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Developmental Stress and Lead (Pb): Effects of Maternal Separation and/or Pb on Corticosterone, Monoamines, and Blood Pb in Rats

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Abstract

The level of lead (Pb) exposure in children has decreased dramatically since restrictions on its use were implemented. However, even with restrictions, children are exposed to Pb and still present with cognitive and behavioral deficits. One prominent aspect of the exposome of these children is that many come from low social economic status (SES) conditions, and low SES is associated with stress. In order to compare the combined effects of early stress and Pb, Sprague-Dawley rats were exposed to vehicle or Pb either alone or in combination with maternal separation stress during brain development (i.e., postnatal day (P)4 to P11, 19, or P28). Maternally separated/isolated pups had lower body and thymus weights during exposure and had increased levels of blood Pb compared with vehicle controls. Isolation, but not Pb, affected the response to an acute stressor (standing in shallow water) when assessed on P19 and P29, but not earlier on P11. Interactions of Pb and isolation were found on monoamines in the neostriatum, hippocampus, and hypothalamus on turnover but not on levels, and most changes were on dopamine turnover. Isolation had greater short-term effects than Pb. Interactions were dependent on age, sex, and acute stress.

Keywords

Lead (Pb); maternal separation; acute shallow water stress; corticosterone; dopamine; serotonin; development; rat

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Conflict Statement

The authors declare no conflicts of interest

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1. Introduction

Lead (Pb) continues to be an environmental health risk despite restrictions on its use. Children are especially susceptible to Pb exposure (Lidsky and Schneider, 2003), and this exposure is associated with altered cardiovascular function (Gump et al., 2005), encephalopathy (Patel and Athawale, 2009), genotoxicity (Mendez-Gomez et al., 2008), and cognitive and behavioral deficits, the latter including but not limited to decreased IQ (Chiodo et al., 2007; Needleman et al., 1979; Surkan et al., 2007; Wang et al., 1989; Winneke et al., 1985), altered language function (Yuan et al., 2006), social problems (Chiodo et al., 2007; Burns et al., 1999), and increased juvenile adjudication (Dietrich et al., 2001). Even levels of Pb below the Centers for Disease Control's 10 µg/dL level of concern (CDC, 2005) result in neuropsychological and IQ reductions (Neal and Guilarte, 2010; Bellinger, 2011). Because of the persistence of Pb in the environment, it continues to be found in children, albeit at low levels (Bellinger, 2008; Lanphear et al., 2000). However, these levels have not been modeled often in animals and represent a gap in understanding how such levels affect brain development.

Pb exposure also has effects on stress mechanisms (Gump et al., 2005; Gump et al., 2008). Interactions between Pb and stress have been reported in children (Tong et al., 2000) and in animals (Weston et al., 2014; Cory-Slechta et al., 2013b; Cory-Slechta et al., 2010; Cory-Slechta et al., 2012; Cory-Slechta et al., 2009; Cory-Slechta et al., 2013a; Rossi-George et al., 2009; Rossi-George et al., 2011; Virgolini et al., 2006; Virgolini et al., 2008b; Virgolini et al., 2008a; Cory-Slechta et al., 2004). The latter are from a laboratory primarily using a paradigm in which female Long-Evans rats are given Pb (50 or 150 ppm in drinking water) for 2 months prior to mating, throughout gestation, and up to weaning or through adulthood. Restraint stress in this model is to the dam and consists of three 45 min episodes given on two days of gestation: embryonic (E) days 15 and 16 (where evidence of mating is counted as E0) and may be characterized as an acute stress paradigm. This model, and its variations, show a number of interactions in which the effects of Pb are increased by the prenatal stress exposure. However, this model does not address the effects of chronic developmental stress.

Chronic stress can cause neurotoxicity (McEwen and Stellar, 1993; McEwen, 1998) and hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Lupien et al., 1999; McEwen et al., 1992). In young children and rodents, the HPA axis passes through a stage of reduced responsiveness to stress (stress hyporesponsive period; SHRP). The SHRP is hypothesized to be a neuroprotective mechanism to prevent excitotoxicity when glucocorticoid receptors are developing (De Kloet et al., 1988; Sapolsky and Meaney, 1986). Stressors that exceed the buffering capacity of the SHRP result in adverse consequences (Anisman et al., 1998; Gos et al., 2008; Gruss et al., 2008).

A number of stressors may interfere with child developmental markers, one of which is low socioeconomic status (SES). Children in such settings face a variety of difficulties that may include impoverishment, family disruption, overcrowding, poor diet, threats, violence, and other challenges that result in elevated cortisol levels and other markers of stress (Gump et al., 2005; Lupien et al., 2000; Lupien et al., 2001). These conditions can co-occur when

children live in older housing that tend to have higher levels of Pb paint, dust, and or soil contamination (Goyer, 1996; Jacobs et al., 2002; Lanphear et al., 1998; Levin et al., 2008; Meyer et al., 2003; Muntner et al., 2005; Schnaas et al., 2004).

In the present study, we tested the interaction of Pb exposure in combination with the chronic stressor of maternal separation (pup isolation; ISO). We hypothesized that the combination would be more detrimental than either factor alone. ISO has been used in many studies. It consists of isolating pups from the dam and littermates during a period that spans the SHRP (Kosten and Kehoe, 2005). The ISO model alters stress markers (McCormick et al., 1998), brain monoamines (Kehoe et al., 1998; Kosten et al., 2004), fear conditioning, and DNA methylation (Kao et al., 2012). Here, offspring were separated on P4 for 4 h/day until the day prior to assessment on P11, P19, or P29. These days were chosen to match a previous experiment of similar design but using barren-cage stress instead of maternal separation (Graham et al., 2011). The P4–28 period of brain development in rats is approximately analogous to late gestation to early childhood in humans (Bayer et al., 1993; Clancy et al., 2007a; Clancy et al., 2007b). In combination with ISO, pups were treated every other day with 1 or 10 mg/kg of Pb acetate by gavage from P4–10, P4–18, or P4–28. The day after the last treatment, pups were challenged with an acute stressor or left undisturbed. Pb doses used here were designed to produce blood Pb (BPb) concentrations similar to those observed in humans (Lanphear et al., 2000; Bellinger, 2008). The effects of chronic stress and Pb were assessed on brain monoamines, organ weights associated with stress and immunity, and corticosterone. The ultimate purpose was to develop a combination model suitable for studies on long-term behavioral effects and test whether maternal separation induces effects similar to or different from barren cage rearing as used in our previous experiment (Graham et al., 2011).

