Interactions of Chronic Lead Exposure and Intermittent Stress: Consequences for Brain Catecholamine Systems and Associated Behaviors and HPA Axis Function

Miriam B. Virgolini,* Kevin Chen,* Doug D. Weston,† Mark R. Bauter,† and Deborah A. Cory-Slechta*†

*Environmental and Occupational Health Sciences Institute, a Joint Institute of the Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey and Rutgers University, 170 Frelinghuysen Road, Piscataway, New Jersey 08854, and †Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, Rochester, New York 14642

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Elevated lead (Pb) burden and high stress levels are co-occurring risk factors in low socioeconomic status (SES) children. Our previous work demonstrated that maternal Pb exposure can permanently alter hypothalamic-pituitary-adrenal (HPA) axis function and responsivity to stress challenges in offspring. The current study sought to determine the consequences of chronic Pb exposures initiated later in development combined with variable intermittent stress challenges. Male rats were exposed chronically from weaning to 0, 50, or 150 ppm Pb acetate drinking solutions (producing blood Pb levels of <5, 9–15, and 23–27 µg/dl, respectively). Pb itself decreased basal plasma corticosterone, with greater effects at 50 than 150 ppm; 150 ppm reduced both cytosolic and nuclear glucocorticoid receptor binding. Responsivity to stress challenges including novelty, cold, and restraint, was measured as changes in Fixed Interval (FI) schedule-controlled behavior in a subset of rats within each group. FI performance was modified by novelty stress only in Pb-treated rats, whereas cold and restraint stress effects were comparable across groups. Novelty elevated corticosterone equivalently across groups, but cold stress markedly increased corticosterone only in Pb-treated groups. The pattern of Pb-induced changes in serotonin (5-HT) or its metabolite 5-HIAA in frontal cortex, nucleus accumbens, striatum, and hypothalamus resembled that observed for basal corticosterone levels indicating a relationship between these variables. In addition to suggesting the potential for HPA axis-mediated effects of Pb on the central nervous system, these findings also raise questions about whether single chemicals studied in isolation from other relevant risk factors can adequately identify neurotoxic hazards.

Key Words: lead; stress; HPA axis; catecholamines; corticosterone; Fixed Interval.

Although blood lead (PbB) levels of U.S. children have continued to decline since the removal of Pb from paint and gasoline in the 1970s, some segments of the population still remain disproportionately affected by this neurotoxicant. Levels of elevated blood Pb in children differ markedly in relation to race/ethnicity, income level, and residence type. The percentage of elevated blood Pb values in African-American children, for example, at 21.9%, is five times higher than that of the general population; for children from low-income families living in pre-1946 homes, residences that typify large U.S. inner cities, the corresponding figure is 16.4%, almost four-fold higher than the overall population (Pirkle et al., 1998). Thus, Pb exposure has become a demographically circumscribed public health problem, preferentially targeting low socioeconomic status (SES) inner city minority children who reside in old housing with Pb-based paints.

Low SES is itself a significant risk factor for numerous adverse health outcomes and various behavioral and neurological dysfunctions, even after controlling for other pertinent covariates. For example, risk of mortality, prevalence of disease, increased blood pressure, and cancer have all been shown to be inversely related to employment grade, occupational status, income, and/or years of education (Pincus et al., 1987). Low SES has also been associated with an increased prevalence of schizophrenia and depression (e.g., Dohrenwend, 1990). For children, adverse outcomes associated with low SES include vision problems, otitis media, hearing loss, cyanotic gallic inclusion disease, and iron deficiency anemia (Starfield, 1982). Lower SES children also evidence higher levels of mental retardation, learning disorders, emotional and behavioral problems, and deficits in language, memory, and attentional capacities (Anderson and Armstead, 1995).

This link between low SES and increased disease incidence has been attributed to the greater stresses associated with low SES environments that presumably results in a corresponding chronic elevation of glucocorticoids (Lupien et al., 2001). Indeed, like stress per se, chronic elevation of glucocorticoids has been reported to increase adverse health outcomes. Elevated stress levels have been associated with cardiovascular disease and death, chronic illness, altered immune function (Calabrese et al., 1987; Wyler et al., 1971), adverse effects on mood and cognitive function (Lupien and McEwen, 1997), anxiety and...
depression (Vinokur and Selzer, 1975), and poorer academic achievement. Similarly, chronic elevation of glucocorticoids increases resistance to insulin, and can lead to hypertension, hypercholesterolemia, arteriosclerosis, and immunosuppression (Munck et al., 1984). Circulating glucocorticoids can also act on central nervous system (CNS) steroid receptor sites, with prolonged elevation resulting in impaired cognitive function, hippocampal neuronal dysfunction, neuronal loss, and increased density of microglia (Sapolsky et al., 1986).

The co-occurrence of elevated Pb burden with higher levels of environmental stress raises the possibility of interactions of these risk factors. Further support for such a possibility derives from the fact that both Pb exposure and stress stimuli (through the hypothalamic-pituitary-adrenal [HPA] axis) act on dopamine/glutamate mesocorticolimbic systems of the brain (e.g., Pokora et al., 1996; Rouge-Pont et al., 1998). These common effects may serve as the basis for the fact that Pb and low SES similarly impair behavioral functions mediated by these systems (Anderson and Armstead, 1995; Schwartz, 1994).

Recent experimental studies in our laboratory confirmed Pb and stress interactions in offspring of rat dams subjected to maternal stress, maternal Pb exposure, or the combination (Cory-Slechta et al., 2004). In addition to gender-related differences in outcomes, these studies demonstrated that the profile of effects produced by combined maternal Pb and stress, as exemplified in levels of monoamines in various brain regions and associated behavioral processes, as well as altered HPA axis function and responsivity to stress challenges, differed notably from that produced by either Pb or stress alone. Moreover, maternal Pb exposure permanently altered plasma corticosterone levels, with increases in both male and female offspring when measured at nine months of age or later, with Pb exposure having ended at 21 days of age. Collectively, these findings underscore the critical need to consider interactions of relevant risk factors with environmental chemical exposures in defining human health hazards, particularly since environmental stress is by no means restricted to low SES populations.

