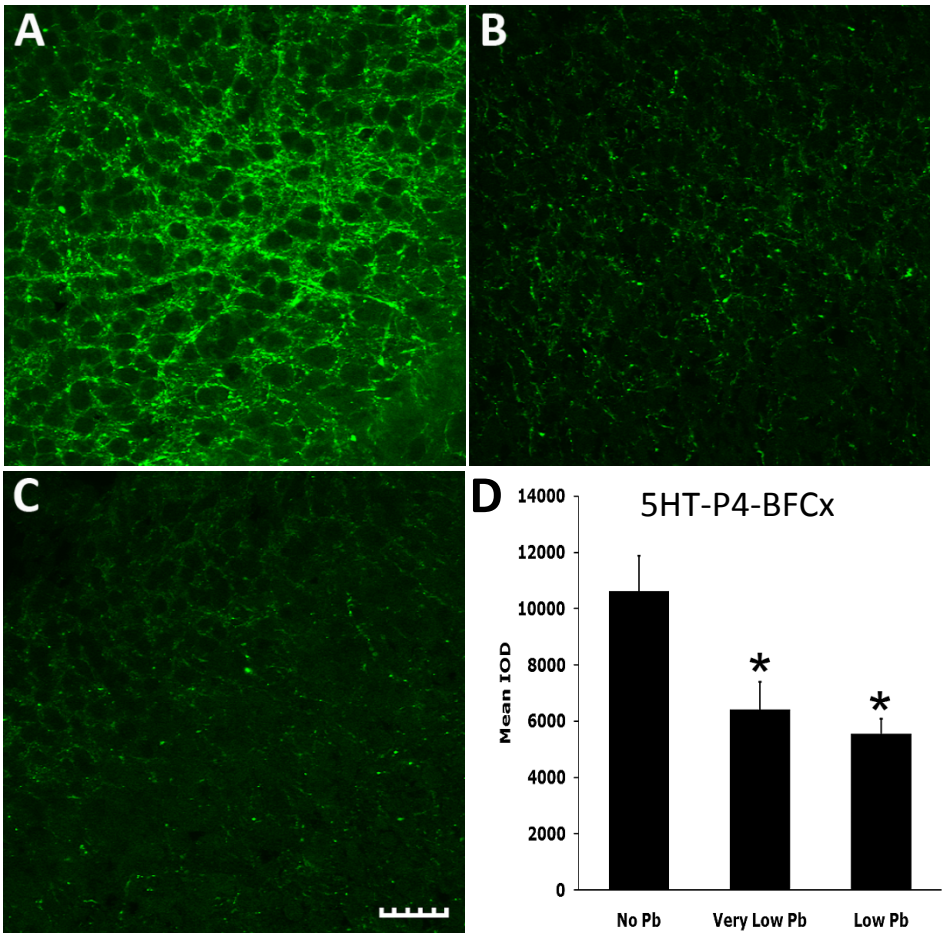


Figure 20. Pb exposure decreases 5-HT immunofluorescence (green) labeling in the area of the somatosensory cortical barrel fields (BFCx) in postnatal day (P) 4 mice. **A-C:** Both the very low (B) and low (C) Pb exposure decreases 5-HT expression in the BFCx compared to control mice (A). D**:** Quantification of 5-HT immunostaining in the BFCx confirms this decrease is statistically significant in Pb exposed mice. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 µm.

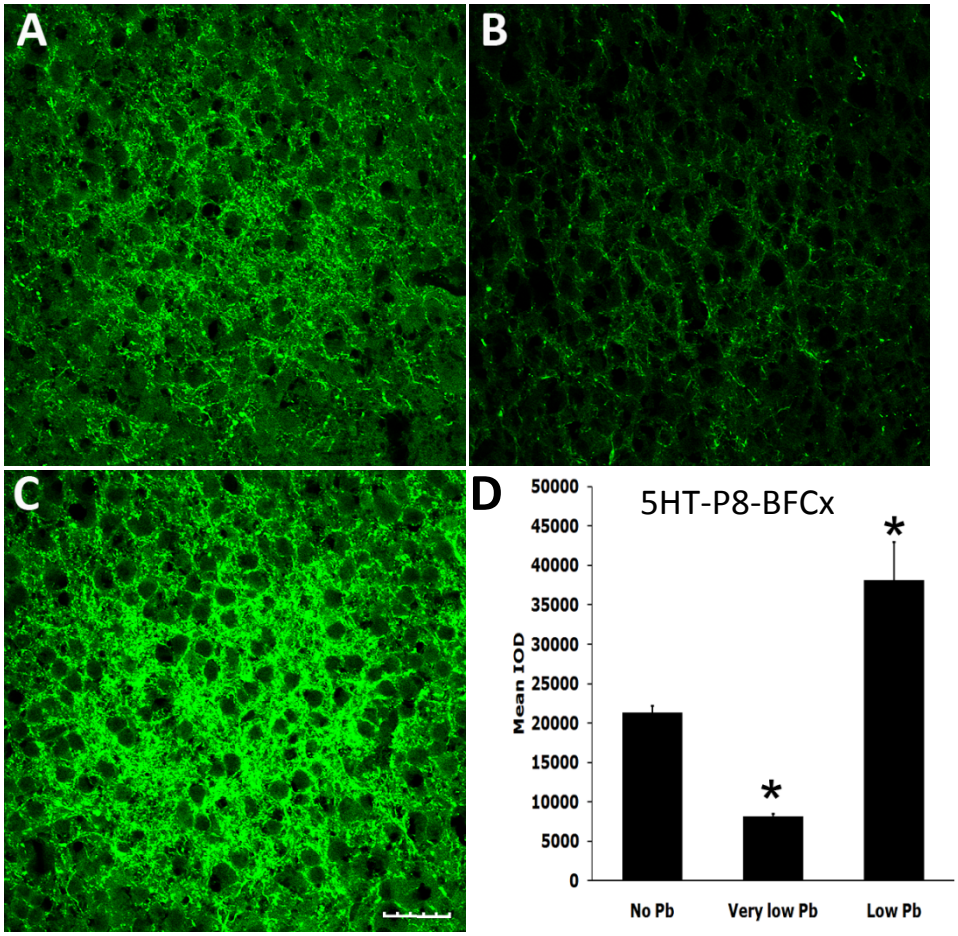


Figure 21. Pb exposure alters 5-HT immunofluorescence labeling (green) in the region of the somatosensory cortical barrel fields (BFCx) in P8 mice. **A-C:** Very low Pb exposure (B) decreases 5-HT expression in the BFCx compared to control mice (A). In contrast, low Pb exposure (C) increases 5-HT expression in the BFCx. D**:** Quantification of 5-HT immunostaining in the BFCx confirms these changes are statistically significant. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 μm.