2. Materials and Methods

2.1 Animals

Male and nulliparous female Sprague-Dawley Crl:CD (IGS) rats (strain 001, Charles River Laboratories, Raleigh, NC) were acclimated for at least 1–4 weeks in the vivarium prior to breeding. The vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and care was provided in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were maintained on a 14:10 h light:dark cycle (lights on at 600 h) with controlled temperature (19 ± 1 °C) and humidity ($50\% \pm 10\%$). To control exposure to other metals in the diet, animals were maintained on an NIH-07 diet throughout the experiment in which metal content was certified by assay to be below preset values (PMI Corp., Lab Diet Auto 5018). Food and water were given ad libitum. For breeding, a male and female were cohabitated until the presence of a sperm plug was detected (E0). On E1, females were transferred to polycarbonate cages ($46 \times 24 \times 20$ cm) containing woodchip bedding and housed singly. Day of birth was designated P0, and on P1 litters were culled to 12 pups (6 males and 6 females) using a random number table. If litters had less than 12 pups on P1, 1 or 2 pups from another litter born on the same day were added to attain a uniform litter size of 12; this occurred in 17 litters (17.7%) of the 96 litters in the experiment. Of the 17 litters that had

pups added, 13 received 1 pup (13.5%) and 4 received 2 pups (4.2%). The experiment was approved by the Institutional Animal Care and Use Committee. Personnel conducting the study were blind to the group membership of the pups.

2.2 Pb Exposure and Rearing Conditions

The experiment had five independent variables: 2 rearing conditions, 3 exposure groups, 3 test ages, 2 levels of the acute stressor, and 2 sexes. The rearing conditions were maternal separation or no maternal separation. The three exposure groups were 0, 1, or 10 mg/kg Pb acetate. The three ages were P11, 19, or 29. The four levels of the acute stressor were no Shallow Water Stress (SWS) and 0, 30, and 60 min after SWS. The two sexes were males and females. Hence, the study design was a $2 \times 3 \times 3 \times 4 \times 2$ design, the experiment had 144 cells, hence $96 \text{ litters} \times 12 \text{ offspring per litter} = 1152 \text{ pups}$; 1152 pups divided by 144 cells resulted in 8 males and 8 females in each cell of the study stratified by litter. The dependent variables were body and organ weights, monoamines, and corticosterone.

Pb administration and ISO began on P4. Pb was given in a vehicle of 0.01 M anhydrous sodium acetate; VEH). Two male and female pairs per litter were gavaged with 0, 1, or 10 mg/kg Pb acetate (1Pb and 10Pb, respectively) in a volume of 3 mL/kg VEH as in our earlier experiment, and in that experiment we also showed that gavaging per se did not increase plasma corticosterone (Graham et al., 2011). Rats were gavaged every other day from P4 until P10, 18, or 28. Offspring assessed on P29 had their dams removed from the cage on P28 at which time the weanlings were housed by sex, 3 rats/cage (1 from each exposure group).

From P4 until assessment, ISO offspring were removed from their dam and isolated individually in a quiet room for 4 h in a small clean paper container with lid (14 cm height $\times$ 13 cm diameter at the bottom and 16 cm diameter at the top). Control animals remained with their dam throughout this period (Standard rearing: STD).

On P11, P19, or P29 separate subsets within each group were exposed to an acute stressor (shallow water stressor, SWS) or left undisturbed. The SWS animals were placed for 30 min in clean cages (28 $\times$ 16 $\times$ 12 cm polycarbonate cages) filled with room temperature water to a depth of 2 cm for P11, 3 cm for P19, and 4 cm for P29 animals. During the middle of the light cycle (approximately 1000–1400 h), one pup from each condition at each age was decapitated 0, 30, or 60 min after removal from SWS or directly upon removal from their home cage (baseline). Trunk blood was collected in 12 $\times$ 75 mm polyethylene tubes containing 0.05 mL of 2% EDTA for corticosterone assay. An additional blood sample was taken from animals on P29 for BPb analysis. Monoamine determinations were from rats not subjected to SWS. The neostriatum, hypothalamus, and hippocampus were dissected over ice using a brain block (Zivic-Miller, Pittsburgh, PA) as described (Grace et al., 2010). Spleen, thymus, and adrenals were removed and weighed. Body weights were obtained before euthanasia. Samples were stored at -80°C until assayed.

2.3 Blood Pb

BPb was measured by anodic stripping voltammetry using an ESA Lead Analyzer 3010B (Chelmsford, MA). Whole blood (100 μL) was mixed with Metexchange® reagent, and the

integration set point was -480 mV. The limit of detection was 1 $\mu\text{g/dL}$; readings below this level were assigned this value (a total of 29 samples: 28 from the control group and 1 from the 1 mg/kg Pb group). Because of the small volume of blood in younger animals, corticosterone and BPb analyses were only performed on P29 rats that did not undergo SWS. Values are reported in $\mu\text{g/dL}$.

2.4 Corticosterone

Blood was centrifuged at 4 $^{\circ}\text{C}$ for 25 min at 1300 RCF. Plasma was diluted 3:1 in buffer and assayed for corticosterone using a commercially available EIA kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ). The limit of detection was 0.55 ng/mL; samples that fell below this (two samples) were assigned this value. Each age $\times$ sex $\times$ Pb exposure $\times$ ISO $\times$ time after stress group had sample sizes of $N = 8$. Values are reported in ng/mL.

2.5 Monoamines

Monoamines were assessed by high performance liquid chromatography with electrochemical detection (HPLC-ECD). Frozen tissues were weighed, thawed, and sonicated in appropriate volumes of 0.1 N perchloric acid (Fisher Scientific, Pittsburgh, PA). Samples were centrifuged for 14 min at 13,000 RCF at 4 $^{\circ}\text{C}$. The supernatant sample was transferred to a new vial for injection on a Supelco SupelcosilTM LC-18 column (150×4.6 mm, 3 μm ; Sigma-Aldrich Co., St. Louis, MO). The HPLC system consisted of a Waters 717plus autosampler (Waters Corp., Milford, MA), ESA 584 pump (ESA, Inc., Chelmsford, MA), and ESA Coulochem III electrochemical detector. The potential settings were -150 mV for E1 and $+250$ mV for E2, with a guard cell potential of $+350$ mV. MD-TM mobile phase (ESA, Inc.) was used and consisted of 75 mM sodium dihydrogen phosphate (monohydrate), 1.7 mM 1-octanesulfonic acid sodium, 100 $\mu\text{L/L}$ triethylamine, 25 μM EDTA, and 10% acetonitrile, with a final pH of 3.0 . The pump flow rate was set at 0.7 mL/min, and the samples were run at 28 $^{\circ}\text{C}$. Standards for dopamine (DA), 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) (Sigma-Aldrich Co., St. Louis, MO) were prepared in 0.1 N perchloric acid. All monoamines were run on a single chromatogram. Each age $\times$ sex $\times$ Pb exposure $\times$ ISO group had a final sample size of $N = 4-8$. Values are reported in pg/mg tissue wet weight.