The current study was designed to further elaborate the nature and potential mechanisms of Pb-stress interactions. It specifically asked whether such interactions are restricted to exposures mediated by the dam, or would also be observed when Pb exposures are initiated at a later period in development and when exposure is chronic in duration. In addition, the current study examined responsivity to variable intermittent stress challenges across the course of Pb exposure to model the combination of risks that characterize the human environment. A well-described postweaning Pb exposure model was utilized to examine the interaction of Pb with stress on a behavioral performance with well documented sensitivity to Pb (Fixed Interval schedule-controlled operant behavior), with outcome measures that included determinations of catecholamine levels in various brain regions, as well as alterations in HPA axis function and behavioral and corticosterone responses to varied intermittent stress challenges.

MATERIALS AND METHODS

Animals. Male Long-Evans rats, 21 days of age, were obtained from Taconic Farms (Germantown, NY) and randomly divided into three groups of equivalent mean body weights. They were provided with drinking water solutions containing 0 (n = 21), 50 (n = 20), or 150 ppm (n = 21) Pb acetate, an exposure protocol that continued for the duration of the experiment (approximately nine months). Rats were individually housed in a vivarium room maintained at 70 ± 2°F with a 12 h light-dark cycle and constant humidity and provided with unrestricted access to food and drinking solutions until reaching approximately 55–60 days of age. At that time, behavioral training was initiated, and rats were permitted to gain 3–5 g/day until body weights reached 300 g, where weights were stabilized through regulation of food availability. In our experience, the 300 g body weights are consistent with extended good health and the absence of excess body fat for this strain of rat. Body weights were recorded at least weekly until the initiation of behavioral testing, after which they were obtained prior to each behavioral test session. All procedures were carried out in accordance with NIH guidelines and with approval from the University of Rochester Animal Care and Use Committee where these experiments were carried out.

Pb exposure. Pb acetate was dissolved in distilled deionized water for drinking solutions and prepared fresh on a weekly basis as in our previous studies (Cory-Slechta et al., 2004).

Experimental sequence and rationale. The sequence of manipulations and measures carried out across the nine months of the experiment are shown in Figure 1. Blood Pb (PbB) measurements were made at three different time points during the course of the experiment, using different subsets of rats from each treatment group at each time point. Because PbBs obtained from the first time point of measurement (one month of exposure) were higher than intended, the diet was switched from a semipurified diet (with lower levels of metals such as Ca that compete with Pb for oral uptake) to a regular breeding diet (after two months of exposure) for the remainder of the experiment. Additional PbB determinations were carried out at 3 and 8.5 months of exposure. These time points were based on the half-life of Pb in blood (approximately one month) and the intention of determining levels across the course of the experiment. All blood collections for these determinations, obtained from tail nicks, were carried out where applicable on Friday afternoons and after behavioral testing to allow a weekend for recovery.

Training on the Fixed Interval (FI) schedule of reinforcement began after approximately two months of Pb exposure and evaluation of FI performance continued for the duration of the experiment. It was preceded by an approximate two-week period during which overnight sessions in the operant

FIG. 1. Sequence of experimental determinations across the course of the nine-month Pb exposure. It shows the three time points of measurement of blood Pb, the change of diet from semipurified to regular lab chow, the measurement of basal corticosterone, the initiation of FI schedule-controlled behavior testing, and the imposition of stress challenges and post-stress determinations of corticosterone.
chambers took place using automated procedures to shape the lever press response in all rats in all treatment groups. This typically involves one overnight session for each rat.

Blood from a tail nick was obtained for determination of basal corticosterone values after approximately five months of Pb exposure, and prior to the imposition of pre-FI session stress challenges. This was carried out on a Friday afternoon and after behavioral test sessions to provide a weekend for recovery from any associated stress. Basal corticosterone levels were determined at five months of Pb exposure so as to be determined as close in time as possible to the initiation of variable stress challenges.

The stress challenge component was intended to be both intermittent and variable, to try to mimic the human environment and to prevent adaptation or habituation through repetition of the same stressor. The specific stressors were chosen because they are widely used in the experimental literature, as well as having been used in our previous study of maternal Pb and stress (Cory-Slechta et al., 2004). Restraint stress was used as the final challenge as it tends to be a stronger stressor than either cold or novelty and as such could make determinations of group differences more difficult to detect. Moreover, if, as a strong stressor, it produced some conditioning effects, these might be conveyed to subsequent stress challenges if it occurred first in the sequence. Each stress challenge was presented only once to an animal.

The first pre-FI session stress challenge involved novelty (measurement of locomotor activity) and was imposed approximately two weeks following determination of basal corticosterone (week 12 of FI testing). All rats in all treatment groups were subjected to novelty stress. Cold stress (39°F for 20 min) was imposed during week 18 of FI testing and a 45 min restraint stress occurred before a session during week 22. Only half of the rats in each Pb treatment group were subjected to cold and restraint stress, while the remaining half provided corresponding control values. Rats were randomly selected within each treatment group for this purpose, with the stipulation that group mean baseline FI overall response rates for the stressed vs. non-stressed sub-groups were equivalent. The same rats in each treatment group were subjected to both the cold and restraint stress challenges.

Tail blood was collected within 15 min after the FI session that followed the novelty stress and the cold stress for determinations of stress-induced corticosterone levels. Corticosterone was not determined following restraint stress as, in our experience this stressor yields extremely high levels of corticosterone relative to other stressors we have used and thus hinders the ability to detect treatment-related differences, while both novelty and cold stress produce milder elevations by comparison. The time required for the pre-FI session stress challenge as well as for the subsequent FI test session and blood collection was designed to encompass the several hour window during which corticosterone levels are elevated following stress challenges (Paris et al., 1987). All rats were sacrificed approximately one month after the final stress challenge and various brain regions and adrenal glands harvested for determinations of catecholamine levels and glucocorticoid receptor (GR) expression in hippocampus.