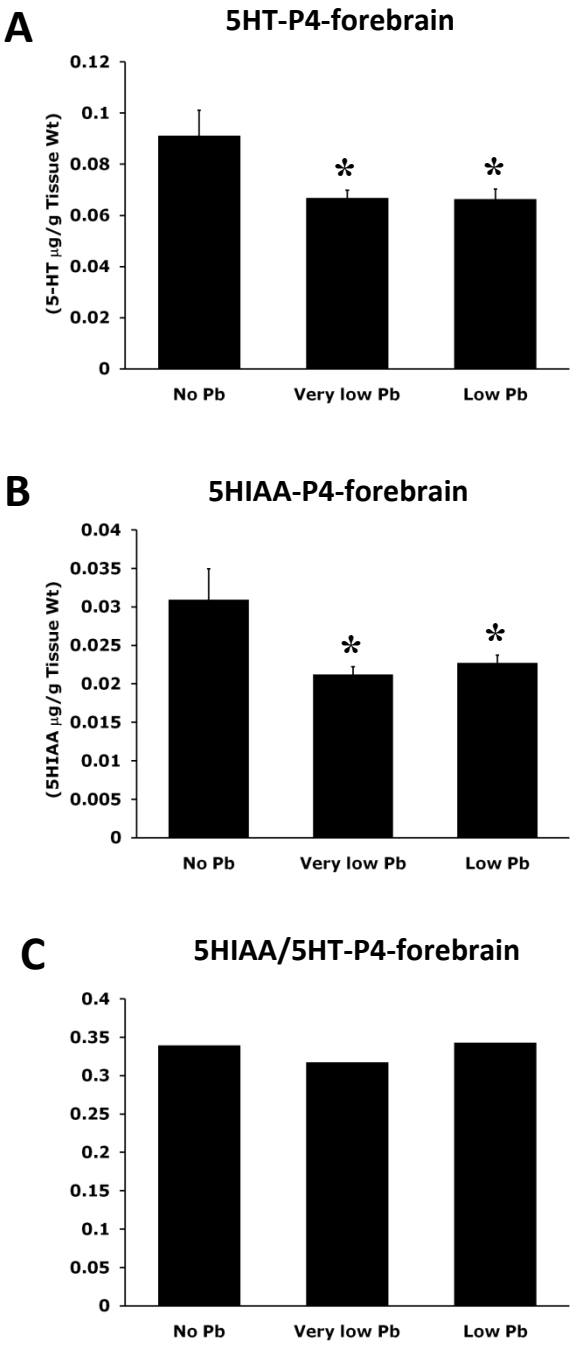


Figure 22. Pb exposure decreases total forebrain levels of 5-HT and 5HIAA in P4 mice. **A, B:** Total brainstem levels of 5-HT (A) and 5HIAA (B) decreases with Pb exposure in P4 mice. **C:** 5-HT turnover ratio (5HIAA/5-HT) does not change with Pb exposure. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 5).

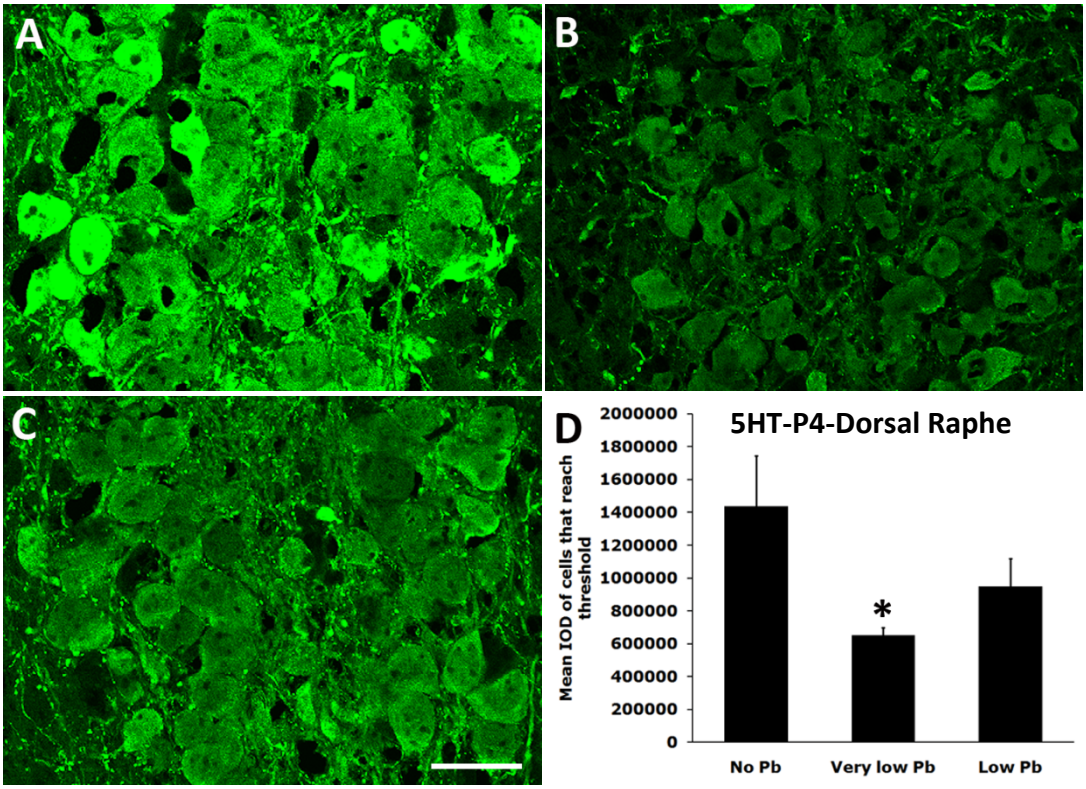


Figure 23. Pb exposure decreases 5-HT immunofluorescence labeling (green) in the dorsal raphe (DR) at P4 mice. **A-C:** Both the very low Pb (B) and low (C) Pb exposure decreases 5-HT expression in the DR compared to no Pb control (A). D**:** Quantification of 5-HT immunostaining in the DR confirms that decrease is statistically significant in very low Pb exposed mice. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 µm.