2.6 Statistical Analysis

Data were analyzed using mixed linear factorial analysis of variance (ANOVA; Proc Mixed, SAS v9.2, SAS Institute, Cary, NC) except for body weight. Factors in Mixed models were exposure (Pb dose), sex, developmental stressor (ISO vs STD housing), and time following the acute stressor (SWS). Each age was analyzed separately. In order to control for litter statistically, it was included in models as a randomized block factor. Significant interactions were further analyzed using slice-effect ANOVAs. Body weight was analyzed using general linear model ANOVA (Proc GLM, SAS) because before SWS pups were not subdivided and therefore sex was a repeated measure. Because there were only two levels of sex and developmental stressor, no *a posteriori* pairwise comparisons were needed when these were significant. Where there were three groups, as for Pb, slice-effect ANOVAs were used with Pdiff used within Mixed model ANOVAs for pairwise comparisons. This is equivalent to an

LSD and does not distort alpha when only 3 groups are compared. Mortality was analyzed using Fisher's test for uncorrelated proportions. Significance was set at $p = 0.05$. Data are presented as least square mean $\pm$ SEM for Mixed analyses and ordinary mean $\pm$ SEM for GLM data. Because data were analyzed by mixed model ANOVA, SEMs are used in figures for purposes of inference. Because of the complexity of the ANOVA models we show only those main effects and interactions that pertain to the objectives of the study, i.e., Pb, ISO, or their combination and any interactions of Pb, ISO, or their combination with sex that were present. Other effects, such as main effects of sex, time, or interactions of secondary factors (such as time $\times$ sex, for example) are generally not presented graphically.

3. Results

3.1 Mortality

Twenty out of the 96 litters in the study had at least one death. This resulted in a total of 29 deaths out of 1152 pups (2.5%). There was no sex difference among the 29 pups that died, however, 55% of the deaths were in the 10Pb group. This resulted in some cells of the study having a total of only 6 or 7 females or males per cell rather than 8.

3.2 Body weight

Pup weight was recorded on P1, P4, and every other day until the day of tissue collection. Using the group that was tested on P29, the ISO groups had decreased body weight (main effect: $F(1,1065)=6.67$, $p<0.01$) compared with STD groups, regardless of Pb (Fig. 1A). Pb had no independent effect on pup weight or any interaction with ISO.

3.3 Adrenal weight

Adrenal weights were unaffected by Pb or ISO at P11, P19, or P29. However, at P29, there was a Pb $\times$ ISO interaction, $F(2,73)=2.67$, $p<0.05$, in which ISO Pb pups (1Pb and 10Pb) had lighter adrenal weights than ISO VEH animals ($p<0.03$; VEH: $24 \pm 2 \mu\text{g}$ vs. 1Pb: $19 \pm 2 \mu\text{g}$ and 10Pb: $18 \pm 2 \mu\text{g}$, respectively). Males had heavier adrenals than females at P29, ($F(1,73)=4.16$, $p<0.05$). When evaluated as a percentage of body weight, there were no effects of Pb, ISO (Table 1), or their interactions.

3.4 Spleen weight

Spleen weights were decreased by ISO compared with STD at P11 ($F(1,93)=4.15$, $p<0.05$; Table 1), and female spleens were lighter than males ($F(1,93)=16.86$, $p<0.0001$). A significant sex $\times$ Pb $\times$ ISO interaction showed that 10Pb ISO males had lighter spleens than STD 10Pb males at this age ($F(1,93)=2.57$, $p<0.02$). At P19, a significant Pb $\times$ ISO interaction ($F(2,74)=4.82$, $p<0.01$) showed that among ISO pups, spleen weights in 10Pb animals were decreased compared with 1Pb, but did not differ compared with STD Pb animals (ISO 10Pb = $0.17 \pm 0.03 \text{ mg}$ vs. ISO 1Pb = $0.19 \pm 0.03 \text{ mg}$ vs. STD = $0.17 \pm 0.03 \text{ mg}$). At P29, female spleens were lighter than males ($F(1,25)=4.43$, $p<0.05$), however there were no effects of Pb or ISO. As a percentage of body weight, the only effect was at P11 and was an ISO main effect ($F(1,30)=7.62$, $p<0.01$) in which spleen to body weight ratios of all ISO groups were smaller than in STD groups (Table 1).

3.5 Thymus weight

ISO pups had decreased thymus weights, regardless of age or Pb, compared with STD pups (P11: $F(1,55)=6.67$, $p<0.01$; P19: $F(1,64)=31.03$, $p<0.0001$; P29: $F(1,73)=5.96$, $p<0.05$, Table 1). When expressed as a percentage of body weight, the reduction in ISO animals remained significant at P11 and P19, but not at P29 (Fig. 1B).

3.6 Blood Pb (BPb)

Pb exposure increased BPb at P29 ($F(2,72)=68.31$, $p<0.0001$). Both the 1Pb and 10Pb animals had increased BPb levels compared with VEH animals (1Pb, $p<0.05$; 10Pb, $p<0.0001$, Fig. 2). BPb in the 10Pb group was significantly higher than in the 1Pb group ($p<0.0001$).

ISO animals had increased BPb levels ($F(1,72)=12.3$, $p<0.0008$) as a function of Pb dose (ISO $\times$ Pb interaction, $F(2,72)=7.49$, $p<0.001$). As shown in Fig. 2, Pb-exposed ISO animals had increased BPb levels in the 10Pb group compared with 10Pb STD animals ($p<0.0001$).