Fixed Interval performance. Lever press responding was shaped in overnight sessions using automated procedures developed in our laboratory. Subsequently, an FI 1 min schedule of reinforcement was imposed. On this schedule, a 45 mg food pellet delivery followed the first lever press response occurring at least 1 min after the preceding food delivery, with responses occurring during the 1 min interval itself having no programmed consequences. Food delivery (reinforcement) also initiated the next 1 min fixed interval. Sessions ended following the completion of the 1 min interval in progress 20 min after the session began, or after a total of 22 min, whichever occurred first. Behavioral sessions were carried out 5–6 days per week between 10:00 a.m. and 5:00 p.m.

Standard performance measures were computed from each session for each rat: (1) overall response rate, i.e., total number of responses divided by total session time; (2) mean postreinforcement pause time, i.e., the mean time to the occurrence of the first response in an interval; and (3) mean running rate, i.e., the rate of responding calculated with the postreinforcement pause time subtracted out.

Stress challenges. Three pre-FI session stress challenges were carried out as noted above. Novelty stress involved measurement of locomotor activity for 10 min in automated locomotor activity chambers, an environment with which subjects had no prior experience. For the cold stress challenge, rats were placed, in their home cages, in a cold room with a temperature of 39°F for a total duration of 20 min. The final stress challenge involved a restraint stress in which rats were placed in a plastic transparent cylindrical device (IITC restrainer model 81; 7 cm diameter, 19 cm long; IITC Life Sciences, Woodland Hills, MA) for a period of 45 min. Immediately after the stress challenges, rats were placed in the operant chambers and the FI schedule of reinforcement was initiated. Non-stressed rats remained in home cages until transported for FI testing. The data from FI sessions preceded by stress challenges are plotted as a percent of the mean of the two preceding non-stress FI sessions.

Apparatus. FI performance was measured in operant chambers (Coulbourn Instruments, Inc., Model E10-10) housed in individual sound-attenuated boxes. Each chamber was equipped with three levers located 9 cm above the grid floor. Only the right lever was active in these experiments. Reinforcement consisted of the delivery of 45 mg food pellets (P.J. Noyes, Inc.). Behavioral responses were programmed and recorded using the SKED-11 operating system on a PD-11 Digital Equipment computer with a resolution of 10 milliseconds.

Automated locomotor activity chambers (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity for novelty stress. Each chamber was equipped with infrared photoeams (3 mm in diameter) separated by 24.4 mm on a horizontal plane 39 mm from the floor of the chamber. A second set of photoeams that divided the chamber vertically were located 57 mm above the horizontal photoeams. Photoeams breaks were recorded at 1 min intervals for 10 min for determination of horizontal, vertical, and ambulatory movements.

Catecholamine determinations. Levels of dopamine (DA) and its associated metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as serotonin (5-HT) and its metabolite 5-HIAA, and norepinephrine (NE) and epinephrine (E) were variously measured (depending upon sensitivity) in frontal cortex, nucleus accumbens, striatum, hypothalamus, and adrenal cortex. Following rapid decapitation, sections were dissected and placed in 0.1 N perchloric acid. The tissues were sonicated and centrifuged twice for 20 min at 10,000 rpm. The supernatants were stored at –80°C until analyzed for the concentrations of DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA), epinephrine (E), and norepinephrine (NE) by HPLC-EC. The pellets were digested in 1 ml of 0.5N NaOH for measurements of protein concentration using Bio-Rad assay reagents. For HPLC-EC analysis, samples were loaded onto a Waters 717 plus autosampler (Waters, Milford, MA) and the mobile phase was delivered at a constant rate of 1 ml/min using a Waters Model 510 pump through a C18, 5 mm, 250 mm × 4.6 mm analytical column (Alltech, Deerfield, IL) placed in a column heater (35°C). The LC amperometric potential was set to 0.75 V with reference to an Ag-AgCl reference electrode and the sensitivity of the detector was modified according to the content of amines in the sample. The mobile phase consisted of 0.1 M monosodium phosphate, 0.15 mM EDTA, 2 mM NaCl, 1.4 mM octyl sodium sulfate, and 12% methanol. The signal from the detector was recorded and the data was analyzed using a Waters Millennium Chromatography Manager. The concentrations of the neurotransmitters were expressed in terms of mg/mg protein. DA turnover was calculated using intracellular (DOPAC/DA), extracellular (HVA/DA), and combined intracellular and extracellular metabolites (DOPAC + HVA/DA).

Corticosterone determinations. For serum corticosterone determinations, blood was obtained following tail nicks and measured by competitive enzymeimmunoassay using a rabbit polyclonal corticosterone antibody (Octeia Corticosterone; Alpco Diagnostics, American Laboratory Products). Sensitivity of the assay is 0.25 ng/ml.

Blood Pb determinations. Blood Pb levels were analyzed using anodic stripping voltammetry as in our previous studies (Cory-Slechta et al., 2004). Whole blood samples were obtained by a small incision in the tail and collected...
in metaexchange reactive tubes (Environmental Science Associates, Inc.). The limit of sensitivity of the assay is 5 µg/dl.

Glucocorticoid Receptor Level Determinations

**Tissue preparation and protein extraction.** For the determination of cytosolic and nuclear GR receptor levels, hippocampus was quickly dissected on an ice plate, immediately snap frozen on dry ice, and stored at −80°C. On the day of the experiment, the tissue was homogenized in a temperature-controlled manner (4°C) according to procedures described by Spencer et al. (2000) with modifications.

