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Effects of Developmental Stress and Lead (Pb) on Corticosterone after Chronic and Acute Stress, Brain Monoamines, and Blood Pb Levels in Rats

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Abstract

Despite restrictions, exposure to lead (Pb) continues. Moreover, exposure varies and is often higher in lower socioeconomic status (SES) families and remains a significant risk to cognitive development. Stress is another risk factor. Lower SES may be a proxy for stress in humans. When stress and Pb co-occur, risk may be increased. A few previous experiments have combined Pb with intermittent or acute stress but not with chronic stress. To determine if chronic developmental stress affects outcome in combination with Pb, we tested such effects on growth, organ weight, brain monoamines, and response to an acute stressor. Sprague Dawley rats were gavaged with Pb acetate (1 or 10 mg/kg) or vehicle every other day from postnatal day (P)4-29 and reared in standard or barren cages. Subsets were analyzed at different ages (P11, 19, 29). Chronic stress did not alter blood Pb levels but altered HPA axis response during early development whereas Pb did not. Pb treatment and rearing each altered organ to body weight ratios, most notably of thymus weights. Both Pb and rearing resulted in age- and region-dependent changes in serotonin and norepinephrine levels and in dopamine and serotonin turnover. The model introduced here may be useful for investigating the interaction of Pb and chronic developmental stress.

Keywords

Maternal stress; Lead (Pb); corticosterone; serotonin; dopamine; barren cage rearing

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1. Introduction

Chronic stress is a known modifier of physiological and neural function, especially when present during development. Many studies have shown that children from lower socioeconomic status (SES) households show evidence of elevated stress. For example, it has been demonstrated that children from lower SES backgrounds have higher cortisol levels and other markers of increased stress (Cohen et al., 2006; Lupien et al., 2000; Lupien et al., 2001) compared to children from higher SES backgrounds. While moderate, periodic stress is normal, chronic stress has detrimental effects on physiological function and brain development (McEwen and Stellar, 1993; McEwen, 1998). Stressors activate the hypothalamic-pituitary-adrenal (HPA) axis resulting in release of a cascade of hormones including corticotropin releasing factor, adrenocorticotropin hormone, and corticosterone (CORT; rodents)/cortisol (humans). The HPA axis maintains homeostasis and responds to threats after activation through negative feedback pathways that return the system to steady-state (Lupien et al., 1999; McEwen et al., 1992). However, chronic or severe exposure to stressors results in HPA axis dysregulation and neuronal damage (De Kloet et al., 1988). One phase of development, the stress hyporesponsive period (SHRP), is known to be vulnerable to elevated CORT levels. This stage (postnatal day (P) 4-14 in rodents; late gestation-early childhood in humans) is characterized by a blunted adrenal response to stress during neuronal development (De Kloet et al., 1988; Sapolsky and Meaney, 1986). It has been hypothesized that stressors that elevate CORT during this period result in long-term CNS damage, especially in the hippocampus (Anisman et al., 1998; Gos et al., 2008; Gruss et al., 2008).

In addition to exposure to elevated stress, children from lower SES environments are more likely to be exposed to higher levels of lead (Pb) because older inner city housing has higher Pb 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). Pb is known to cause neurotoxicity in humans and animals, especially during development. Despite restrictions on its use in the United States, it is still used in some industrial settings and is less restricted in other parts of the world (Goyer, 1996; Levin et al., 2008; Meyer et al., 2003). A number of studies examining the effects of environmental and occupational Pb exposure in adults show that it is associated with elevated levels of oxidative stress (Ergurhan-Ilhan et al., 2008), kidney and peripheral arterial diseases and hypertension (Muntner et al., 2005), white matter lesions and altered brain volumes (Stewart et al., 2006), persistent cognitive impairment (Shih et al., 2006; Stewart and Schwartz, 2007; van Wijngaarden et al., 2009), and, at high levels, can contribute to mortality (Menke et al., 2006).

While exposure to Pb at any age can be harmful, children are particularly susceptible. Developmental Pb exposure is associated with genotoxic damage (Mendez-Gomez et al., 2008), altered cardiovascular function (Gump et al., 2005), encephalopathy (Patel and Athawale, 2009; Sahu et al., 2009), and abnormal responses to stressors (Gump et al., 2005; Gump et al., 2008). In addition, children exposed to Pb prenatally and/or postnatally exhibit attention deficits (Chiodo et al., 2007; Nigg et al., 2008; Plusquellec et al., 2007; Surkan et al., 2007), sociability and behavioral problems (Burns et al., 1999; Chiodo et al., 2007), decreased reaction times (Chiodo et al., 2007; Winneke et al., 1985), hyperactivity (Chiodo et al., 2007), poorer performance on math and reading tests (Min et al., 2009; Surkan et al., 2007), altered language function (Yuan et al., 2006), decreased gray matter volume (Cecil et al., 2008), and lower IQ scores (Chiodo et al., 2007; Needleman et al., 1979; Surkan et al., 2007; Wang et al., 1989; Winneke et al., 1985). Recent reports from the Cincinnati Lead Study (CLS), a long-term prospective project that includes a cohort of children exposed to Pb in their homes, have shown that developmental exposure to Pb is associated with increased rates of delinquent behavior (Dietrich et al., 2001) and higher arrest rates for non-

violent and violent offenses (Wright et al., 2008). Moreover, many of the recent studies (Jusko et al., 2008; Lanphear et al., 2000; Vega-Dienstmaier et al., 2006) suggest effects of Pb at blood Pb (BPb) levels below the CDC's "action" level of 10 μg/dl (CDC, 1991). Efforts to understand the effects of low level Pb exposure may depend on how it interacts with other risk factors.