3.7 Corticosterone

At P11, corticosterone was significantly altered by sex ($F(1,308)=6.00$, $p<0.05$; females had higher levels) and SWS ($F(3,308)=9.17$, $p<0.0001$). Corticosterone levels were highest immediately after SWS but were unaffected by ISO or Pb (Fig. 3A). At P19, corticosterone was affected by time after SWS ($F(3,319)=52.40$, $p<0.0001$) and there was an interaction of ISO and time after SWS ($F(3,319)=4.77$, $p<0.01$, Fig. 3B). ISO animals had increased levels of corticosterone compared with STD animals immediately after the SWS, but not at later intervals. There was no effect of Pb at this age. At P29, there was an effect of time after SWS ($F(3,330)=180.80$, $p<0.0001$) and an interaction of ISO with post-SWS time ($F(3,330)=2.91$, $p<0.05$, Fig. 3C). Basal levels were similar among groups, but ISO animals had a blunted response immediately after SWS at this age. Sex interacted with time after SWS ($F(3,330)=4.60$, $p<0.01$); females had higher corticosterone immediately after SWS than males. As with P11 and P19 animals, there was no effect of Pb.

3.8 Monoamines

3.8.1 Neostriatum—At P11, neither Pb nor ISO had main effects on monoamine or metabolite concentrations; however, there were interactions. There was a P11 sex $\times$ Pb interaction for DOPAC/DA ($F(2,59.1)=3.52$, $p<0.04$). By slice ANOVA the effect was only in females ($p<0.04$), but there was a trend in males ($p<0.10$). The STD 10Pb females had increased turnover compared with STD VEH females and ISO 10Pb males had reduced turnover compared with STD VEH males (Fig. 4A). For HVA/DA, there was a sex $\times$ Pb ($F(2,59.2)=4.14$, $p<0.03$) and sex $\times$ Pb $\times$ ISO interaction at P11 ($F(2,59.2)=3.58$, $p<0.04$, Fig. 4B). STD 10Pb females had increased HVA/DA ratios compared with STD VEH females. In males, HVA/DA ratios were decreased in ISO 1Pb and 10Pb and in STD 10Pb compared with STD VEH males. There was a similar pattern in total metabolite turnover (DOPAC+HVA)/DA; there was a sex $\times$ Pb ($F(2,59.1)=4.01$, $p<0.03$) and sex $\times$ Pb $\times$ ISO interaction ($F(2,59.1)=3.37$, $p<0.05$, Fig. 4C) in which STD 10Pb females had higher turnover compared with STD VEH females. In males ISO VEH, 1Pb, and 10Pb and STD

10Pb males had decreased turnover compared with STD VEH males. The 5-HIAA/5-HT ratio was also decreased in all ISO groups compared with STD groups (310.0 ± 12.3 vs. STD: 373.0 ± 12.3 , $F(1,60)=13.09$, $p<0.01$).

At P19, neither Pb nor ISO had main effects on DA or NE. There was a sex $\times$ ISO interaction for HVA/DA ($F(1,68.2)=3.88$, $p=0.05$). ISO males had reduced turnover compared with STD males regardless of Pb exposure (ISO males 35.0 ± 7.1 vs. STD males 58.0 ± 7.0). 5-HT was higher in females compared with males ($F(1,68)=4.52$, $p<0.05$) but there were no interactions with Pb or ISO. For NE there was an interaction of sex $\times$ ISO ($F(1,29.3)=5.49$, $p<0.03$); STD males had lower NE than STD females (females = 102.6 ± 20.2 pg/mg vs. males = 54.9 ± 19.7 pg/mg) and NE was decreased in ISO females compared with STD females (ISO females 49.5 ± 20.1 vs. females = 102.6 ± 20.2 pg/mg).

At P29, there were no main effects for DA, NE, or 5-HT. There was a sex $\times$ Pb $\times$ ISO interaction ($F(2,68.2)=3.79$, $p<0.05$) for 5-HIAA/5-HT ratios. ISO 1Pb males had a decreased ratio compared with ISO VEH males and STD 1Pb males (ISO 1Pb: 247.9 ± 27.6 vs. ISO VEH: 278.7 ± 27.6 , $p<0.05$ and STD 1Pb: 356.5 ± 27.6 , $p<0.01$).

3.8.2 Hypothalamus—At P11, there were no main effects of Pb or ISO on DA, NE, or 5-HT. DOPAC had an ISO $\times$ Pb interaction ($F(2,60)=5.29$, $p<0.01$): in ISO pups 10Pb animals had decreased levels compared with VEH animals (88.4 ± 20.4 pg/mg vs. 129.4 ± 20.4 pg/mg), but not 1Pb animals (103.1 ± 20.4 pg/mg). Pb $\times$ ISO interacted on HVA levels ($F(2,54)=3.99$, $p<0.05$), such that ISO 10Pb pups (142.8 ± 23.4 pg/mg) and ISO 1Pb (143.1 ± 23.8 pg/mg) pups had decreased levels compared with ISO VEH animals (187.3 ± 23.7 pg/mg), but there were no differences in STD animals. A sex $\times$ Pb interaction was found for 5-HIAA/5-HT ($F(2,59.1)=5.85$, $p<0.01$), such that 10Pb males had decreased ratios compared with 1Pb and VEH males (179.8 ± 21.0 vs. 228.3 ± 21.0 , $p<0.03$ and 234.8 ± 21.5 , $p<0.05$, respectively) with no effects in females.

At P19, there were no main effects on DA, NE, or 5-HT. There was a Pb $\times$ ISO interaction on DA ($F(2,66.6)=6.68$, $p<0.01$). By slice ANOVA the effect was only significant for the STD condition ($p<0.03$), not for ISO. The STD 1Pb group had increased DA compared with the STD VEH group (STD VEH 205.9 ± 29.9 vs. STD 1Pb 299.5 ± 30.9). There was a Pb $\times$ ISO interaction on DOPAC/DA ($F(2,66.3)=3.6$, $p<0.04$) but further analyses failed to show any meaningful group differences. The DOPAC+HVA/DA ratio showed an ISO main effect ($F(1,14)=5.6$, $p<0.04$) and a Pb $\times$ ISO interaction ($F(2,63.4)=4.57$, $p<0.02$). ISO decreased the ratio compared with STD regardless of Pb exposure (75.5 ± 11.3 vs. 113.2 ± 11.2) and the interaction showed the ratio was decreased in 10Pb and 1Pb groups in relation to the STD VEH group (VEH: 135.3 ± 13.6 vs. 10Pb: 106.5 ± 13.6 , $p<0.04$ and 1Pb: 97.7 ± 13.3 , $p<0.005$, respectively).