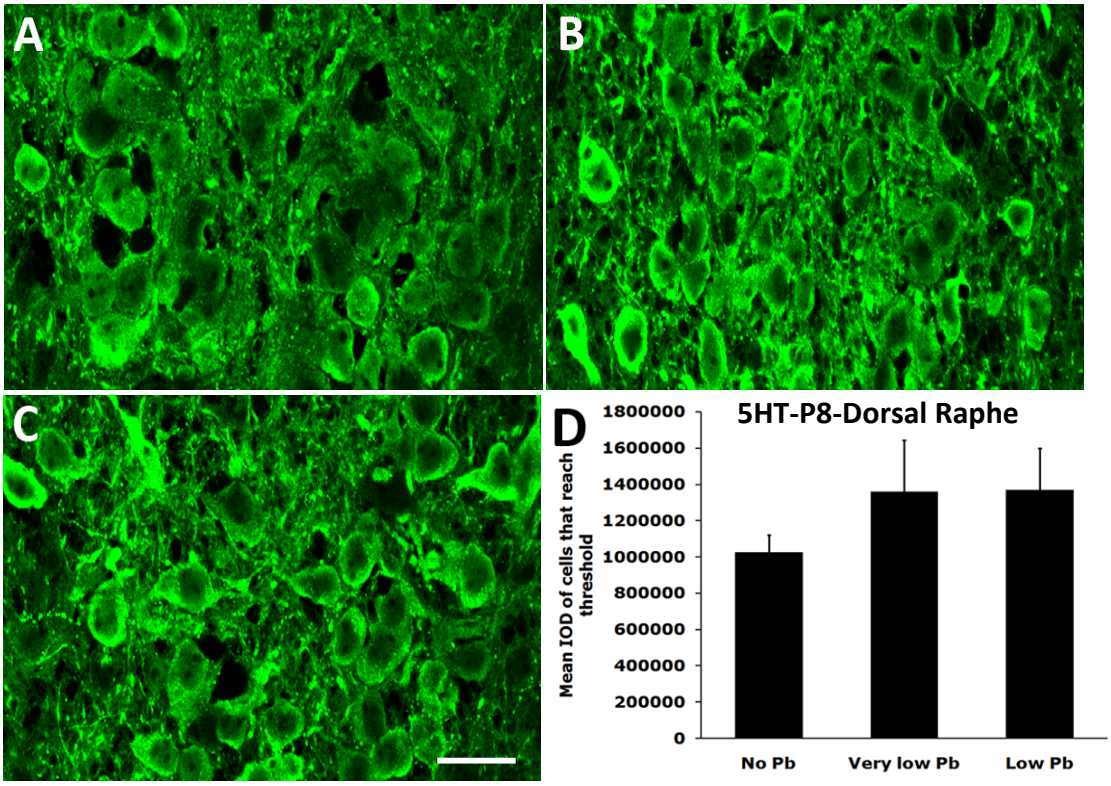


Figure 24. Pb does not affect 5-HT expression levels in the DR at P8 mice. Both the very low Pb (B) and low (C) Pb dose do not significantly change 5-HT immunofluorescent labeling (green) compared to controls (A). Quantification of the immunostaining (D) confirms that Pb has no significant effect on 5-HT staining in the DR. Graphs represent mean ± SEM. ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 µm.

Pb increases immunoreactivity for TPH in the Dorsal Raphe nuclei

To examine whether the Pb induced decreases in 5-HT levels in the DR and forebrain are correlated with altered synthesis of 5-HT by raphe neurons, brainstem sections from control and Pb exposed mice were immunostained for tryptophan hydroxylase (TPH), a rate limiting enzyme in 5-HT synthesis. Pb exposure resulted in significant increases of TPH immunolabeling in the DR of both P4 (Figure 25) and P8 (Figure 26) mice. Quantification of TPH staining in the DR demonstrated that the staining intensity for TPH in the low Pb exposed mice at P4 (Figure 25D) and in the very low and low Pb exposed mice at P8 (Figure 26D) was significantly increased compared to control mice. The increase in TPH immunostaining suggests a compensatory response by DR neurons to Pb-induced decreases in 5-HT. However, these results are puzzling in light of the fact that Pb did not seem to affect degradation of 5-HT (Figure 22C). It could be that Pb decreases the function of TPH and therefore more TPH is synthesized to compensate for impaired activity of the enzyme. Further studies are needed to fully define the effect of Pb on TPH activity.