Since stress alone can have deleterious effects, it has been postulated that the combination of stress and Pb may be additive (Tong et al., 2000). A number of studies, including those by Cory-Slechta and colleagues, have investigated this interaction in rats. Using a gestational stressor (restraint stress on two consecutive days) and Pb administration via maternal drinking water, they report that these treatments, both singly and in combination, result in HPA axis dysfunction, neurochemical changes in catecholamine and serotonin levels, and altered responses on fixed interval (FI) schedule-controlled operant behavior when these animals are examined as adults (Cory-Slechta et al., 2004; Cory-Slechta et al., 2009; Rossi-George et al., 2009; Virgolini et al., 2005; Virgolini et al., 2006; Virgolini et al., 2008a; Virgolini et al., 2008b). These studies focused on the effects of Pb-stress interactions during gestation which is equivalent to the first half of human gestation in terms of brain development (Clancy et al., 2007). Most children are exposed to Pb over longer periods of development (Melnyk et al., 2000; Toscano and Guilarte, 2005) and lower SES may be a proxy for chronic stress.

BPb levels in children in many of the current clinical studies are under 10 μg/dl. It has been shown that cognitive deficits occur in this range (Bellinger, 2008; Lanphear et al., 2000). Accordingly, the purpose of this experiment was to determine the effects of chronic stress and moderate to low level Pb exposure during stages of brain development (P4-29) analogous to late gestation through early childhood. Specifically, BPb levels, brain monoamine measurements, organ weight of organs associated with the stress and immunity, and stress responsivity were assessed. BPb levels in the range of 3–10 μg/dl were our target values. In addition, the Pb was administered directly to the pups rather than to the dam (i.e., Pb in drinking water). A chronic rather than acute or intermittent stressor was used. For this, we used rearing in cages without standard bedding (barren cage), a method adapted from previous studies (Gilles et al., 1996) but with significant modification. In the original use of this method it was shown to be an effective stressor in rodents that results in elevated basal and stress-induced CORT levels, corticotrophin-releasing hormone and vasopressin expression, glucocorticoid receptor number, reduced body weights, and learning and memory impairment (Avishai-Eliner et al., 2001; Gilles et al., 1996; Rice et al., 2008). Here, animals were examined at different ages for basal and acute stressor-induced changes. Brain monoamines were determined in hypothalamus, hippocampus, entorhinal cortex, and neostriatum. The hypothalamus and hippocampus were included because they are important in stress responses (Herman et al., 1999; McEwen, 2007) and the latter in spatial (allocentric) learning; Pb has been shown to cause spatial deficits ((Jett et al., 1997; Zhou and Suszkiw, 2004); see also (Gilbert et al., 2005)). The entorhinal cortex was chosen for its role in spatial learning as well (Fyhn et al., 2004; Hafting et al., 2005). The neostriatum was included because stress and Pb were previously shown to affect monoamines in this region (Virgolini et al., 2005), and also because of its involvement in locomotor activity, which has also been shown to be altered by stress (Merrett et al.) and Pb (Reiter et al., 1975; Szczerbak et al., 2007). Furthermore, Pb induces monoamine changes in the striatum in animals exhibiting altered operant behavior (Cory-Slechta et al., 2002). Organs sensitive to stress and/or Pb were also assessed: adrenals because of their importance in HPA axis regulation (Sapolsky and Meaney, 1986), and thymus and spleen because of their role in immunity, stress (Dhabhar and McEwen, 1997; Miller and O'Callaghan, 2002), and Pb effects (Bunn et al., 2001b; Lee and Dietert, 2003; Talcott and Koller, 1983).

2. Materials and Methods

2.1 Animals

Male (250–275 g) and nulliparous female (175–200 g) Sprague-Dawley (IGS) rats (Charles River Laboratories, Raleigh, NC), were bred singly in-house following a minimum of 1 week of habituation in the vivarium (AAALAC-accredited). The 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% ± 10%). In order to minimize exposure to dietary Pb and maintain regular mineral consumption, animals were maintained on NIH-07 rodent chow, which has known and consistent levels of metals and minerals. Food and tap water were freely available. Presence of a sperm plug was designated embryonic day 0 (E0), and on E1, impregnated females were transferred to polycarbonate cages (46 × 24 × 20 cm) containing woodchip bedding. Day of birth was designated P0, and on P1 litters were culled to 12 pups (6 males and 6 females). In the cases of litters <12, 1 or 2 pups from other litters born on the same day were added to attain uniform litter sizes of 12; this occurred in 13/96 litters (<14%). Of the 13 litters that had pups added, 9 received 1 pup (9.4%) and 4 received 2 pups (4.2%). Pups were identified via ear punches on P4. The experiment was approved by the Institutional Animal Care and Use Committee.

2.2 Pb Treatment and Rearing Condition

Pb administration and differential rearing conditions began on P4. A total of 96 litters were used; 48 litters with standard cage bedding and 48 in barren cages using the method of (Gilles et al., 1996) with modification. For the barren cage condition, the woodchip bedding was omitted from the cage, and a single paper towel provided. A clean cage and towel were provided each day. Two male and female pairs per litter were gavaged with 0 (0.01 M anhydrous sodium acetate), 1, or 10 mg/kg Pb acetate in a volume of 3 ml/kg. Gavage was used to avoid maternal exposure. Pb is known to effect adult behavior (Cory-Slechta and Weiss, 1989; Mantovani et al., 1999; Massaro et al., 1986; Yun et al., 2000) which could affect dam behavior, including maternal care. A preliminary experiment on the effect of gavage showed that giving water on P4 did not significantly increase CORT levels 30 min later compared to non-gavaged rats. This does not prove that gavage has no significant CORT effect at later ages but suggests that it may not. We previously showed that rats receiving four saline injections subcutaneously have only slightly higher CORT levels than handled controls on P11 (Williams et al., 2000); CORT levels of SAL-injected and handled controls also did not differ on P15 or P20 after multiple days of exposure. Rats were gavaged every other day from P4 until P28. Litters for the P29 time point (see below) had dams removed on P28 and offspring re-housed in separate cages containing standard bedding by sex, 3 rats/cage (1 from each treatment group). Mortality was recorded.