At P29 there were no changes in hypothalamic DA or NE. 5-HT showed interactions of sex $\times$ ISO ($F(1,57.4)=5.96$, $p<0.02$) and sex $\times$ Pb ($F(2,55.1)=3.82$, $p<0.03$). In STD animals, females had decreased 5-HT compared with males (females: 423.4 ± 60.6 pg/mg vs. males: 534.9 ± 58.8 , $p<0.03$), an effect not seen in ISO pups (females: 464.4 ± 55.8 pg/mg vs. males: 411.9 ± 52.9 pg/mg). The sex $\times$ Pb interaction showed that 10Pb females had

decreased 5-HT compared with 10Pb males (females: 423.4 ± 60.6 pg/mg vs. males: 534.9 ± 58.8 pg/mg, $p < 0.03$). 10Pb males also had increased 5-HT compared with males in the 1Pb and VEH groups (569.5 ± 50.6 pg/mg vs. 421.0 ± 50.6 pg/mg, $p < 0.01$ and 429.5 ± 50.6 pg/mg, $p < 0.01$, respectively).

3.8.3 Hippocampus—At P11, there were no main effects on DA, NE, or 5-HT. There was a main effect of Pb treatment on HVA ($F(2,53.2)=4.04$, $p < 0.03$) in which 10Pb animals had increased HVA compared with 1Pb and VEH groups (146.7 ± 10.7 pg/mg vs. 114.3 ± 10.9 pg/mg, $p < 0.04$ and 106.3 ± 10.7 pg/mg, $p < 0.01$, respectively). A sex $\times$ ISO interaction ($F(1,53.2)=6.26$, $p < 0.02$) showed that ISO females had increased HVA compared with STD females and ISO males (153.6 ± 12.8 pg/mg vs. 103.2 ± 11.8 pg/mg, $p < 0.05$ and 110.8 ± 12.8 pg/mg, $p < 0.02$, respectively). 5-HT and 5-HIAA were unaffected by ISO (Fig. 5A and B, respectively). 5-HIAA showed a sex main effect ($F(1,59.1)=8.32$, $p < 0.01$) and a sex $\times$ Pb interaction ($F(2,59.1)=3.20$, $p < 0.05$). Males had lower 5-HIAA levels compared with females. 5-HIAA levels in 10Pb males were lower than in 1Pb and VEH males (576.9 ± 42.4 pg/mg vs. 719.0 ± 43.6 pg/mg, $p < 0.004$ and 673.2 ± 42.4 pg/mg, $p < 0.04$, respectively). Turnover ratios were mostly unchanged, except the 5-HIAA/5-HT ratio for ISO on P11 ($F(1,12)=5.12$, $p < 0.05$) where the ISO groups had lower turnover than STD groups (Fig. 5C).

At P19 there were no main effects on DA, NE, 5-HT, or 5-HIAA (Fig. 5A&B). For NE there was a sex $\times$ Pb interaction ($F(2,65.7)=3.97$, $p < 0.03$). 10Pb females had increased NE compared with 1Pb and VEH females (441.7 ± 27.0 pg/mg vs. 376.2 ± 27.0 pg/mg, $p < 0.03$ and 353.3 ± 26.4 pg/mg, $p < 0.004$, respectively) and compared with 10Pb males (363.0 ± 27.7 pg/mg, $p < 0.05$). The 5-HIAA/5-HT ratio was decreased in all P19 ISO groups compared with all P19 STD groups ($F(1,13.8)=5.09$, $p < 0.05$; Fig. 5C).

At P29, there were no main effects on DA, NE, or 5-HT. There was a Pb $\times$ ISO interaction on 5-HT ($F(2,54)=4.99$, $p = 0.01$). In ISO animals, 1Pb pups had increased 5-HT compared with 10Pb and VEH pups (655.4 ± 86.1 pg/mg vs. 462.7 ± 87.5 pg/mg, $p < 0.01$ and 511.5 ± 86.1 pg/mg, $p < 0.05$, respectively). 5-HIAA showed a sex $\times$ ISO interaction ($F(1,53.9)=4.17$, $p < 0.05$): ISO females had increased 5-HIAA compared with males (918.0 ± 76.0 pg/mg vs. 768.0 ± 76.0 pg/mg, $p < 0.02$), with no difference among the STD groups. A Pb $\times$ ISO interaction was significant for 5-HIAA/5-HT ($F(2,54.1)=3.48$, $p < 0.04$): STD 1Pb animals had lower ratios compared with other STD groups (168.7 ± 36.7 vs. SAL: 223.5 ± 36.7 and 10Pb: 237.5 ± 37.5). No difference was found for 5-HIAA/5-HT ratios between the STD and ISO groups at P29 (Fig. 5C).

4. Discussion

Pb continues to pose risks to children despite mandated decreases in exposure. In children, the primary risk is to neurocognitive and behavioral development. Pb exposure is not uniform and is higher in children living in low SES conditions. Children living in low SES settings often live in older neighborhoods where housing, soil, and plumbing may contain elevated levels of Pb. Low SES may also be used as a marker for conditions associated with a variety of stressors. Hence, Pb and stress may interact to increase the effect of each when

they co-occur. Hence, our hypothesis was that developmental stress, as modeled by maternal separation, would interact with developmental Pb exposure to increase effects compared with either factor alone on organ weights, corticosterone, and brain monoamines.

One of the objectives of this experiment was to determine if maternal separation produced more or larger interactions with Pb than another developmental stressor that we had used previously, barren cage rearing. While these two developmental stressors are different we wanted to determine if one had a greater effect in combination with Pb on corticosterone, selected organ weights, and monoamines. Our intent was only to make this comparison in a global sense since it was impractical to use both stressors in a single experiment. Therefore, the comparison is a relative one focusing on areas of consistency or difference.

We previously tested the same doses of Pb in combination with barren-cage rearing in an experimental design identical to the present one (Graham et al., 2011). We hypothesized that maternal separation stress would induce greater interactions than Pb and barren-cage rearing. We found that neither barren-cage nor maternal separation stress interacted with Pb on adrenal or splenic weights, nor did they interact with Pb to affect thymic weight but both developmental stressors reduced thymic weights independently of Pb exposure. Barren-cage rearing reduced thymus weights only on P19 whereas maternal separation reduced thymus weight on P11 and P19 (see Fig. 1B).