Tissue was processed in a solution of 0.5 ml buffer/100 mg tissue using a hand-held 2 ml dounce glass-on-glass grinder in homogenization buffer consisting of 6 mM MgCl₂, 1 mM EDTA, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml antipain, 1 µg/ml aprotinin, 1 µg/ml soybean trypsin inhibitor, and 50 mM Tris at a final pH of 7.2. The homogenate was centrifuged at 2000 × g for 5 min in a refrigerated centrifuge. The resulting supernatant and pellet were further processed to obtain the cytosolic and nuclear fractions, respectively. The supernatant was ultracentrifuged at 105,000 × g for 30 min yielding the cytosolic fraction. For the nuclear extract, the pellet from low speed centrifugation was re-suspended twice in 0.25 ml of homogenization buffer containing 0.5 M NaCl, incubated in an ice bath for 1 h with frequent shaking, and finally centrifuged at 8000 × g for 10 min. The resulting supernatant was collected as the nuclear extract. Protein levels were determined in an aliquot of each fraction using a commercially available Bio-Rad kit. Protein concentrations ranged from 4.5 to 7.5 mg/ml for cytosol and 2.0 to 4.0 mg/ml for the nuclear preparation.

**Western blotting.** Sample loading was counterbalanced across groups and adjusted to a final protein concentration of 30 and 60 µg/protein for cytosol and 40 and 80 µg/protein for nucleus. Samples were denatured by mixing and boiling with 100 µl of sample containing 10% SDS, 2.5 M sucrose, 0.2 M Tris, 0.02 M EDTA, pH 8.0, 10% 2-mercaptoethanol and bromophenol blue. Proteins were separated by electrophoresis on 3% Bis-polyacrylamide gels and adjusted to a final protein concentration of 30 and 60 µg/ml for the nuclear preparation. The membranes were then washed with TBS-TX for 35 min and proteins visualized on X-ray film (X-Omat AR Autoradiography film, Kodak, Rochester, NY) using enhanced chemiluminescence reagents (West-Pico substrate for cytosol and West-Femto for nucleus; Pierce Biotecnoity Inc., Rockford, IL). The bands obtained were compared with Kaledioscope prestained standard (BioRad Laboratories, Hercules, CA) to ensure that the most prominent band visualized corresponded to 97 KDa representing GR molecular mass.

Semi-quantitative densitometric analysis of GR signals was carried out using Scion, a Macintosh-driven imaging processing software. Relative measurements of GR protein levels were determined in the darkest region of the band with the background intensity of the film subtracted-out.

**Statistical analyses.** Body weights of treated groups were compared across time using repeated measures ANOVA (RMANOVA) with Pb exposure as a between-groups factor and time as a within-groups factor. Fixed Interval schedule-controlled behavior was also evaluated using RMANOVA, with Pb exposure as a between-groups factor and session as a within-groups factor. In cases of significant main effects or interactions, subsequent one-way ANOVAs were carried out as appropriate to further define the nature of the effect.

One-way ANOVAs with Pb exposure as the between-groups factor were used to evaluate differences in PbB, basal corticosterone levels, and the impact of novelty stress on FI schedule-controlled behavior. One way ANOVAs rather than RMANOVAs were used for PbB data since different subsets of rats were used at each of the three different time points of measurement. In the event of main effects, Fisher’s post-hoc assessments were utilized to further define the nature of the Pb-related differences.

Two factor ANOVAs were used for evaluating the effects of cold and restraint stress challenges on FI schedule-controlled behavior, on cold-stress induced changes in corticosterone levels, on levels of various neurotransmitters in brain regions and adrenal cortex, and on GR densitometry. In these analyses, Pb exposure and stress challenge served as between-groups factors. In the event of main effects or interactions, subsequent one-factor ANOVAs were carried out as appropriate to further define the nature of the effect. For all analyses, a p < 0.05 was considered statistically significant.

**RESULTS**

**Body Weights**

Group mean ± SE body weights, as recorded at 21, 28, 36, 41, 50, 100, and 275 days of age, are presented in Table 1 for the three Pb exposure groups. Body weights increased across time until reaching 300 g (by 100 days of age), where they were stabilized. RMANOVA confirmed a significant interaction of Pb by time (F(12,366) = 2.09, p = 0.017) which subsequent one way ANOVAs carried out at each time point showed was due to

<table>
<thead>
<tr>
<th>Pb (ppm)</th>
<th>Days of age</th>
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<tr>
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<td>21</td>
</tr>
<tr>
<td>0</td>
<td>46.7 ± 1.1</td>
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<tr>
<td>50</td>
<td>48.3 ± 1.2</td>
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<td>150</td>
<td>45.0 ± 1</td>
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*n = 21, 20, and 23, for the 0, 50, and 150 ppm groups, respectively.

*Significantly greater than corresponding 0 ppm body weight.
increases in body weights of the 50 ppm group relative to the 0 ppm group at 41 days of age (218.3 g vs. 205.6 g). No other Pb-related changes in body weight were found at any time point.

**Blood Pb Levels**

Blood Pb levels (PbBs) measured after 1, 3, and 8.5 months of Pb exposure are presented in Figure 2. As can be seen, significant Pb exposure-related increases were observed at all three time points (F(2,20) = 74.4, p < 0.0001; F(2,23) = 68.7, p < 0.0001; F(2,20) = 84.7, p < 0.0001 for the 1, 3, and 8.5 months time points, respectively), and in each case, levels of both the 50 and 150 ppm groups exceeded controls, and levels of the 150 ppm group were significantly higher than those for the 50 ppm group. At the one-month determination, rats had been maintained on a semipurified diet which resulted in PbBs higher than intended. Consequently, all rats were switched to a regular laboratory chow diet, and, as expected, PbBs had decreased to the preferred levels by the three-month time point. Thus, 50 ppm exposures were associated with PbBs of 9–15 μg/dl and 150 ppm with 23–27 μg/dl from three months of exposure until the termination of the experiment.

**Baseline FI Performance**

As in our previous studies (Cory-Slechta et al., 1985), postweaning Pb exposure increased baseline FI overall response rates (top row) in a concentration-dependent manner, as can be seen in Figure 3. Assessment of FI performance prior to the imposition of the first pre-FI session stress challenge (left column; weeks 1–12) shows that increased overall response rates emerged after approximately 10 weeks (50 sessions) on the schedule (Pb × time, F(20,590) = 1.96, p = 0.008) with increases of approximately 10–23% in the 50 ppm group and 27–34% in the 150 ppm group across weeks 10–12 relative to the 0 ppm group. These increases in overall rates were the result of significantly shorter postreinforcement pause times (bottom row; Pb × time, F(20,590) = 2.45, p = 0.0005), with reductions ranging from 6–17% in the 50 ppm group and 17–28% in the 150 ppm group relative to control during weeks 10–12. No significant changes in run rate (middle row) were found during this period.