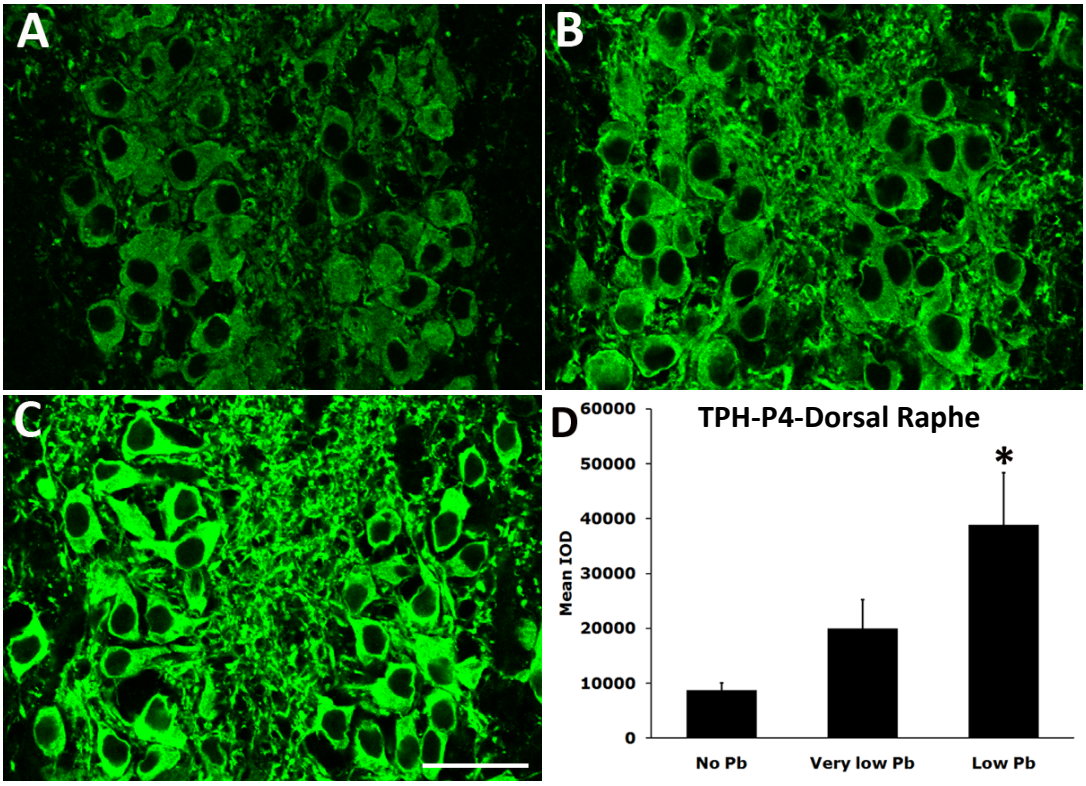


Figure 25. Pb increases TPH expression levels (green) in the DR at P4 mice. **A-C:** Very low Pb shows an increasing trend of TPH immunostaining (B) while the low (C) Pb exposure significantly increases TPH immunostaining in the DR compared to control (A). D**:** Quantification of TPH immunostaining. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 μm.

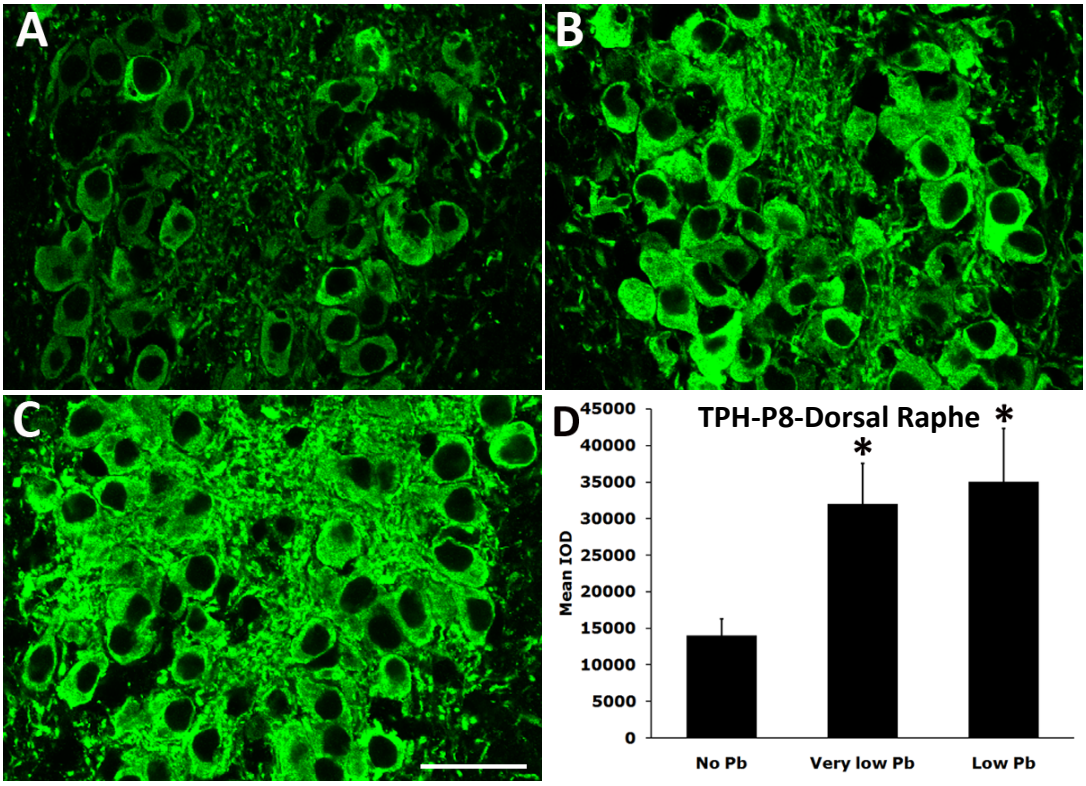


Figure 26. Pb increases TPH expression levels in the DR at P8 mice. **A-C:** Both the very low Pb (B) and low (C) Pb exposure increases TPH immunostaining in the DR compared to control (A). D**:** Quantification of TPH immunostaining in the DR confirms that increase is statistically significant. Graphs represent mean ± SEM. *$p<0.05$; ANOVA with Dunnett's post hoc test (n = 6). Scale bar = 30 μm.

Discussion

The current study demonstrates that Pb decreased 5-HT immunoreactivity in the TCAs in the region of the BFCx during the early postnatal period (P4 and P8). This decreased immunoreactivity for 5-HT in the TCAs is correlated with decreased 5-HT immunostaining in the DR and the entire forebrain. Pb also increased TPH expression in the DR, indicating that this could be a compensatory response by DR neurons to Pb-induced decreases in 5-HT.

Depletion of 5-HT by Pb exposure

The findings of the current study demonstrating decreases in 5-HT in the DR and forebrain confirm other studies that show an effect of Pb on 5-HT metabolism. For example, when rats are exposed to Pb-Ac (300 mg/L; blood Pb 17.79 ± 0.4 μg/dL) from gestation through P5, both 5-HT and 5-HIAA levels decrease, and increases in 5-HT turnover (5-HIAA/5-HT) are observed in the entire brain (Antonio et al., 1996). When the same concentration of Pb-Ac (300 mg/L) is used to expose rats for a longer period (from gestation through P12; blood Pb 29.32 ± 8.15 μg/dL) Pb decreases 5-HT and 5-HIAA in the hippocampus, and decreases 5-HT without affecting 5-HIAA levels in the hypothalamus and cerebellum (Antonio and Leret, 2000). Our Pb concentrations in vivo yield blood Pb levels of 8.0 ± 0.4 μg/dL in very low Pb, and 42.3 ± 1.97 μg/dL in low Pb exposed mice at P21 (Fortune and Lurie, 2009) and are in a similar range to these studies.