At each evaluation age (P11, P19, or P29), separate sets of animals were exposed to an acute stressor (shallow water stressor, SWS) or not prior to tissue collection (Mineur et al., 2003; Mineur et al., 2006). The SWS group was placed individually in clean cages filled with wading-depth (2 cm at P11; 3 cm, P19; 4 cm, P29), room temperature water for 30 min. Subsets were sacrificed via rapid decapitation at 0, 30, or 60 min after removal from the water. Rats not sacrificed at the 0 min time point were placed back in the home cage until the designated time. Blood was collected in 12 × 75 mm polyethylene tubes containing 0.05 ml of 2% EDTA for CORT assay. An additional blood sample was taken from animals on P29 for BPb analysis. Neostriatum (caudate and putamen), hypothalamus, hippocampus, and entorhinal cortex were dissected over ice using a 1 mm brain block (Zivic-Miller, Pittsburgh, PA) from non-SWS exposed rats for monoamine determinations (Grace et al., 2010). Spleen, thymus, and adrenals were removed and weighed, such that each age × sex × treatment × rearing condition group had sample sizes of N = 6–8. Body weights from these

rats were obtained in order to determine relative weight. Samples were stored at −80°C until assayed.

2.3 Corticosterone Assessment

Blood was centrifuged at 4°C for 25 min at 1300 RCF. Plasma was diluted 3:1 in buffer and assayed for CORT using a commercially available EIA kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ). The limit of detection for this assay was 0.55 ng/ml; samples that fell below this (two samples) were assigned this value. Single samples were run since we have found that the variation in duplicates is below 5% for this assay. Each age × sex × treatment × rearing condition × time after stress group had sample sizes of N = 6–9.

2.4 Blood-Pb Levels

BPb levels were measured using an ESA Lead Analyzer 3010B (Chelmsford, MA), which utilizes anodonic stripping voltammetry. Whole blood (100 μl) was mixed with Metexchange® reagent, and the integration set point was −480 mV. Values were recorded in μg/dl. The limit of detection was 1 μg/dl; readings below this level (a total of 29 samples: 28 from the control group and 1 from the 1 mg/kg Pb group) were assigned this value. Due to difficulty obtaining sufficient blood for both CORT and BPb in younger animals, these analyses were performed only on P29 animals that did not undergo the SWS (N = 7–8 per treatment × rearing × sex group).

2.5 Neurochemical Analyses

Monoamines were assessed via 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°C. The supernatant sample was transferred to a new vial for injection onto a Supelco Supelcosil™ 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 salt, 100 μl/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°C. Standards for dopamine (DA), 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) (all obtained from Sigma-Aldrich Co., St. Louis, MO) were prepared in 0.1 N perchloric acid. All neurotransmitters were run on a single chromatogram. Each age × sex × treatment × rearing condition group had a final sample size of N = 4–8 which varied due to sample loss or technical malfunctions.

2.6 Statistical Analyses

Most data were analyzed using mixed linear factorial analysis of variance (ANOVA; Proc Mixed, SAS v9.1, SAS Institute, Cary, NC). Factors were treatment (Pb (1 or 10 mg/kg) and acetate control), sex, rearing conditions (rearing), age (i.e., P11, P19, or P29), and time following SWS (0, 30, or 60 min). In order to account for litter effects, litter was a randomized block factor. Significant interactions were further analyzed using slice-effect ANOVAs. Body weights collected prior to each gavage administration from animals killed at the P29 time point only (N = 7–8 per rearing × treatment × sex group) were analyzed using general linear model ANOVA (Proc GLM, SAS). Treatment, day, and sex were within

factors, while rearing was a between factor. Mortality was analyzed using Fisher's test for uncorrelated proportions. Significance was set at $p \leq 0.05$. Data are presented as mean ± SEM.

3. Results

3.1 Body Weights

Body weights were significantly altered by rearing conditions (Rearing main effect). When collapsed across age, barren-reared rats were lighter than those raised in standard cages [(35.2 ± 1.5 vs. 38.7 ± 0.8), ($F(1,26) = 4.23$, $p<0.05$)]. Treatment had no significant effect. A significant Sex × Treatment × Day interaction was obtained on P26 such that vehicle-treated males weighed significantly more than males treated with either dose of Pb ($p < 0.05$; not shown).

3.2 Mortality

Mortality was analyzed without regard to SWS exposure. P19 male barren-reared 10 mg/kg Pb group had elevated mortality relative to their controls ($p < 0.05$; Table 1). Between rearing conditions, barren-reared 10 mg/kg Pb males also had higher mortality than 10 mg/kg Pb males reared in standard conditions ($p < 0.05$; Table 1).

3.3 Organ Weights

3.3.1 Adrenals—When adrenal weights were expressed relative to body weight, a significant main effect of Age was found ($F(2,179)=3.37$, $p<0.05$); P19 rats had significantly larger relative adrenal weights than P11 or P29 rats (0.032 ± 0.002 % vs. 0.023 ± 0.002 % or 0.025 ± 0.003 % body weight, respectively). No other main effects or interactions were observed for relative adrenal weights, although in terms of *absolute* adrenal weights, adrenals of barren-reared rats weighed significantly less than those raised in standard conditions, regardless of age (Rearing main effect, ($F(1,192) = 6.73$, $p\leq0.01$); 12.0 ± 0.8 mg vs. 14.8 ± 0.8 mg).