While overall response rates of the groups began to merge following imposition of the novelty stress challenge (top row; weeks 13–18), the Pb-related reductions in postreinforcement pause time generally persisted across the duration of the experiment. Significant effects of Pb alone on postreinforcement pause time were found for weeks 13–18 (F(2,59) = 3.61, p = 0.033), with similar magnitudes of reduction as seen prior to the novelty challenge. With the further subdivision of each treatment group following the cold stress (week 18, stressed vs. non-stressed), Pb-based differences were marginally significant in RMANOVAs thereafter, i.e., during weeks 19–22 and 23–24.

**Pre-FI Session Stress Challenges**

Changes in FI performance in sessions preceded by stress challenges are shown in Figure 4. While novelty stress (top panel) had no impact on control rats, it did modify behavior of Pb-treated groups. Specifically, locomotor activity had no influence on either response rate measures or postreinforcement pause time in control rats, but significantly decreased response rates and lengthened the postreinforcement pause times in a concentration-dependent manner in Pb-treated groups (overall rate: F(2,55) = 4.4, p = 0.017; run rate: F(2,60) = 3.61, p = 0.033; postreinforcement pause time: F(2,60) = 17.0, p < 0.0001). Decreases in overall rates and run rates of approximately 10–12% were observed at 50 ppm and 13–24% at 150 ppm. Increases in postreinforcement pause times were statistically significant only in the 150 ppm group, and were approximately 30%.

Cold stress challenge preceded the FI session during week 18 (middle panel). In contrast to the effects of Pb uncovered by novelty stress, the cold stress, while decreasing response rates by approximately 10% (stress: F(1,55) = 6.02, p = 0.017) and increasing postreinforcement pause times by 20–30% (stress: F(1,56) = 11.52, p = 0.001) did not affect Pb groups differentially.

During week 22, a restraint stress was imposed prior to the FI session (bottom panel). This stress challenge resulted in dramatic reductions in both overall rates (F(1,55) = 91.4, p < 0.0001) and run rates (F(1,55) = 94.0, p < 0.0001) on the order of 55–85%. In the case of run rates, these reductions were also influenced by Pb exposure, such that combined no stress + stress Pb groups had significantly greater reductions than did the combined no stress + stress control group. Corresponding to the reductions in response rates on the FI schedule, marked increases in postreinforcement pause time were produced by restraint stress (F(1,56) = 11.52, p = 0.001) ranging from approximately 200–400%.

![FIG. 2. Group mean ± SE blood Pb levels across the three Pb exposure groups as determined at 1, 3, and 8.5 months of exposure. Diet was switched from semipurified to regular lab chow between the first and second blood Pb determination. Sample sizes were 8–9 for each treatment group for each time point. *Differs from corresponding 0 ppm; #differs from 50 ppm.](image-url)
statistically equivalent across Pb exposure groups, they appear to be larger in the 50 ppm Pb exposure group.

**Corticosterone Levels**

Basal corticosterone levels were determined at approximately five months of exposure, prior to the imposition of the first stress challenge, and corresponding levels are shown in Figure 5. Postweaning Pb exposure decreased basal corticosterone levels ($F(2,58) = 3.46, p = 0.038$), with reductions resulting in a U-shaped concentration effect curve. This was the result of significant decreases of 27% in the 50 ppm group and of 15% reductions in the 150 ppm group that were only marginally different from control.

While novelty stress increased corticosterone levels, it did so equivalently across Pb-treated groups. In contrast, a notable interaction between Pb and stress was found for corticosterone levels following cold stress (Pb: $F(2,55) = 6.37, p = 0.003$; stress: $F(1,55) = 17.31, p = 0.0001$; Pb × stress: $F(2,55) = 3.37, p = 0.042$). This was due to the prominent increases in corticosterone levels produced by cold stress in the 50 and 150 ppm treated groups, on the order of 74–77% of corresponding non-stress Pb values, in the absence of any significant increase (7%) in controls that underwent the cold stress challenge.

**CNS Catecholamine Determinations**

Changes in levels of catecholamines in frontal cortex are shown in response to Pb exposure and stress challenges in Figure 6. Pb exposure per se reduced levels of 5-HT in a concentration-dependent fashion ($F(2,56) = 5.59, p = 0.006$), with reductions of 16–18% in the 50 ppm group and 21–30% in the 150 ppm group. Reductions in NE and in DOPAC turnover were also seen ($F(2,56) = 3.97, p = 0.025$ and $F(2,56) = 3.19, p = 0.049$), which in both cases reflected differences between the 50 and 150 ppm groups based on slight increases at 50 ppm and slight decreases at 150 ppm. Pb exposure increased the 5-HT metabolite 5-HIAA ($F(2,56) = 7.16$, $p = 0.0003$).
an effect due to an increase observed at 50 ppm in comparison to both 0 and 150 ppm (from 18–61%). Interactions of Pb and stress were found in alterations in the DA metabolite, DOPAC (Pb: $F(2,52) = 3.4$, $p = 0.041$; stress: $F(1,52) = 4.34$, $p = 0.042$; Pb x stress: $F(2,52) = 2.62$, $p = 0.083$). This was due to the fact that control rats showed a substantial reduction in DOPAC levels following stress ($\approx 45\%$), whereas no reductions were seen in the 50 ppm group ($\approx 9\%$), and reductions were blunted in the 150 ppm group ($\approx 25\%$).