The mechanism underlying Pb induced changes in 5-HT levels is poorly understood. The current study shows that Pb increases TPH expression in the DR, with no changes in 5-HT degradation, suggesting that Pb may interfere with 5-HT synthesis.

In the CNS, 5-HT is synthesized exclusively in neurons of the rostral (B6-B9; corresponding to the dorsal and median raphe nuclei) and caudal (B1-B5; corresponding to the raphe pallidus, magnus, obscures, and pontis) raphe nuclei (Gaspar et al., 2003). TPH is an initial, rate-limiting enzyme of 5-HT synthesis, responsible for converting tryptophan into 5-hydroxytryptamine, and TPH gene expression has shown to be regulated by 5-HT levels. For example, depletion of 5-HT in rat brain by *p*-chlorophenylalanine (PCPA), a potent and specific inhibitor of TPH, has been shown to produce a rapid reduction in TPH activity followed by increased TPH mRNA expression in the raphe and hypothalamus (Park et al., 1994). Similarly, lesioning of serotonergic neurons by 5,7-dihydroxytryptamine (DHT) also has been shown to increase TPH mRNA expression in the raphe (Bendotti et al., 1990). Therefore, the results of the present study showing increased TPH expression in the DR following Pb exposure appears to be a compensatory response to Pb-induced decreases in 5-HT levels. In addition, the Pb-induced decreases in 5-HT in the DR seems to be transient, because 5-HT levels in the DR recover to normal levels by P8, and we also observed that DR 5-HT levels do not change with Pb exposure in P21 mice (data not shown). To our knowledge, this is the first study demonstrating increased TPH expression in the DR with developmental Pb exposure.

The mechanism by which Pb initially produces decreases in 5-HT and 5HIAA levels in the P4 forebrain remains to be elucidated. Previous studies have shown that reductions in 5-HT levels are correlated with increased 5-HT degradation following Pb exposure (Antonio et al., 1996), but we did not observe any changes in the degradation of 5-HT. However, our study uses mice instead of rats and we analyzed the forebrain region

only as opposed to the entire brain that was analyzed by Antonio et al, 1996. One possible explanation for the Pb induced decreases in the 5-HT and 5HIAA observed in current study is that Pb could inhibit the function of TPH and therefore more TPH is synthesized to compensate for impaired activity of the enzyme. Further studies are necessary to define the effect of Pb on TPH activity.

Functional implications of Pb induced decreases in 5-HT levels in early cortical development

Serotonergic neurons are generated early in development, on embryonic day (E) 12 to 15 in the rat (Gaspar et al., 2003). One day after their generation, raphe neurons are able to synthesize 5-HT and begin to send axons towards the telencephalon. Thus the serotonergic raphe neurons and their processes play a role in early cortical development by regulating diverse events such as proliferation, migration, differentiation, and synaptogenesis of cortical neurons (Vitalis and Parnavelas, 2003). In the somatosensory barrel field cortex, "non-serotonergic" thalamocortical neurons have been shown to accumulate 5-HT through transient expression of the SERT transporter during early development, and precise regulation of 5-HT levels is critical for the proper formation of cortical barrel fields (Cases et al., 1996; Cases et al., 1998; Lebrand et al., 1998; Vitalis et al., 2002). For example, in MAO k/o mice, where brain 5-HT levels are 6-9 fold higher than wild type mice during the first few postnatal weeks, barrels are not formed, as the clustering of both the thalamocortical axons and the cortical granular neurons around thalamocortical axon endings are disrupted (Cases et al., 1995; Lebrand et al., 1996; Vitalis et al., 1998; Upton et al., 1999). In contrast to the complete loss of barrel fields

induced by excessive amounts of 5-HT, depletion of 5-HT by *p*-chloramphetamine (PCA) or DHT results in barrel fields that are either smaller in size or are developmentally delayed, but does not completely prevent the normal pattern of barrel field formation (Blue et al., 1991; Bennett-Clarke et al., 1994; Turlejski et al., 1997). In addition to the effect on barrel field formation, depletion of 5-HT has also been shown to affect cortical maturation by reducing both dendritic arborization and the complexity of the pyramidal neurons of layer III and V in the somatosensory cortex and delaying incorporation of interneurons into the cortical plate (Vitalis et al., 2007). Thus, depletion of 5-HT appears to impair cortical development in general, and is not restricted to a specific effect on thalamocortical neurons that transiently take up 5-HT (Persico et al., 2000; Gaspar et al., 2003; Luo et al., 2003). In the current study, we found that Pb decreases 5-HT levels in the DR and forebrain fractions, and decreases the transient uptake of 5-HT by thalamocortical axons. These results suggest that Pb could delay cortical maturation including the formation of somatosensory cortical barrel fields. In support of this, low levels of Pb exposure (1-31 μg/dL) from P1 through P10 in rats have been shown to decrease the area of cortical barrel field with increasing doses of Pb (Wilson et al., 2000). Future studies, including a detailed morphometric analysis of barrel field pattern and size are needed to confirm that the Pb induced deficits in the development of the BFCx in rodents are associated with Pb induced alterations in 5-HT levels in the somatosensory cortex.

Summary and Conclusion

The current study demonstrates that Pb exposure decreased the uptake of 5-HT by TCAs during a critical period of cortical development. Pb also decreased 5-HT levels in the forebrain and dorsal raphe (DR), suggesting that Pb may delay cortical development in general, and is not restricted to a specific effect on thalamocortical neurons. Pb also increased TPH expression in the DR with no changes in 5-HT degradation, suggesting that Pb might inhibit the function of TPH resulting in an upregulation of TPH synthesis in order to compensate for impaired activity of the enzyme. To our knowledge, this is the first study to demonstrate that 1) low levels of Pb result in decreased expression of 5-HT within TCAs and 2) TPH expression within the DR decrease with low level Pb exposure. It will be exciting to further define the mechanism and determine whether these changes in 5-HT underlie the behavioral and cognitive impairments induced by developmental low-level Pb exposure.