3.3.2 Spleens—Relative splenic weights showed a main effect of Age ($F(2,180) = 18.61$, $p<0.0001$) with the P11 rats exhibiting greater relative weight than P19 and P29 animals (0.49 ± 0.02 % vs. 0.38 ± 0.02 % or 0.38 ± 0.02 % body weight, respectively). The Treatment main effect was not significant ($p<0.08$). Treatment was significant for absolute splenic weight [($F(2,193)=3.32$, $p<0.05$), 195.2 ± 5.4 mg (10Pb) > 206.3 ± 5.4 mg (controls) or 207.4 ± 5.3 mg (1Pb)]. Barren rearing had no significant effect on relative spleen weight ($p=0.22$), although spleens of animals reared in barren conditions were lighter than those raised in standard conditions [($F(2,193)=10.97$, $p<0.01$), 188.1 ± 6.3 mg vs. 217.8 ± 6.4 mg]. Additionally, there was also a significant Sex × Treatment × Rearing interaction ($F(2,193) = 4.57$, $p<0.05$) for absolute splenic weight; none of the slice-effect ANOVAs were significant.

3.3.3 Thymus—Relative weight showed a main effect of Age ($F(2,180)=10.77$, $p<.0001$; P11 < P19 = P29) and Sex ($F(1,180)=19.78$, $p<0.001$; female > male). Also, a significant main effect of Treatment occurred ($F(2,180)=5.06$, $p<0.01$); control group weights were significantly greater than those treated with 1 ($p<0.05$) or 10 mg/kg Pb ($p<0.01$). Barren rearing also resulted in a significant decrease in relative weight compared with standard rearing [Rearing main effect ($F(1,180)=4.01$, $p<0.05$)]. A significant Rearing × Age interaction occurred, such that barren-reared rats had lower relative weight compared with standard-reared rats on P19 ($F(2,180)=3.21$, $p<0.05$) but not on P11 or P29.

3.4 CORT Assessment

The levels reported below show no evidence of gavage treatment altering CORT levels compared with age-matched rats that were injected with saline (Graham et al., 2010). CORT levels in rats not exposed to SWS showed no effect of treatment. There was a main effect of Rearing ($F(1, 903)=11.98$, $p<0.001$); animals raised in barren cages exhibited elevated basal CORT levels compared to standard-reared animals. In analyses that included SWS as a factor there was a main effect of Age ($F(2, 903)=90.20$, $p<0.0001$) (P11 < P29 <P19); and Stress ($F(3, 903)=155.93$, $p<0.0001$) (basal < 60 min < 30 min < 0 min). A significant Rearing × Age × Stress interaction ($F(6,903)=10.47$, $p<0.0001$) was also seen (Fig. 1). At P11, animals raised in barren cages had significantly higher basal (i.e., prior to the SWS) levels of CORT compared with animals raised in standard cages (Fig. 1A). SWS significantly increased CORT in standard-reared rats at all time points, but not in barren-reared rats (that had higher basal CORT initially). No differences in basal CORT levels were seen on P19, however following SWS differences emerged. Barren-reared rats had significantly higher CORT at all time points compared to standard-reared rats. In addition, CORT was elevated at 0 and 30 min after the SWS regardless of rearing condition (Fig. 1B). At 60 min, CORT returned to basal levels in standard-reared rats, however, CORT remained elevated in barren-reared rats and was significantly higher than at 30 min ($p<0.05$). No difference between rearing conditions was seen at P29; SWS increased CORT at 0 and 30 min in both rearing groups (Fig. 1C).

3.5 Blood-Pb Levels

There was a significant Treatment main effect on P29 ($F(2,69)=40.98$, $p<0.0001$); both the 1 mg/kg and 10 mg/kg Pb treatments increased BPb compared with vehicle controls ($p<0.05$ and $p<0.0001$, respectively, Fig. 2); rats treated with the 10 mg/kg Pb dose had greater BPb levels than those treated with 1 mg/kg ($p<0.0001$). The interactions of Sex × Rearing and Sex × Rearing × Treatment were not significant ($p=0.09$ and $p=0.07$, respectively).

3.6 Monoamine Measurements

Monoamine levels are shown in Tables S1–S8. For all neurotransmitters and their metabolites, there were significant main effects of Age.

3.6.1 Neostriatum—For DA there was no significant effect of Treatment or Rearing (Table S1). Likewise, treatment had no significant effect on DOPAC levels. Treatment altered HVA levels ($F(2,177)=8.50$, $p<0.001$); the 10 mg/kg Pb treatment had higher HVA levels (1077.4 ± 40.0 pg/mg) than the 1 mg/kg Pb dose ($p<0.01$, 944.3 ± 39.3 pg/mg) or controls ($p<0.001$, 907.6 ± 39.6 pg/mg). A Treatment × Rearing interaction ($F(2,177)=3.06$, $p<0.05$) was also obtained. It showed that standard-reared 1 mg/kg Pb animals had higher HVA levels than barren-reared 1 mg/kg Pb animals (1022.3 ± 55.7 pg/mg vs. 866.3 ± 55.5 pg/mg, $p<0.05$). Barren-reared 10 mg/kg Pb animals had significantly higher HVA levels (1105.5 ± 57.6 pg/mg) than barren-reared 1 mg/kg Pb (866.3 ± 55.5 pg/mg, $p<0.001$) or controls (886.8 ± 55.4 pg/mg, $p<0.001$). Similarly, standard-reared 10 mg/kg Pb animals had higher HVA levels than standard-reared controls (1049.2 ± 55.5 pg/mg vs. 928.3 ± 56.6 pg/mg, $p<0.05$).

DA turnover relative to HVA (HVA/DA ratio) was significantly affected by Treatment ($F(2,173)=4.73$, $p\leq0.01$), with the 10 mg/kg Pb group exhibiting higher turnover (0.37 ± 0.03) than controls (0.29 ± 0.03, $p<0.01$) or the 1 mg/kg Pb group (0.31 ± 0.03, $p<0.05$). A significant interaction of Treatment × Age was found ($F(4,173)=2.86$, $p<0.05$); on P11, rats in the 10 mg/kg Pb group (0.49 ± 0.05) had greater turnover than controls (0.30 ± 0.05, $p<0.0001$) or the 1 mg/kg Pb group (0.38 ± 0.04, $p<0.01$). No effects were observed for DA

turnover relative to DOPAC (DOPAC/DA ratio) or for both metabolites [(DOPAC+HVA)/DA].