For corticosterone, neither barren-cage rearing nor maternal separation interacted with Pb, but both induced changes independent of Pb. The pattern was somewhat different in response to the acute stressor inasmuch as barren cage reduced basal corticosterone on P11 (but not on P19 or P29) and reduced the rise in corticosterone 60 min after SWS. However, on P19, barren-cage induced a greater increase in corticosterone 0, 30, and 60 min after SWS. No changes were found on P29. Maternal separation had no effects on corticosterone at P11, increased the response to SWS at P19, and reduced the SWS increase in corticosterone at P29. Collectively, these data do not support the hypothesis that maternal separation was more stressful than barren-cage rearing on basal corticosterone or on corticosterone in response to an acute stress (SWS).

In terms of neurotransmitters, the models showed similarities and differences. Barren-cage increased hypothalamic NE (males) and 5-HT (males and females) at P19 but not at P11 or P29. Pb exposure increased hypothalamic NE and 5-HIAA in the 1Pb but not the 10Pb group at P29 in females. Maternal separation had no effects on hypothalamic monoamines, but reduced DA turnover in the striatum; 10Pb also reduced DA turnover in the striatum but did not interact with the stressor. In the hippocampus, barren-cage rearing interacted with 1Pb to increase 5-HT at P29 whereas 10Pb reduced 5-HT at P19 in females but did not interact with the stressor. Maternal separation reduced 5-HT utilization in the hippocampus at P11 and P19 but did not interact with Pb; Pb itself had no effect on 5-HT. Based on these outcomes, we conclude that there is no evidence that maternal separation is more stressful than barren-cage rearing on brain monoamines

Overall, our findings are in broad agreement with data reported by Cory-Slechta et al. in which Pb is given in drinking water during development at relatively high doses (see below)

alone or in combination with two-days of prenatal restraint stress. Restraint in these experiments was given on gestational days (G)16 and G17 three times, 45 min each time, spaced 3 h apart (where G1 = embryonic day E0, hence G16–17 is the same as E15–16). In these studies a number of Pb and prenatal stress effects were reported on behavior, corticosterone, and monoamines, but relatively few stress $\times$ Pb interactions were found (Cory-Slechta et al., 2004;Virgolini et al., 2008a;Cory-Slechta et al., 2009;Rossi-George et al., 2009;Cory-Slechta et al., 2010;Rossi-George et al., 2011;Cory-Slechta et al., 2012;Cory-Slechta et al., 2013b;Cory-Slechta et al., 2013a;Weston et al., 2014). In the first study, interactions between prenatal restraint stress and monoamines were found in the high dose group Pb Group (150 ppm/day Pb) on frontal cortex DA utilization (DA/HVA but not DA/DOPAC) and in nucleus accumbens (increased DOPAC and HVA) and reduced 5-HT (Cory-Slechta et al., 2004). However, in a follow-up experiment using a similar design the effects were only in the frontal cortex in groups given prenatal and postnatal stress combined with Pb and were on NE rather than DA or 5-HT (Virgolini et al., 2008a). Effects of Pb and stress on monoamines were essentially absent in the next experiment of the same design with the same doses of Pb and the same prenatal stress paradigm (Cory-Slechta et al., 2009). In the next experiment, using 50 ppm/day Pb and the same stressor, there were also almost no effects on monoamines in the frontal cortex, striatum, or nucleus accumbens, but this time monoamines were measured in midbrain and hypothalamus and Pb $\times$ stress interactions were obtained. However, the interactions were not seen in the stressed-Pb group but in the Pb non-stressed and stressed-no Pb groups for 5-HT and 5-HT utilization; there were no effects on DA or NE (Cory-Slechta et al., 2010). In a further study using the same model with both 50 and 150 ppm/day Pb doses, a few interactions on brain monoamines were seen but not in frontal cortex. Three interactions were found in the nucleus accumbens and one in the striatum. Of those in the nucleus accumbens, DOPAC was reduced in the 50 ppm Pb-stress group but not in the 150 ppm Pb-stress group; DA utilization was increased in the no Pb-prenatal stress group; and NE was increased in the no Pb-prenatal stress group. The interaction in the striatum was in the 150 ppm Pb no stress group in which DOPAC was higher than the no Pb no stress control group (Cory-Slechta et al., 2012). In the next experiment using the same model but only the 50 ppm Pb dose, no interactions were found in the frontal cortex on monoamines, but reductions in the Pb-stress group were found in frontal cortex glutamate and GABA (not measured in previous studies) and in midbrain 5-HT and 5-HIAA. However, in all these latter instances the effects were hemisphere-specific, being found on only one side. Moreover, these effects were mostly in Pb groups, regardless of stress, or in stress groups regardless of Pb, very few were found in Pb-stress combination groups (Cory-Slechta et al., 2013a). In the most recent report using the same model and the 50 ppm Pb exposure with or without the same prenatal restraint stress, almost no effects were found on monoamines in the frontal cortex, nucleus accumbens, striatum, hypothalamus, or midbrain. In this experiment brain derived neurotrophic factor (BDNF), NMDA-NR2A, and 5-HT transporter (SERT) were examined in frontal cortex, nucleus accumbens, hippocampus, and hypothalamus. There were no Pb-stress interactions on BDNF in any brain region, but there were Pb main effects (reduced BDNF in the frontal cortex) and Pb and stress main effects in the hippocampus. For NMDA-NR2A receptors, there were Pb main effects in frontal cortex and hypothalamus (reductions) but no interactions with stress. For SERT there were Pb-stress interactions in the frontal cortex and

hippocampus. Pb without stress increased SERT in the frontal cortex. In the hippocampus, Pb and stress increased SERT relative to the stress no Pb and Pb no stress groups, although these groups had decreased SERT compared with control. Interestingly, all of the Pb-stress interactions in these studies were not sorted statistically by *a posteriori* methods, but shown as significant interactions with no pairwise group comparisons. Hence, while these experiments provide some limited evidence of interactions between prenatal stress and Pb, the effects are modest with limited consistency across experiments, not unlike what we report in this and our previous Pb-stress experiment. In the studies by Cory-Slechta et al. most of the interactions were found on behavior rather than on neurotransmitters. We have not assessed behavior using our Pb and two stress models.

Maternal separation in our ISO groups reduced body and organ weights and this has not been reported (Knuth and Etgen, 2005;Knuth and Etgen, 2007), but the effects found here replicate our findings using the Pb + barren cage combination (Graham et al., 2011). Chronic stress, whether in neonatal or adult rats is known to decrease thymus weight (Tuchscherer et al., 2002;Felszeghy et al., 2000;Bakker et al., 1995). Decreases in thymus weight suggests immune disruption but we did not measure other immune markers, but this should be done in future experiments.