Overall summary and Conclusions

In the current study, we investigated the effect of developmental Pb exposure on the transient uptake of 5-HT by LSO neurons and thalamocortical processes of early postnatal mice. Our major finding is that Pb exposure delays the normal developmental uptake of 5-HT by LSO neurons in P4 and P8 mice. This effect of Pb is specific for the transient uptake of 5-HT because Pb does not alter 5-HT immunostaining in the processes within the LSO that originate from the raphe nuclei. Results from double immunofluorescence staining of 5-HT and SERT indicate that Pb delays the expression of SERT in the LSO neurons. SERT appears to be a target for the Pb induced delay in transient uptake of 5-HT by LSO neurons, because Pb does not affect either synthesis or degradation of 5-HT in the brainstem. In addition, Pb specifically targets SERT expressed by LSO neurons because it has no effect on its expression in the processes within the LSO. We also found that Pb decreases 5-HT within the dorsal raphe, the forebrain, and the processes of thalamocortical neurons terminating within somatosensory cortex.

Pb and the developing LSO

Our studies reveal that neurons such as LSO neurons that transiently take up 5-HT during development may be specific cellular targets of Pb. The delayed uptake of 5-HT by LSO neurons is correlated with the decreased expression of SYP and VMAT2 within the LSO, suggesting that the delayed uptake of 5-HT by LSO neurons may be one mechanism by which Pb impairs synaptic maturation within the LSO during the critical period of LSO development . Interestingly, total brainstem levels of DA and DOPAC

decreased with Pb exposure, suggesting that Pb may affect the dopaminergic synapses. Future studies that include double-label immunofluorescence for tyrosine hydroxylase (TH, a marker for dopaminergic neurons) and SYP or VMAT2 are needed to determine whether the decreased SYP and VMAT2 staining that we observe in the Pb-treated P8 LSO reflects a decrease in synapses of dopaminergic neurons. The changes in GAP-43 immunostaining further support the hypothesis that Pb disrupts axonal arborization and synaptic maturation in the LSO, and also suggest that GAP-43 may be a target molecule for Pb.

Precise regulation of transient uptake of 5-HT by "non-serotonergic" sensory neurons has been shown to be critical for the proper formation of highly topographically organized sensory maps (Gaspar et al., 2003). Studies suggest that this transient uptake of 5-HT by non-serotonergic sensory neurons is necessary to clear 5-HT away from the extracellular space, thereby maintaining proper extracellular concentrations of 5-HT during a critical period of development. Because 5-HT functions as a neurotrophic factor during brain development prior to the time when it plays a role as a neurotransmitter (Luo et al., 2003), increased uptake of 5-HT by these non-serotonergic sensory neurons may limit the amount of 5-HT necessary for the development of these systems. Our results showing that Pb delays the normal uptake of 5-HT by LSO neurons indicate that Pb could shift the extracellular concentrations of 5-HT during a critical window of LSO development, thereby resulting in decreased synaptic density as evidenced by decreases in SYP and VMAT2 imuunostaining within the LSO of P8 mice (Figure 27). This raises the question: what is the factor that mediates this 5-HT depletion induced impaired synaptogenesis? One possible candidate is GAP-43. GAP-43 is expressed early in

development and plays a role in axonal outgrowth and synaptic plasticity in response to extracellular signals (Luo et al., 203). GAP-43 has shown to be upregulated during the period of barrel field formation, and is then subsequently downregulated (Maier et al., 1999). In GAP-43 k/o mice, thalamocortical axons fail to innervate the cortex, thus barrels are not formed, and forebrain levels of 5-HT and 5HIAA are decreased (Donovan et al., 2002). These studies suggest that GAP-43 may be a factor that mediates the 5-HT induced regulation of sensory neuron development. In the current study, we found that Pb decreases GAP-43 expression in the LSO at P4 and P8, the time when the highest levels of GAP-43 are necessary for the maturation of LSO synapses. Thus, modulation of GAP-43 in response to changes in extracellular 5-HT levels is one potential mechanism by which Pb impairs synaptic maturation within the LSO.

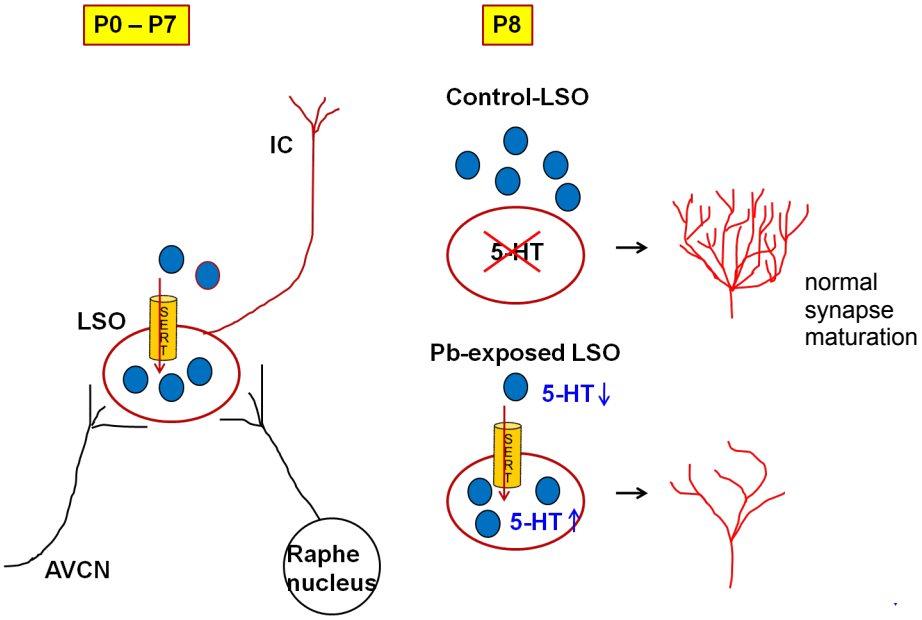


Figure 27. Summary of the effect of Pb on the developing LSO. LSO neuronal somata transiently take up 5-HT from the extracellular environment through transient expression of SERT during early postnatal development (P0-P7). By P8, control (No Pb) LSO neurons lose their ability to take up 5-HT due to the loss of SERT. However, Pb-exposed LSO neurons continue to express SERT, and thus continue to take up 5-HT from the extracellular space. This extended uptake of 5-HT by LSO neurons likely leads to decreases in the extracellular levels of 5-HT necessary for the proper maturation of the LSO. This could ultimately lead to decreased arborization (in red) and synapse formation (decreases in SYP, VMAT2, and GAP-43) within the LSO.