3.6.2 Hypothalamus—There was a Sex × Rearing × Age interaction ($F(2,157)=3.71$, $p<0.05$) for NE; barren-reared males had higher NE levels on P19 compared with standard-reared males (Fig. 3A). No effect was observed in females (Fig. 3B). In addition, a significant Sex × Treatment × Age interaction was obtained [($F(4,157)=2.58$, $p<0.05$); Fig. 3C-D, Table S2]. On P29, females in the 1 mg/kg Pb group had higher NE levels than control or 10 mg/kg Pb animals (Fig. 3D); no differences were observed in males (Fig. 3C). There were no treatment or rearing effects obtained for hypothalamic DA (Table S3) or DOPAC; HVA was below the limit of quantification.

There were no significant main effects of Treatment or Rearing on 5-HT (Table S4) or 5-HIAA levels or turnover. There was a Sex × Rearing interaction for 5-HIAA, $F(1,150)=3.94$, $p<0.05$) that was apparent only in barren-reared groups (females: 1927.7 ± 157.7 pg/mg vs. males: 1534.2 ± 154.3 pg/mg). A Treatment × Rearing × Age [($F(4,150)=2.43$, $p<0.05$), Fig. 4A-C] interaction showed that barren-reared animals exhibited higher 5-HIAA levels than standard-reared animals on P29 in the 1 mg/kg Pb group (Fig. 4B). There was also a Sex × Treatment × Rearing interaction ($F(2,150)=3.53$, $p<0.05$) (Fig. 4D–E), that showed that barren-reared 1 mg/kg Pb females had greater 5-HIAA levels than barren-reared female controls (Fig. 4E). No other differences were obtained.

3.6.3 Entorhinal Cortex—No effects of Treatment, Rearing, or interactions of these factors were obtained for any neurotransmitter, metabolite, or turnover in this region.

3.6.4 Hippocampus—Hippocampal NE showed no effects of Treatment, Rearing, or their interactions (Table S7). For 5-HT, there was a Treatment × Rearing × Age interaction [($F(4,152)=2.74$, $p<0.05$); Fig. 5A–C, Table S8]; barren-reared 1 mg/kg Pb rats had higher 5-HT levels than standard-reared 1 mg/kg rats on P29 (Fig. 5B). A significant Sex × Treatment × Age ($F(4,149)=2.67$, $p<0.05$) interaction for 5-HIAA was also seen (Fig. 5D–E); P19 female 1 mg/kg Pb rats had higher levels of 5-HIAA than P19 female 10 mg/kg Pb rats (Fig. 5E). Finally, there was a significant Treatment × Rearing × Age interaction ($F(4,145)=2.69$, $p<0.05$) in 5-HT turnover (5-HIAA/5-HT). Further analyses did not show differences between groups at any age.

4. Discussion

Both stress and Pb have been shown to be developmental risk factors. Human studies show that even low level Pb exposure (i.e., below the 10 μg/dl) disrupts CNS development (Mendola et al., 2002). There are fewer animal studies that utilize BPb levels in this range. In the present experiment, we tested doses that produced average BPb levels of 3.3 and 12.6 μg/dl in rats on P29 after exposure from P4-28. These levels are lower than those reported by several laboratories (Cory-Slechta et al., 2009; Heidmets et al., 2006; Nihei et al., 2000; Verina et al., 2007; Virgolini et al., 2005; Virgolini et al., 2006; Virgolini et al., 2008b), but are similar to what is found in recent human studies where neurocognitive deficits are reported (Dietrich et al., 2001; Lanphear et al., 2000; Nigg et al., 2008; Wright et al., 2008) and in a few animal studies (Cory-Slechta et al., 1985; Rice, 1985). This experiment is unique in that Pb was administered directly to the pups (via gavage), whereas in most animal studies Pb was administered indirectly (via maternal drinking water). Direct administration resulted in BPb levels within a somewhat narrower range compared with drinking water-based models and maternal Pb exposure was avoided, eliminating Pb effects on dam behavior. However, other indirect effects on the dams' behavior cannot be ruled out such as disturbance caused by removal of pups during gavaging. The present experiment introduces

a model for developmental Pb treatment and barren cage rearing that may be used in future investigations of these two independent variables.

Barren cage rearing was used as a chronic stressor in order to mimic conditions proposed as a risk factor in families where elevated BPb levels are frequently reported (Baum et al., 1999; Ivy et al., 2008; Rice et al., 2008). Both Pb exposure and chronic stress have been shown to be harmful in humans and animals (Cecil et al., 2008; Chiodo et al., 2007; Gilbert and Lasley, 2007; McEwen, 1998; Toscano and Guilarte, 2005). However, the extent to which they interact is unknown. This experiment sought to determine the effects of exposure to low level Pb and chronic stress during development in rodents at ages analogous to late gestation and early childhood on growth and developmental parameters and reactivity to an acute stressor (SWS).

Similar to other studies (Cory-Slechta et al., 2009; Rossi-George et al., 2009; Virgolini et al., 2006), the chronic stressor did not interfere with Pb pharmacokinetics on P29, as there was no difference in BPb levels between barren- and standard-reared rats. Whether rearing condition affects BPb at earlier ages was not determined. Pb has a long biological half-life, ~35 d in blood and decades in bone, in humans (Rabinowitz et al., 1976). Therefore, interactions of BPb levels may not occur until later when it is redistributed following cessation of exposure. It would also be of interest to examine BPb at earlier ages; one study has shown that BPb levels peak at P21 using a drinking water model (Zhu et al., 2005). An aspect that was not examined here is the ability of stress to alter the concentration of Pb in different brain regions. Future studies should consider this since we found regional differences in the effects of Pb and stress on neurotransmitters.