BPb concentrations were dose-dependent. However, ISO 10Pb pups had higher BPb compared with STD 10Pb rats at P29. The fact that stress-induced heightened BPb above that seen in non-stressed Pb exposed rats has not been reported (Graham et al., 2011;Virgolini et al., 2008a;Virgolini et al., 2008b). If similar effects occur in children, this could be important in understanding why some children have higher BPb levels than others even of the same SES level. The mechanism of how ISO stress interacts to increase BPb is unknown but stress mobilizes energy reserves, such as glycogen and lipids to generate more glucose, and this may cause Pb stored in lipids to be released faster than in non-stressed Pb-exposed animals.

ISO animals showed similar levels of basal corticosterone, but altered responses to acute stress (SWS) compared with STD animals. ISO animals exhibited exaggerated responses to SWS at P19 but not at P29. The differential P19 responses were transient and disappeared 30 min after SWS exposure.

For neostriatum, DA turnover was decreased in ISO pups at P11, but not at later ages. STD females had increased NE compared with males at P19, a sexually dimorphic effect eliminated by ISO. A related effect was seen in the hypothalamus: ISO decreased male 5-HT levels.

ISO also affected hippocampal 5-HT turnover. ISO animals had decreased 5-HT turnover on P11 and P19, but not on P29. Chronic stress and elevated corticosterone are known to affect hippocampal development in other models (Ivy et al., 2010;Brunson et al., 2003;Gilles et al., 1996) and this may be why hippocampal 5-HT turnover was affected.

As noted, neurotransmitter effects of Pb differed from our previous study using barren cage rearing. One noteworthy difference was that ISO altered Pb levels whereas barren cage rearing did not. Since the aim of this experiment was to test for interactions of Pb and ISO, it

is of interest that most of the ISO*Pb interactions were reductions in DA turnover. There were also ISO*Pb*sex interactions and most of these were in the neostriatum and hypothalamus and also involved reduced DA turnover preferentially in one sex, although in a few cases an increase was observed such as in the female STD-10Pb group. Hence, DA appears to be the most sensitive monoamine to Pb-stress interactions.

Limitations of the study include the use of only two doses of Pb. A greater range might have shown more effects. To model current levels of Pb found in children, we used doses of Pb lower than those used in many studies. While the use of lower doses has relevance to humans, detecting effects at these levels is more difficult. Interestingly, if one compares administered versus internal doses of Pb after developmental exposure in rats compared with non-human primates, species differences are apparent. While comparisons across studies cannot be precise, as a first approximation, we compared data from two laboratories that have published extensively on the developmental effects of Pb exposure. In rats we focused on studies from the laboratory of Cory-Slechta (Cory-Slechta et al., 2004;Virgolini et al., 2008a;Rossi-George et al., 2011;Cory-Slechta et al., 2009;Rossi-George et al., 2009;Cory-Slechta et al., 2010;Rossi-George et al., 2011;Cory-Slechta et al., 2012;Cory-Slechta et al., 2013b;Cory-Slechta et al., 2013a;Weston et al., 2014) and for non-human primates on studies from the laboratory of Rice (Rice et al., 1979;Rice, 1984;Rice and Gilbert, 1985;Truelove et al., 1985;Rice, 1985;Gilbert and Rice, 1987;Rice, 1988b;Rice and Karpinski, 1988;Rice, 1988a;Reuhl et al., 1989;Rice and Gilbert, 1990b;Rice and Gilbert, 1990a;Rice, 1990;Rice and Willes, 1979;Rice, 1992c;Rice, 1992a;Rice, 1992b;Rice, 1992a;Foster et al., 1993;Rice, 1993;Foster et al., 1996b;Foster et al., 1996a;Rice, 1997;Rice, 1998;Rice, 1996b;Rice, 1996a). Rice expresses doses as $\mu\text{g/kg/day}$ given once orally per day in monkey infant formula. Cory-Slechta expresses doses in rats as ppm/day in drinking water. In order to compare these it is first necessary to estimate how much water rats drank/day. Using water consumption data from the literature, adult SD rats (males or females) consume 80–100 mL/day (Slone et al., 2012;McGivern et al., 1996). Cory-Slechta gives 50 or 150 ppm/day in drinking water which is the same as 50 and 150 $\mu\text{g/mL/day}$. Using the lower end of the drinking volume range of 80 mL/day, 50 $\mu\text{g/mL}$ means an adult rat consumes 50×80 or 4000 $\mu\text{g/day}$. Assuming a body weight of 320 g, the resulting dose would be $4000 \times 0.32 = 1280 \mu\text{g/kg/day}$. Similarly, the higher dose would be 150 $\mu\text{g/mL/day} \times 80 \text{ mL/day}$ which is 150×80 or 12000 $\mu\text{g/day}$; again using a body weight of 320 g, this amounts to $12000 \times 0.32 = 3840 \mu\text{g/kg}$ as the dose. We can then compare what the doses given to monkeys and rats produce in terms of BPb concentrations. Cory-Slechta (Cory-Slechta et al., 2009) reports that the 50 ppm/day (i.e., 1280 $\mu\text{g/kg/day}$) dose results in BPb = 12–20 $\mu\text{g/dL}$ and the higher dose of 150 ppm/day (i.e., 3840 $\mu\text{g/kg/day}$) results in BPb = 25–36 $\mu\text{g/dL}$. Rice reports four doses (50, 100, 500, and 2000 $\mu\text{g/kg/day}$). The 50 $\mu\text{g/kg/day}$ dose results in BPb = $\sim 10 \mu\text{g/dL}$; the 100 $\mu\text{g/kg/day}$ dose results in BPb = $\sim 13 \mu\text{g/dL}$; 500 $\mu\text{g/kg/day}$ dose results in BPb = $\sim 19 \mu\text{g/dL}$; and 2000 $\mu\text{g/kg/day}$ dose results in BPb = $\sim 26 \mu\text{g/dL}$ (with a sharp rise in BPb when the animals get older). In the present study if we express our doses of 1 and 10 mg/kg in micrograms, we gave 1000 and 10000 $\mu\text{g/kg/day}$ every other day which averages out to 500 and 5000 $\mu\text{g/kg/day}$ by gavage. These doses resulted in BPb values in the unstressed group of 3 and 10 $\mu\text{g/dL}$, respectively, values in the range of BPb levels found in Cory-Slechta's studies. Comparing the rat and monkey doses relative to

BPb concentrations shows that rats must be given 1–2 orders of magnitude higher doses to obtain BPb levels comparable with non-human primates. While only approximate, this comparison suggests that rats are less sensitive than non-human primates based on administered dose. There are also differences in internal dose in terms of effect, but this is difficult to compare because the outcomes measured in non-human primates and rats are very different. Nevertheless, it may be that had we given doses that produce BPb concentrations similar to the higher end of Cory-Slechta's range of 12–36 µg/dL, perhaps we would have seen larger effects. That rats are less sensitive to neurotoxins than primates is not unique to Pb. For example, rats are at least an order of magnitude less sensitive to the prenatal effects of ethanol on brain development compared with primates (Gohlke et al., 2008), suggesting that this may be a more general phenomenon.