Analyses of catecholamine levels in nucleus accumbens revealed several Pb by stress interactions related to the DA metabolite, DOPAC (Pb: $F(2,55) = 3.76$, $p = 0.03$; DOPAC turnover, $F(2,55) = 3.24$, $p = 0.047$; and DOPAC + HVA turnover, $F(2,54) = 3.06$, $p = 0.055$), all of which showed the same pattern of effect (Fig. 7). Specifically, these were the result of substantial decrements in levels of DOPAC in the 50 (32–71%) and 150 ppm (25–56%) non-stressed groups relative to non-stressed control, coupled with the absence of a stress-related reductions in either the 50 or 150 ppm groups ($+9\%$), and reductions were blunted in the 150 ppm group ($-25\%$).

Analyses of catecholamine levels in nucleus accumbens revealed several Pb by stress interactions related to the DA metabolite, DOPAC (Pb: $F(2,52) = 3.4$, $p = 0.041$; stress: $F(1,52) = 4.34$, $p = 0.042$; Pb x stress: $F(2,52) = 2.62$, $p = 0.083$). This was due to the fact that control rats showed a substantial reduction in DOPAC levels following stress ($-45\%$), whereas no reductions were seen in the 50 ppm group ($+9\%$), and reductions were blunted in the 150 ppm group ($-25\%$).

Analyses of catecholamine levels in nucleus accumbens revealed several Pb by stress interactions related to the DA metabolite, DOPAC (Pb: $F(2,55) = 3.76$, $p = 0.03$; DOPAC turnover, $F(2,55) = 3.24$, $p = 0.047$; and DOPAC + HVA turnover, $F(2,54) = 3.06$, $p = 0.055$), all of which showed the same pattern of effect (Fig. 7). Specifically, these were the result of substantial decrements in levels of DOPAC in the 50 (32–71%) and 150 ppm (25–56%) non-stressed groups relative to non-stressed control, coupled with the absence of a stress-related reductions in either the 50 or 150 ppm groups that was seen in the control group. (Levels of detection were not sufficient to produce reliable measures of NE or 5-HIAA in this region.)

In striatum (Fig. 8), changes in catecholamines were largely driven by Pb exposure per se. Pb exposures reduced levels of DA ($F(2,56) = 22.4$, $p < 0.0001$), HVA ($F(2,54) = 3.58$, $p = 0.035$), and 5-HT ($F(2,52) = 22.7$, $p < 0.0001$) by 26% (50 ppm HVA) to 59–65% (500 ppm, DA and 5-HT). Prominent increases were seen in other catecholamines in response to Pb.
Most notable were increases in DOPAC turnover, HVA turnover, and DOPAC + HVA turnover, which were seen at 150 ppm and approximated 700% (F(2,55) = 16.1, p < 0.0001, F(2,55) = 16.6, p < 0.0001; F(92,52) = 14.8, p < 0.0001, respectively). Concentration-related increases were seen in 5-HIAA (F(2,55) = 13.16, p < 0.0001) with elevations of 64 and 216% for 50 and 150 ppm exposures, respectively. Only one Pb stress interaction was noted in striatum, for 5-HT (F(2,52) = 4.35, p = 0.018), which consisted of an attenuation by cold stress of the reduction in 5-HT levels produced by 150 ppm Pb exposure, as well as a lower level of 5-HT in 50 ppm treated rats compared to the corresponding non-stress 50 ppm group. (Levels of detection were not sufficient to produce reliable measures of NE in this region.)

Corresponding changes in hypothalamus are presented in Figure 9. Like striatum, effects in hypothalamus were primarily related to Pb exposure and not modified by the stress challenge experience. In hypothalamus, reductions were observed in DA (F(2,55) = 7.63, p = 0.001), NE (F(2,54) = 21.18, p < 0.0001) and 5-HT (F(2,55) = 46.17, p < 0.0001) that ranged from 31–52% in the 150 ppm Pb group, while reductions of 22% were seen in HVA turnover in the 50 ppm group (F(2,53) = 7.56, p = 0.0013). The higher Pb exposure concentration also resulted in marked increases in some catecholamines, including DOPAC (F(2,55) = 12.53, p < 0.0001), 5-HIAA (F(2,54) = 29.7, p < 0.0001), DOPAC turnover (F(2,54) = 55.8, p < 0.0001), and DOPAC + HVA turnover (F(2,53) = 7.56, p = 0.0013) that ranged from 172–305%.

**Adrenal Cortex Catecholamines**

Levels of NE and E in adrenal cortex are depicted in Figure 10. While marginal Pb-related reductions were observed in E, Pb by stress interactions were found in NE (Pb x stress: F(2,56) = 5.29, p = 0.008). This interaction was the result of differential changes in the 50 ppm group. While reductions in NE were produced by 50 ppm Pb alone, no such reductions were seen in 50 ppm Pb-treated rats that also experienced stress challenges. Adrenal cortex weights were virtually unaffected.
by either Pb or stress, with group mean ± SE mg values of: 17.8 ± 1.12 for 0/NS, 18.9 ± 1.06 for 0/S, 18.7 ± 0.77 for 50/NS, 18.95 ± 0.85 for 50/S, 18.7 ± 1.26 for 150/NS, and 19.0 ± 1.2 for 150/S.

**Hippocampal Glucocorticoid Receptor Density**

Changes in GR immunoreactivity and density in hippocampal nuclear and cytosolic fractions are shown in Figure 11, panels A and B, respectively. Pb exposure tended to reduce levels of both nuclear and cytosolic GR receptors at the 150 ppm exposure, although effects of Pb in the statistical analysis were marginally significant and observed only for the nuclear fraction (F(2, 41) = 2.77, p = 0.075), with subsequent post-hoc evaluations confirming a significant difference between the 0 and 150 ppm groups (p = 0.029). Western blots (panel C) depict these reductions in the 150 ppm group. Although these decrements appeared to be larger in the 150 ppm Pb group that underwent stress relative to the 150 ppm non-stress group, no Pb by stress interactions were found in the statistical analyses.