Pb has a permanent effect on synpatogenesis in the LSO

We have previously reported that Pb decreases 5-HT immunostaining in the adult LSO (P21) and this decrease is correlated with decreased VMAT2 and SYP expression (Fortune and Lurie, 2009). In the current study, we confirmed that the decrease in 5-HT immunostaining observed in the adult LSO is not the result of an acute effect of Pb on 5-HT metabolism, because Pb did not change synthesis, degradation, or re-uptake of 5-HT in the LSO of P21 mice. In addition, the Pb induced decreases in SYP and VMAT2 expression (Fortune and Lurie, 2009) were correlated with changes in the GAP-43 staining pattern in the adult LSO. Taken together, this data further supports the hypothesis that developmental Pb exposure has lasting effects on synaptic maturation within the LSO of adult mice (figure 28).

Pb and the IC

LSO neurons provide a major afferent input to the central nucleus of inferior colliculus (CNIC), contributing to the tonographically organized laminar formation within the IC (Thompson and Schofield, 2000). We found that only our very low dose of Pb (0.01mM), increased 5-HT immunostaining in the adult CNIC (P21), but did not affect synthesis, degradation, or re-uptake of 5-HT in the IC. Thus, it is likely that Pb acts to modulate the number of 5-HT processes within the IC and future studies will address this issue. Interestingly, our low dose (0.1mM) of Pb increased VMAT2 expression within the IC, suggesting that different doses of Pb might have two different effects on axonal maturation of the IC, with very low doses increasing the number of 5-HT positive processes, and low Pb doses increasing synaptogenesis of monomaminergic

neurons. To our knowledge, this is the first evidence in vivo that differing doses of Pb modulate different aspects of neuronal maturation and additional studies are needed to further elucidate this process.

The fact that Pb increases the number of 5-HT immunoreactive processes within the IC, but decreases the number of these processes in the LSO suggests that Pb modulates process arborization differentially in the two nuclei. Furthermore, Pb did not affect total expression levels of SYP in the IC, indicates that Pb subtly impairs the precise organization of the topographic map in the IC, instead of causing a general increase or decrease in the total number of synapses (Figure 28). In addition, we did not observed IC neurons that transiently take up 5-HT during development in our mouse model, thus the changes in 5-HT and VMAT2 expression in the IC following Pb exposure could be a secondary effect of Pb on the LSO. The mechanism by which Pb differentially regulates axon arborization and synaptogenesis in the two different nuclei remains to be characterized. Investigating the effect of Pb on early postnatal development of IC will be necessary to fully understand how Pb regulates the synaptogenesis of the IC differentially compared to that of LSO.

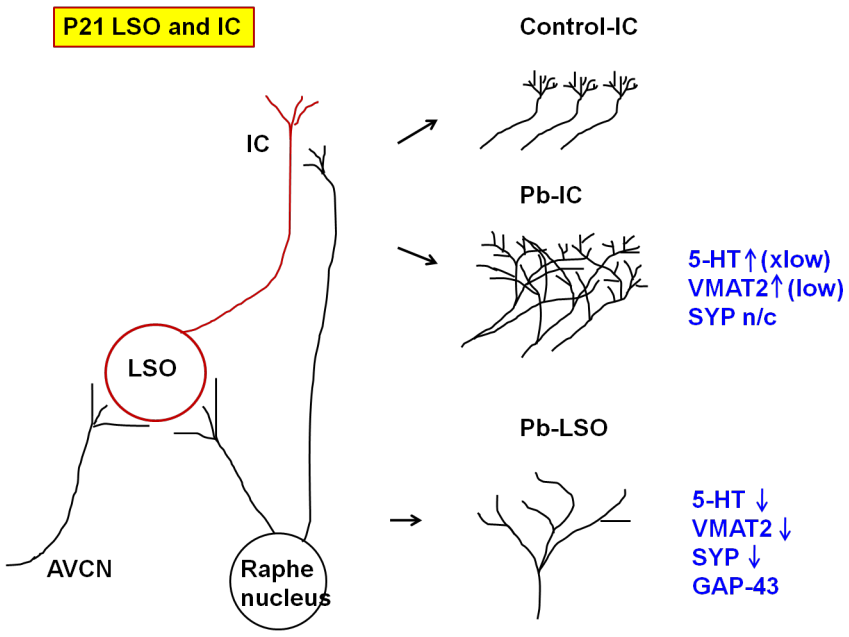


Figure 28. **Summary of the effect of Pb on postnatal day 21 LSO and IC.** Developmental Pb exposure has lasting effects on process arborization and synapse formation within the LSO as evidenced by decreases in 5-HT, SYP, and VMAT2 and altered staining patterns of GAP-43 in the adult LSO. In the IC, a major afferent target of LSO, Pb increases the number of 5-HT positive processes at the very low dose (0.01mM), and increases VMAT2 expression with the low dose (0.1 mM). Pb increases VMAT2 expression without changing SYP expression, suggesting that Pb subtly impairs the precise organization of the topographic map in the IC, rather than causing a general increase or decrease in the total number of synapses.