We hypothesized that Pb and maternal separation stress would exacerbate the effects of Pb on corticosterone (basal and following acute stress (SWS)), monoamines, and organ weights. The data showed such effects, but there is no easily overriding pattern and the interactions were not large. Furthermore, the interactions were often with age, dose, and sex and more on dopaminergic markers of turnover. The data suggest that monoamine metabolism (turnover) may be more sensitive to Pb and ISO compared with levels of the neurotransmitter itself. Children exposed to Pb are often exposed to stressors, hence, interaction models may be useful for better understanding how Pb affects brain development but it is not yet clear what the optimal stress model is.

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Highlights

- Effects of neonatal Pb, maternal separation and the combination were evaluated
- Pups were evaluated on postnatal day 11, 19, and 28
- Early stress-Pb interactions were present but complex
- Maternal separation, Pb, age, sex, and acute stress interacted on neurotransmitters
- Maternal separation reduced body and thymus weight and corticosterone after shallow water stress

Fig. 1. Effects of ISO on Body and Thymus Weights

(A) ISO pups (regardless of Pb exposure) weighed less than STD pups. N by age and group collapsed across sex (VEH, 1Pb, 10Pb): **P11** STD: basal=12/12/12, t0=12, t30=,t60=; **P11** ISO: basal=13/14/14, t0=,t30=,t60=. **P19** STD: basal=16/16/16, t0=12, t30=,t60=; **P19** ISO: basal=14/14/12, t0=,t30=,t60=. **P29** STD: basal=14/14/11 (2 male/female pairs were inadvertently placed in the ISO group, thus reducing the numbers in this group and increasing them in the basal group), t0=16/16/16, t30=,t60=; ISO: basal=, t0=,t30=,t60=. **P29** ISO: basal=18/18/18, t0=12, t30=,t60=; ISO: basal=, t0=,t30=,t60=. (B) Thymus weight

as a proportion of body weight. **P11** STD: basal=12/12/12, t0=12, t30=,t60=; **P11** ISO: basal=14/14/14, t0=,t30=,t60=. **P19** STD: basal=16/16/16, t0=12, t30=,t60=; **P19** ISO: basal=14/14/13, t0=,t30=,t60=. **P29** STD: basal=14/14/13, t0=16/16/16, t30=,t60=; ISO: basal=18/18/17, t0=16/16/16, t30=,t60=. **P29** ISO: basal=18/18/18, t0=12, t30=,t60=; ISO: basal=, t0=,t30=,t60=. *p<0.05, **p<0.01, ***p<0.001 compared with STD.

Fig. 2. Blood Pb Levels

Blood was taken from animals not exposed to SWS. BPb was determined only in the P29 age groups. There were no differences between males and females, therefore, their data were combined. N = total (males, females): VEH-STD = 16 (8,8); VEH-ISO = 18 (9,9); 1Pb-STD = 16 (8,8); 1Pb-ISO = 16 (8,8); 10Pb-STD = 15 (8,7); 10Pb-ISO = 17 (9,8). * $p < 0.05$ vs. VEH, # $p < 0.05$ vs. 1Pb in the same stressor group, +++ $p < 0.0001$ 10Pb ISO vs. 10Pb STD.

Fig. 3. Plasma corticosterone before and after acute stress (SWS)

Pups at all three ages showed increased corticosterone in response to SWS regardless of rearing condition. There were no effects of ISO rearing at P11 (A). ISO increased the rise in corticosterone at time-0 after SWS at P19 (B). ISO decreased the rise in corticosterone at time-0 after SWS at P29 (C). *p<0.05, **p<0.01 vs. STD rearing. Group sizes were balanced for sex; no significant sex differences were found. N/group at all 3 ages were as in Fig. 1.

Fig. 4. Effects of ISO and Pb on P11 DA

(A) Striatal DOPAC/DA was reduced in all ISO groups regardless of Pb or sex. In addition, this ratio was reduced in the male STD 10Pb-treated group and increased in the female STD 10Pb-treated group. (B) Striatal HVA/DA ratio was also reduced on all ISO groups regardless of Pb or sex. In addition, and in 10Pb-treated males. (C) P11 striatal (HVA +DOPAC)/DA ratio was reduced only under the ISO rearing condition but not by 10Pb treatment. N per group were 7–8 per sex per Pb exposure per rearing condition per assessment age. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with VEH within sex. # $p < 0.05$, ### $p < 0.001$ ISO compared with STD.

Fig. 5. Effects of ISO on hippocampal 5-HT

(**A**) Effects on 5-HT, (**B**) on 5-HIAA, and (**C**) 5-HIAA/5-HT; the latter was reduced by ISO with no effect of Pb at P11 and P19. N per group were 7–8 per sex per treatment per rearing condition per assessment age. * $p < 0.05$ vs. STD animals.

Absolute organ weight ratios for adrenals and spleen and thymus weights (mg) as a function of rearing condition and age

Table 1

Age	Adrenals		Spleen		Thymus	
	STD	ISO	STD	ISO	STD	ISO
P11	0.007±0.0006	0.006±0.005	0.26±0.030	0.17±0.031 *	0.09±0.005	0.07±0.005 **
P19	0.017±0.003	0.011±0.003	0.20±0.032	0.18±0.030	0.19±0.006	0.14±0.007 *****
P29	0.022±0.002	0.020±0.002	0.24±0.090	0.18±0.055	0.39±0.018	0.34±0.015 **

* p<0.05,

** p<0.01,

***** p<0.0001