This experiment tested the effects that Pb and stress on organ weights associated with stress and immunity. Stress is a known modulator of the immune system (Dhabhar and McEwen, 1996; Dhabhar et al., 1997; Miller and O'Callaghan, 2002; Selye, 1937; Wilson and Warise, 2008), and Pb has been shown to have effects on immune function in humans (Lutz et al., 1999) and animals (Bunn et al., 2001a; Bunn et al., 2001b; Lee and Dietert, 2003; Struzynska et al., 2007; Talcott and Koller, 1983). Adrenal glands, an integral part of the HPA axis, were smaller in rats raised in barren compared with standard bedding, but Pb had no effect. Previous reports have shown that chronic variable stress in adult rats results in adrenal hypertrophy (Choi et al., 2008; Ulrich-Lai et al., 2006). Another study using barren-reared rats also demonstrated adrenal hypertrophy at P9 (Avishai-Eliner et al., 2001). However, unlike these studies, we did not see a stress-induced change in relative adrenal weight at a later age (P29), indicating that adrenal hypertrophy did not occur under these conditions or was no longer present by P29. We did note a reduction in absolute adrenal weight, as have others using corticosteroids (Hodges and Mitchley, 1970; Karatsoreos et al., 2010) or chronic noise stress (Alario et al., 1987). Another study investigating the effects of Pb and intermittent stress during later development found that neither of these factors had an influence on adrenal size (Virgolini et al., 2005). These differences may be the result of developmental versus adult stress, the types of stressors, or species used.

As in the adrenals, barren rearing decreased absolute and relative spleen weights as did 10 mg/kg Pb (latter was a trend only). The spleen has a prominent role in immune function, as well as serving as a reservoir for monocytes. Several studies have shown that stress results in decreased splenic weight or size (i.e., involutions) (Johnson et al., 2002; Miller et al., 1991; Pruett et al., 2007) and we show the same effect in juvenile animals. Pb has also been shown to decrease spleen weights (Massaro et al., 1984) and can accumulate in spleen (Sun et al., 2009). Using spleens cultured from mice exposed to Pb, it has been shown that exposed spleens have an altered balance between T helper type-1 cells (Th1) and Th2 activation, indicative of an autoimmune response (Heo et al., 1996). While a Sex ×

Treatment × Rearing interaction was noted for absolute spleen weight, no treatment differences within each sex or rearing condition were obtained. This suggests that the effects of Pb and stress are subtle but these data do not rule out possible effects on spleen function.

Pb and stress both decreased absolute and relative thymic weights. An interaction of age with stress was found as well, since barren-reared rats had significantly lighter thymi than standard-reared rats on P19. The thymus is an important component of the immune system responsible for T-cell maturation. Thymic atrophy is a well-known outcome following stress (Gruver and Sempowski, 2008; Solomon et al., 2010) whereas Pb has been reported to cause hypertrophy (Bunn et al., 2001b). However, thymic weight changes are only an index, as histopathological changes (i.e., cellularity) are more important in determining injury. Taken together with the spleen changes, these data suggest that animals exposed to stress and/or Pb early in development have altered immune responses.

Interactions between Pb and chronic stress were found on neurotransmitters. For example, HVA concentrations were elevated in standard-reared low Pb animals compared with the comparable barren-reared group. Moreover, barren-reared high Pb treatment elevated HVA levels compared to their controls or low Pb treatment. Despite the fact that there were no alterations in either DA or DOPAC, these data indicate that Pb and stress together alter aspects of DA utilization. HVA can serve as a marker of dopaminergic neuronal function (Cooper et al., 2003), primarily as an indication of changes in monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) activity. The variable effects that developmental Pb and stress have on DA, particularly the mesocorticolimbic system, have been examined previously (Cory-Slechta et al., 2004; Gedeon et al., 2001; Ma et al., 1999; Rossi-George et al., 2009; Virgolini et al., 2005; Virgolini et al., 2008a).

The serotonin metabolite 5-HIAA was elevated in the hypothalamus as a result of Pb treatment and stress, as was hippocampal 5-HT. In fact, multiple changes in 5-HT, 5-HIAA, and 5-HT turnover were seen. There is evidence that glucocorticoids influence brain 5-HT levels and 5-HT turnover by acting upon tryptophan hydroxylase (Chaouloff, 1993; Singh et al., 1992). As we have demonstrated that barren rearing alters the HPA axis, it is possible that this perturbation may contribute to the 5-HT changes. Furthermore, the exposure period used here spans part of hippocampal neurogenesis (Bayer et al., 1993), and the hippocampus is important for spatial learning and reference memory (Morris et al., 1982). Thus, alterations to this region could result in learning and memory effects. It has been shown that other treatments that alter hippocampal 5-HT during neonatal development result in spatial learning deficits (Morford et al., 2002; Schaefer et al., 2006).

While Pb had a significant effect upon neurotransmitters, it did not have direct effects upon the HPA axis, as reflected by CORT levels under basal levels and following SWS. Barren rearing, on the other hand, resulted in significant changes in CORT levels, both before and after SWS. At P11, barren rearing resulted in elevated CORT levels compared with standard rearing. While the latter had a typical response with CORT levels peaking immediately after SWS, acute stress did not alter CORT levels in barren-reared rats. Furthermore, barren-reared rats did not show the typical response to SWS, indicating that prior exposure to stress alters the ability of the HPA axis to respond to a subsequent stressor. This is not a ceiling effect as we have previously shown that P11 animals with drug-induced elevated CORT can respond with higher adrenal output (Williams et al., 2000). At P19, barren-reared rats had higher CORT levels following SWS than standard-reared animals. Moreover, CORT levels continued to rise 60 min following SWS. This dysregulation in negative feedback might indicate quantifiable changes in glucocorticoid receptor numbers in the hippocampus where GRs are abundantly expressed and are part of the HPA axis regulatory loop (Fietta, 2007; Herman et al., 2003; McEwen et al., 1968). Given that we found stress-induced changes in

hippocampal 5-HT and 5-HT plays a regulatory role in GR expression (Andrews and Matthews, 2004; Mitchell et al., 1992), this may be worth further investigation. While these effects were no longer apparent on P29, others have found that insults to the HPA axis early in development emerge later (Aisa et al., 2007; Skelton et al., 2007; Williams et al., 2003).