**DISCUSSION**

The current findings clearly demonstrate that interactions between Pb and environmental stress are not restricted to maternally mediated exposure, since Pb treatment beginning only at weaning and continuing well into adulthood in this study also resulted in Pb-stress interactions. Such interactions included: (1) modification of stress responses by Pb (stress-induced responses observed in controls but not in Pb groups), as observed for DOPAC, DOPAC turnover, and DOPAC + HVA turnover in nucleus accumbens (Fig. 7); (2) modification of Pb effects by stress (Pb effects not seen in the Pb + stress groups), as observed for 5-HT in striatum (Fig. 8) and NE in adrenal cortex (Fig. 10); as well as (3) potentiated effects, i.e., Pb + stress effects that occur in the absence of an effect of either variable alone, here observed in the form of a notable cold-stress induced increases in corticosterone levels observed in Pb-treated but not control rats (Fig. 5).

Postweaning Pb exposure per se altered both HPA axis function and stress responsivity. As with maternal Pb exposure...
Cory-Slechta et al. (2004), the outcomes differed with different stress challenges. With respect to behavioral responsivity to stress, novelty stress imposed before behavioral testing impaired FI performance in Pb-treated, but not control rats. In contrast, the subsequent cold and restraint stress challenges, while evoking FI performance changes, did not do so differentially in Pb-treated groups. It is possible that this differential outcome reflects some type of adaptation in Pb-treated groups; tolerance to repeated stress has certainly been described in the stress literature (McEwen, 2001). At least two alternative explanations are also possible. One is that novelty stress may be considered a psychological stressor, while both cold and restraint stress challenges reflect more of a physical stress challenge. Different forms of stress clearly evoke different neurochemical and glucocorticoid responses (Van de Kar and Blair, 1999). Unclear from the current experimental design, however, is whether this Pb-related alteration in behavioral responsivity to novelty stress reflected its sequence in the experimental design (the first stress) and/or its salience as a stimulus.

A contrasting pattern of Pb-induced alterations was noted for HPA axis function as indicated by changes in levels of corticosterone following stress. In this case, all three groups displayed generally equivalent increases in plasma corticosterone following novelty stress, the first stress challenge, whereas, Pb-treated, but not control rats, exhibited marked elevations in corticosterone in response to a subsequent challenge with cold stress (Fig. 5). Such findings could be indicative of a sensitization of the HPA axis response by Pb exposure, as has been noted in other cases of repeated stress (Servatius et al., 1994). Together, these findings show that stress-induced changes in behavior and HPA axis function can be dissociated. Future experiments using varied order of stressor presentations as well as of salience of the stressors coupled with measurement of corticosterone levels will be required to address questions related to adaptation vs. sensitization of these changes in response to Pb exposures.

Both the effects of Pb itself on stress systems, as well as its ability to modify stress responsivity and HPA axis function are interesting to consider in the context of schizophrenia.
Current studies suggest that exposures to various insults during pregnancy can lead to fetal stress and corresponding increases in fetal glucocorticoid exposure. This is proposed to yield a “schizotaxic phenotype” upon which later environmental stresses act to produce full-blown schizophrenia with its associated psychosis in later adolescence. Consistent with this hypothesis, schizophrenics exhibit altered HPA axis function, elevated basal cortisol levels (Breier and Buchanan, 1992), abnormalities of diurnal changes in cortisol release (Van Cauter et al., 1991) and in negative feedback functions of the HPA axis (Tandon et al., 1991). Further, associations between cortisol release and both symptom severity and prognosis are reported in schizophrenics (Kuhs and Folkerts, 1995). Antipsychotics used to treat schizophrenia reduce cortisol release in patients (Tandon et al., 1991), and drugs that increase cortisol release also worsen the symptoms of schizophrenia (Lucas et al., 1990).

Pb exposure and schizophrenia also both act on brain DA systems. Interactions between the HPA axis and the mesocorticocorticolimbic DA pathway have been extensively documented for regions that include prefrontal cortex, nucleus accumbens, ventral tegmental area, and septohippocampus (Rouge-Pont et al., 1998). Postweaning Pb exposure effects include reductions in levels of D2 DA receptor and DA transporter binding in nucleus accumbens that persisted over the course of 12 months of exposure (Pokora et al., 1996) and increased evoked DA release measured using in vivo electrochemistry (Zuch et al., 1998). Pb exposures beginning at parturition caused an impairment of receptor-mediated regulation of DA synthesis in nucleus accumbens but not in caudate putamen (dorsal striatum), and decreased concentrations of DA metabolites in nucleus accumbens and ventral tegmental area (Lasley and Lane, 1988). More recently, Pb exposures beginning at 2–6 days of age were found to be associated with augmented levels of nucleus accumbens DA release in response to KCl perfusion in microdialysis studies (Devoto et al., 2001).

Moreover, like elevated Pb burden, the risk for schizophrenia is higher in low SES populations as reported for several industrialized countries (Warner, 1995). Studies from Denmark and England support the assertion that lower SES can increase the risks for schizophrenia (Byrne et al., 2004) and a U.S. study reported that lower SES status was associated with greater symptom severity levels (Brown et al., 2000).

**FIG. 9.** Group mean ± SE values for various neurotransmitters in hypothalamus in relation to Pb exposure level in subgroups of rats that had experienced no stress (NS) or cold and restraint stress (S). Pb = main effect of stress in the ANOVA, with subsequent post-hoc effects as indicated. Sample sizes of 10, 10, and 10–11 for the 0, 50, and 150 ppm NS groups, and 9–10, 9–10, and 10–11, respectively, for the corresponding S groups.
Collectively, these similarities raise questions as to whether elevated Pb body burden could contribute to the risk of either the etiology (e.g., via maternal exposure) or the progression of schizophrenia through alterations in HPA axis function. In accord with such a possibility is a recent report now suggesting that prenatal Pb exposure increases the risk of schizophrenia (Opler et al., 2004). Interestingly, in the current study the effects of Pb per se resembled those of stress itself, particularly in nucleus accumbens (Fig. 7), where decreases in DOPAC, DOPAC turnover, DOPAC + HVA turnover were produced by stress in controls and were also produced by Pb exposure; thus Pb effects mirrored effects of stress in this region. To a lesser extent, increases in 5-HT produced by stress in controls were also seen with Pb exposure. Prior prospective cohort studies of Pb-exposed children might be particularly useful to further evaluate this possibility.