Pb and the developing somatosensory cortex

In the somatosensory system, "non-serotonergic" thalamocortical neurons transiently take up 5-HT during the period of early postnatal development, and precise regulation of 5-HT levels has been shown to be critical for the proper formation of somatosensory cortical barrel fields (BFCx) (Cases et al., 1996, 1998; Lebrand et al., 1998; Vitalis et al., 2002). Interestingly, early postnatal Pb exposure has been shown to decrease the area of the cortical barrel field (Wilson et al., 2000). This raises the intriguing possibility that the Pb induced deficits in the development of the BFCx in rodents are associated with Pb induced alterations in the transient uptake of 5-HT by thalamocortical neurons. We found that Pb indeed decreases 5-HT immunoreactivity of thalamocortical neurons at their axon terminals (TCAs) in the region of the BFCx during the early postnatal period (P4 and P8). However, the impact of Pb on thalamocortical neurons and the forebrain is different to its effects on the central auditory nuclei (LSO and IC) and the brainstem. In the brainstem, we found that Pb decreased 5-HTimmunostaining in the LSO neuronal somata while having little effect on total brainstem levels. In contrast, we found that Pb decreases 5-HT levels in the TCAs as well as in the entire forebrain and dorsal raphe. These findings suggest that in the cortex, the effect of Pb is not restricted to the transient uptake of 5-HT by thalamocortical neurons, but may delay cortical development in general by decreasing the 5-HT levels available for the maturation of the forebrain (Figure 29). In support of this, depletion of 5-HT by PCA or DHT results in barrel fields that are either smaller in size or are developmentally delayed, but does not completely prevent the normal pattern of barrel field formation (Blue et al., 1991; Bennett-Clarke et al., 1994; Turlejski et al., 1997). In

addition to the effect on barrel field formation, depletion of 5-HT has also been shown to affect cortical maturation by reducing both dendritic arborization and the complexity of the pyramidal neurons of layer III and V in the somatosensory cortex and delaying incorporation of interneurons into the cortical plate (Vitalis et al., 2007). Thus, the subtle effect of 5-HT depletion on barrel field formation may be the result of a 5-HT induced delay in general brain development, rather than a direct effect of 5-HT on cortical barrel field formation itself (Persico et al., 2000; Luo et al., 2003; Gaspar et al., 2003). Future studies, including a detailed morphometric analysis of barrel field pattern and size are needed to confirm that the Pb induced deficits in the development of the BFCx in rodents are indeed associated with the Pb induced decreases in 5-HT levels that we have found in the somatosensory cortex.

Pb and the Dorsal Raphe

Interestingly, Pb decreased 5-HT immunostaining in the DR of P4 mice. This finding is unexpected, because Pb did not change 5-HT levels in the entire brainstem and the serotonergic processes within the LSO. Because the DR is not a major source of serotonergic innervation in the LSO (Thompson and Thompson, 2001), and other raphe nuclei are localized in the brainstem fractions, it may be that decreases in 5-HT levels in the DR are not sufficient to result in decreases in total brainstem levels of 5-HT. In contrast, the DR provides a major afferent input to the forebrain, thus the Pb-induced decrease in 5-HT levels in the DR are the likely explanation for the reduced 5-HT levels that we observe in the forebrain. It will be interesting to examine whether Pb modulates 5-HT levels differentially in other raphe nuclei of the brainstem.

Pb also increased TPH expression in the DR, indicating that this could be a compensatory response by DR neurons to Pb-induced decreases in 5-HT. Because Pb does not alter 5-HT degradation, one potential mechanism is that Pb may inhibit the function of TPH resulting in an upregulation of TPH synthesis in order to compensate for impaired activity of the enzyme. Further studies are necessary to fully elucidate the effect of Pb on TPH activity.

To our knowledge, the current study is the first to demonstrate an effect of low-level Pb exposure on the transient uptake of 5-HT in non-serotonergic neurons of central auditory and thalamocortical neurons during development. Delaying normal transient accumulation of 5-HT in these neurons by Pb exposure during the critical period of axonal/dendritic arborization and synaptogenesis produces changes in process elaboration/arborization and synaptic maturation that persists into adulthood. These changes may be an underlying mechanism for the behavioral and cognitive impairments induced by low level Pb exposure during childhood.

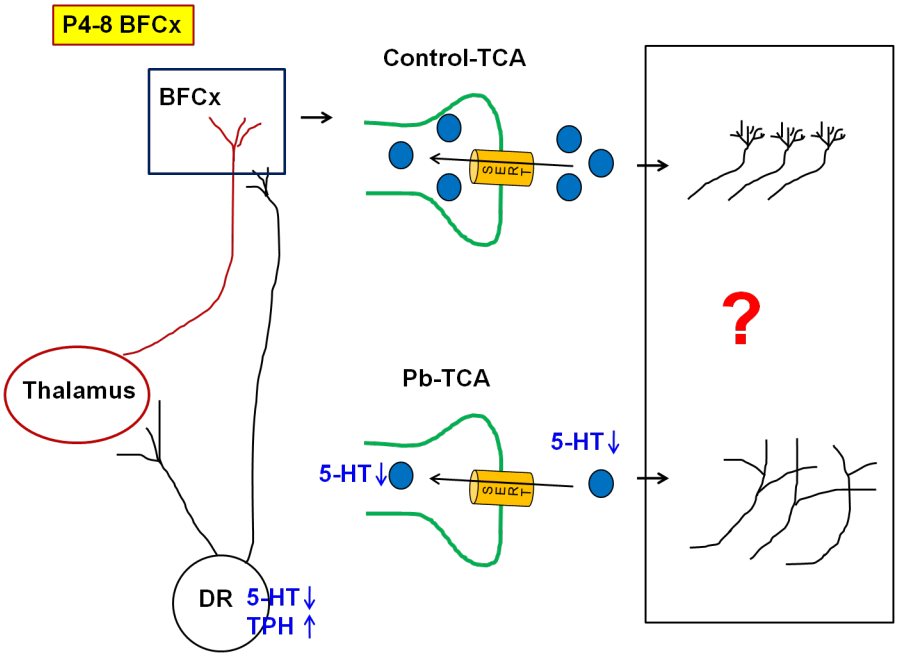


Figure 29. Summary of the effect of Pb on postnatal day 4-8 TCA and cortex. Pb decreases 5-HT levels in in the entire forebrain and dorsal raphe and thus there is potentially less extracellular 5-HT available for uptake by TCAs. These findings suggest that in the cortex, the effect of Pb is not restricted to the transient uptake of 5-HT by thalamocortical neurons, but may delay cortical development in general by decreasing the 5-HT levels available for the maturation of the forebrain. Pb also increases TPH expression in the DR, indicating that this could be a compensatory response by DR neurons to Pb-induced decreases in 5-HT. Future studies are needed to determine whether the Pb induced decreases in the 5-HT levels in the TCAs and forebrain lead to impaired BFCx formation.

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