Unlike previous studies investigating the effects of developmental stress and Pb (Cory-Slechta et al., 2004; Cory-Slechta et al., 2009; Virgolini et al., 2005; Virgolini et al., 2006; Virgolini et al., 2008a; Virgolini et al., 2008b), we did not see an effect of Pb upon CORT levels. However, the studies differ from ours in terms of exposure. The current study used direct administration of Pb to the pups compared with drinking water to the dams. Furthermore, Pb was only administered during the postnatal period and not prenatally. It could be that Pb exposure earlier is required to alter CORT levels in the offspring. Finally, the CORT levels of the developmentally treated rats in the studies of Cory-Slechta and colleagues were oftentimes obtained later (2–8 months of age). It is possible that Pb-induced HPA axis changes only emerge at ages beyond when we measured.

The data suggest that chronic stress is more harmful than low level Pb exposure on the outcomes measured here, supporting the idea that at lower levels other factors may be important for understanding why some children with equal BPb levels are affected and others not. While low BPb levels comparable to humans were obtained, it is possible that higher BPb levels, longer exposures, or use of later time points is required to induce changes in the rat because of species differences in susceptibility. It is also possible that Pb and stress interacted on other systems not assessed here. For example, IL-6 has been shown to impact the HPA axis, in human tissue *in vitro* (Path et al., 1997) and *in vivo* in rodents (Naitoh et al., 1988; Navarra et al., 1991; Path et al., 2000). One study noted that adults of lower SES had elevated IL-6 levels (Brydon et al., 2004). Furthermore, Pb has been shown to induce neural inflammation in the form of increased glial fibrillary acidic protein (GFAP) (Struzynska et al., 2007). This is not surprising, as astrocytes are known to accumulate Pb in the brain (Lindahl et al., 1999). GFAP is also differentially regulated in response to stress (Barros et al., 2006; Kwon et al., 2008). Thus, examining IL-6 and GFAP following developmental Pb and stress may help elucidate combination effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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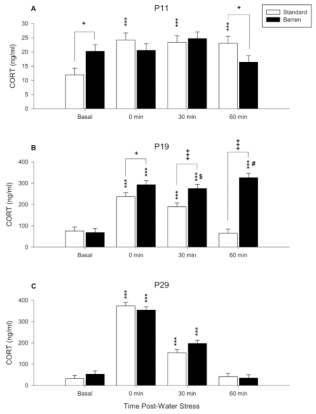

Figure 1.

Mean ± SEM plasma concentrations of CORT both before and after an acute stressor (SWS) at different ages: A) P11, B) P19, and C) P29. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. basal CORT levels within rearing conditions; bars indicate significant differences between rearing conditions $^{+}p < 0.05$, $^{+++}p < 0.001$ vs. basal levels; $^{\#}p < 0.05$ vs. 30 min; $^{\$}p < 0.05$ vs. 60 min.

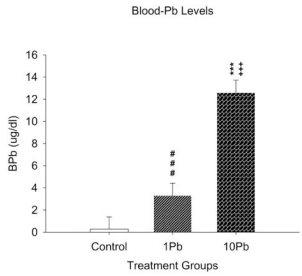

Figure 2.

Mean ± SEM blood Pb (BPb) levels on P29. No effects of chronic stress were found. $^{***}p < 0.001$ vs. vehicle control; $^{+++}p < 0.001$ vs. 1 mg/kg Pb; $^{###}p < 0.001$ vs. 10 mg/kg Pb.

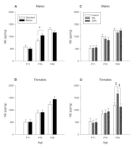

Figure 3.
Hypothalamic NE levels (pg/mg tissue) expressed as mean ± SEM as an effect of rearing condition in A) males and B) females in order to show the Sex × Rearing × Age effect. Effect of Pb exposure in C) males and D) females in order to show the Sex × Treatment × Age effect. $^{*}p < 0.05$, $^{**}p < 0.01$.

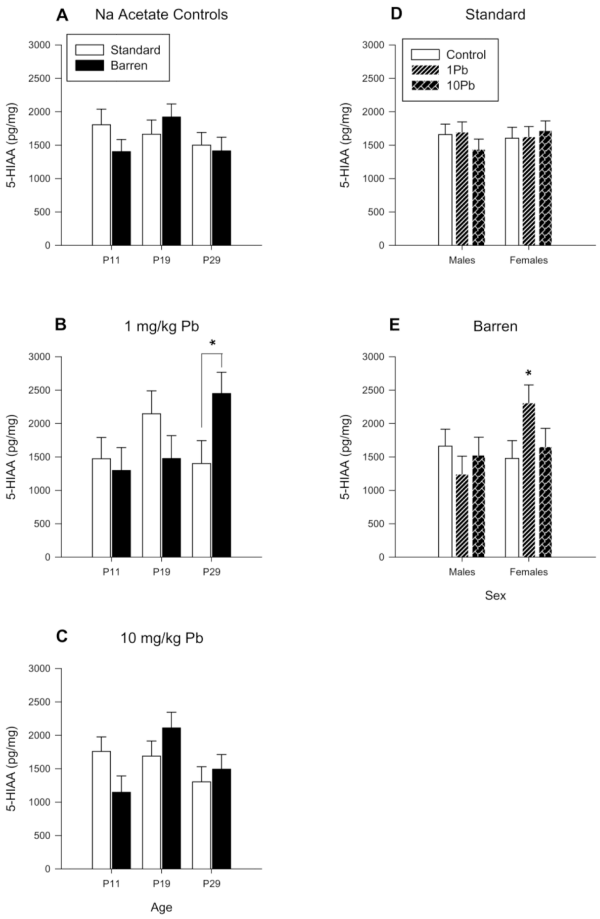

Figure 4.