Comparisons of the current finding to our previous study of maternal only Pb exposure (Cory-Slechta et al., 2004) suggests that the timing of Pb exposure impacts the nature of the changes observed. For example, maternal only exposure increased plasma basal corticosterone levels, while postweaning Pb decreased levels producing a U-shaped dose effect curve, a not infrequent effect with Pb exposure (Davis and Svendsgaard, 1990). Other studies confirm the capacity for adult only Pb exposures to modify HPA axis function. A 70-day exposure of adult male rats to Pb acetate (50 or 250 l g ip)

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**FIG. 10.** Group mean ± SE values for NE and E in adrenal cortex in relation to Pb exposure level in subgroups of rats that had experienced no stress (NS) or cold and restraint stress (S). Pb × stress = interaction in the ANOVA, with subsequent post-hoc effects as indicated. *Indicates significantly different from corresponding 0 ppm value, + indicates significantly different from 50 ppm NS value. Sample sizes of 10, 10, and 11 for the 0, 50, and 150 ppm NS groups, and 10, 10, and 11, respectively, for the corresponding S groups.

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**FIG. 11.** Individual density values for glucocorticoid receptors as determined in nucleus (A) and in cytosol (B) in relation to Pb exposure level and previous stress (NS = no stress; S = previous cold + restraint stress). The bar in each treatment group column depicts the mean value for the indicated group; sample sizes ranged from 7–8. Western blot images (panel C) show corresponding specific immunoreactivity for cytosol and nucleus respectively for protein loadings as indicated for each. Representative blots are shown for an individual rat for each treatment group: 0-NS, 0 ppm + no stress; 0-S, 0 ppm + cold + restraint stress; 50-NS, 50 ppm + no stress; 50-S, 50 ppm + cold + restraint stress; 150-NS, 150 ppm + no stress; 150-S, 150 ppm + cold + restraint stress.
increased plasma corticosterone, with two-fold increases at the lower Pb exposure level, as compared to an approximate 50% increase at 250 μg (Det et al., 1977). Exposures of young adult male rats to 0.1–0.3 mg Pb nitrate ip for 21 days decreased basal plasma corticosterone levels by 35% at the 0.3 mg dose, and also decreased the elevation of corticosterone in response to immobilization stress by 46% (Agrawal and Chansouria, 1989). Exposures of young male BalbC mice for eight weeks to 2 mM Pb acetate in drinking water reduced serum corticosterone levels (Kim and Lawrence, 2000). Systematic studies will be required to clarify the differential impacts of developmental period and duration of Pb exposure on HPA axis function. It is also important to note that Pb-related changes in corticosterone in all of these studies were measured at a single time point. Multiple time points need to be examined to determine whether such changes are static or dynamic across time, and whether Pb exposure has the potential to accelerate age-related changes in HPA axis function.

The mechanism(s) by which Pb exposure alters HPA axis function and stress responsivity have yet to be elaborated. It is possible that Pb has direct effects on the HPA axis. Alternatively, such changes may be indirectly mediated by Pb-induced alterations in neurochemical function, among other possibilities. The complexity of HPA axis function and its interactions with various neurotransmitters and brain regions underscores the difficulties that will be inherent in localizing mechanisms, since multiple brain regions (e.g., septohippocampal system, prefrontal cortex, nucleus accumbens, amygdala, and the raphe nucleus) and neurotransmitter systems (e.g., serotonergic, norepinephrine, dopamine, and GABA) are known to interact with the HPA axis (Carrasco and Van de Kar, 2003; Van de Kar and Blair, 1999).

In this regard, however, it is interesting to consider the patterns of Pb-induced neurochemical changes observed here in relation to the changes in basal plasma corticosterone levels. Basal corticosterone was decreased to a greater extent by 50 than by 150 ppm Pb (Fig. 5). A corresponding pattern of changes in neurotransmitter levels can be generally observed in 5-HT (serotonergic) systems particularly for Pb-treated groups that did not experience novelty and cold stress. In frontal cortex, increases in the serotonin metabolite 5-HIAA were seen in response to Pb, with more pronounced effects at 50 ppm (Fig. 6), even while corresponding decreases in 5-HT were concentration-related. In nucleus accumbens (Fig. 7), Pb (non-stressed Pb groups, p = 0.02) increased 5-HT levels, again with a greater increase at 50 ppm (62%) than seen in response to 150 ppm (31%). Very similar although less pronounced effects can be seen both in striatum (Fig. 8) and hypothalamus (Fig. 9). Further, increases in body weight were observed in 50 ppm but not 150 ppm Pb-treated rats relative to control (Table 1). A substantial body of evidence documents the critical roles played by dopaminergic and serotonergic systems in hypothalamus as well as in other regions, in aspects of eating behavior, meal size, and body weight (Meguid et al., 2000). Another notable correspondence with basal corticosterone can be observed with adrenal cortical NE levels. Future experiments using agonists and antagonists of these neurotransmitter systems as well as genetically engineered animals will be useful to further explore these potential relationships, along with more specific assessments of various hypothalamic nuclei.

Finally, as was also observed in our study of maternal Pb and maternal stress (Cory-Slechta et al., 2004), the profile of effects of combined Pb + stress: (1) differ from those produced by Pb alone, (2) are complex and not systematic in nature, and thus (3) difficult to determine how any experimental stress challenge might be extrapolated to human environments, but it is notable that both the nature of the behavioral effects produced by Pb and the blood Pb concentrations at which they occur show marked similarities in human and experimental studies. For such reasons, these findings raise serious questions not only about whether current experimental approaches based on studies of single chemicals in isolation from other relevant risk factors are adequate approaches for identifying neurotoxic hazards, but also about the utility of current risk assessment methods based on such outcomes.

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