Hypothalamic 5-HIAA levels (pg/mg tissue) expressed as mean ± SEM. Left, effect of rearing condition after A) Na acetate (control), B) 1 mg/kg Pb, or C) 10 mg/kg Pb on P11, P19, and P29 to show the Treatment × Rearing × Age effect. Right, effect of Pb treatment and sex in those reared in D) standard-reared and E) barren-reared conditions to show the Sex × Treatment × Rearing effect. $^{*}p < 0.05$ vs. respective vehicle control.

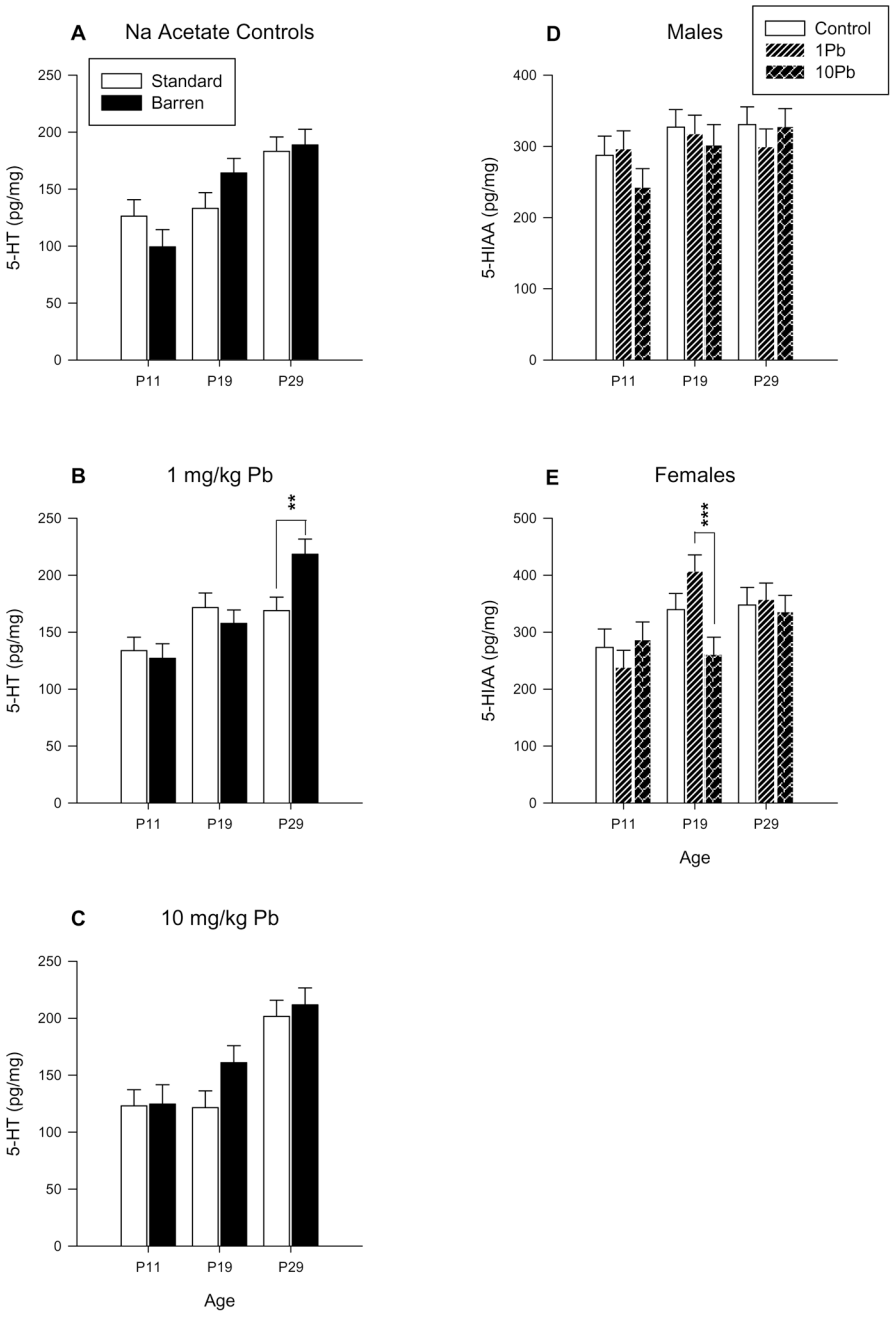

Figure 5.

Hippocampal 5-HT and 5-HIAA levels (pg/mg tissue) expressed as mean ± SEM. Left, 5-HT in each rearing condition following A) Na acetate, B) 1 mg/kg Pb acetate, and C) 10 mg/kg Pb acetate to show the Treatment × Rearing × Age effect. Right, hippocampal 5-HIAA levels for each treatment group at each age examined in D) males and E) females to show the Sex × Treatment × Age effect. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1

Mortality in each Sex × Treatment × Rearing × Age group.

		P11		P19		P29	
		Cage Rearing Conditions					
		Standard	Barren	Standard	Barren	Standard	Barren
Male	Control	0/32	0/32	0/32	0/32	0/32	0/32
	1Pb	1/32	0/32	0/32	3/32	0/32	0/32
	10Pb	2/32	3/32	0/32	4/32[a]	1/32	0/32
Female	Control	0/32	0/32	0/31	0/32	0/32	0/32
	1Pb	0/32	0/32	0/31	1/32	0/32	2/31
	10Pb	2/31	3/32	1/32	3/32	3/32	1/32

[a] denotes significance between both control-treated, barren-reared group as well as 10 mg/kg Pb-treated, standard-reared group within respective sex and age categories ($p < 0.05$).