Published in final edited form as:

Neurotoxicology. 2008 September ; 29(5): 812–827. doi:10.1016/j.neuro.2008.03.003.

CNS EFFECTS OF DEVELOPMENTAL Pb EXPOSURE ARE ENHANCED BY COMBINED MATERNAL AND OFFSPRING STRESS

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Abstract

Lead (Pb) exposure and elevated stress are co-occurring risk factors. Both impact brain mesolimbic dopamine/glutamate systems involved in cognitive functions. We previously found that maternal stress can potentiate Pb-related adverse effects in offspring at blood Pb levels averaging approximately 40 ug/dl. The current study of combined Pb exposure and stress sought to extend those results to lower levels of Pb exposure, and to examine relationships among consequences in offspring for Fixed Interval (FI) schedule-controlled behavior, neurochemistry and corticosterone levels. Dams were exposed to maternal Pb beginning 2 mos prior to breeding (0, 50 or 150 ppm in drinking water), maternal restraint stress on gestational days 16 and 17 (MS), or the combination. In addition, a subset of offspring from each resultant treatment group was also exposed intermittently to variable stressors as adults (MS+OS). Marked “Pb-stress”-related increases in response rates on a Fixed Interval schedule, a behavioral performance with demonstrated sensitivity to Pb, occurred preferentially in female offspring even at mean blood Pb levels of 11 ug/dl when 50 ppm Pb was combined with maternal and offspring stress. Greater sensitivity of females to frontal cortex catecholamine changes may contribute to the elevated FI response rates as mesocorticolimbic systems are critical to the mediation of this behavior. Basal and final corticosterone levels of offspring used to evaluate FI performance differed significantly from those of non-behaviorally tested (NFI) littermates, demonstrating that purported mechanisms of Pb, stress or Pb/stress effects determined in non-behaviorally trained animals cannot necessarily be generalized to animals with behavioral histories. Finally, the persistent and permanent consequences of Pb, stress and Pb+stress in offspring of both genders suggest that Pb screening programs should include pregnant women at risk for elevated Pb exposure, and that stress should be considered as an additional risk factor. Pb+stress effects observed in the absence of either risk factor alone (i.e., potentiated effects) raise questions about the capacity of current hazard identification approaches to adequately identify human health risks posed by neurotoxicants.

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Keywords

lead; stress; corticosterone; dopamine; catecholamines; behavior; fixed interval schedule; gender

Introduction

Exposure to lead (Pb) is associated with numerous adverse effects that have been similarly documented in humans and experimental animals. While most studies examine Pb effects in isolation, Pb exposures always occur in the context of numerous risk modifiers, including genetic, host and environmental factors which have the potential to influence the impact of Pb (Cory-Slechta 2005). To date, with the exception of recent studies of vulnerability conferred by some genetic polymorphisms, almost nothing is known about how other potential risk modifiers may modulate the impact of Pb.

One risk factor likely to interact with Pb exposure is psychological stress. First, Pb and stress can be conceived as co-occurring risk factors. The highest levels of Pb in the U.S. are sustained by low socioeconomic status (SES) inner city minority children. Low SES is itself associated with higher levels of various diseases and disorders (Dohrenwend 1973; Starfield 1982; Pappas et al. 1993; Anderson and Armstead 1995; Stipek and Ryan 1997), a phenomenon that has been hypothesized to arise from chronic elevation of stress and associated stress hormones such as glucocorticoids (Munck et al. 1984; Lupien et al. 2001). Thus, both Pb and stress target low SES populations. Secondly, both Pb and stress act on brain mesocorticolimbic and hippocampal dopamine and glutamate systems (Diorio et al. 1993; Lowy et al. 1993; Piazza et al. 1996; Pokora et al. 1996; Cory-Slechta et al. 1998; Rouge-Pont et al. 1998; Cory-Slechta et al. 1999; Barrot et al. 2000; Moghaddam 2002), which may in fact explain their common consequences, including cognitive dysfunction and attention deficits (Dohrenwend, 1973; Bellinger et al., 1994; Schwartz, 1994; Anderson and Armstead, 1995a; Cory-Slechta, 1995; Needleman et al., 1996; Dietrich et al., 2001; Bradley and Corwyn, 2002). Third, the mesocorticolimbic dopamine system, a key target of Pb effects on behavior (Cory-Slechta 1995; Cory-Slechta et al. 1996; Cory-Slechta et al. 1997a; Cory-Slechta et al. 1997b; Cory-Slechta et al. 1998; 1999; Cory-Slechta et al. 2002; Abbott et al. 2003) has extensive interactions with the hypothalamic-pituitary-adrenal (HPA) axis, the system that coordinates the body's physiological response to stress (Vazquez 1998). Stress, of course, is experienced universally and not exclusively by low SES populations, so that the study of combined Pb and stress more accurately models the human condition in general.

The combined effects of Pb and stress could function in a multi-hit model of neurotoxicity where multiple risk factors target a common system of the brain, but act via different mechanisms. Such a scenario could be postulated to enhance vulnerability, particularly for the central nervous system, when compared to a situation in which multiple concurrent insults act by a single mechanism. The brain may be readily able to compensate for the effects of an individual chemical or risk factor itself acting on a single specific target site of the brain. But when confronted by multiple insults at different functional sites (i.e., by different mechanisms), the ability to evoke homeostatic processes would presumably be compromised, thereby leading to sustained or cumulative damage (Cory-Slechta 2005).

This study focuses on the impact of combined maternal Pb exposure and maternal stress. Maternal blood lead (PbB) levels are highly correlated with fetal and cord blood lead levels (Li et al. 2000; Chuang et al. 2001; Furman and Laleli 2001; Kirel et al. 2005; Iranpour et al. 2007) and clearly contribute to the adverse cognitive deficits observed in children (Canfield et al. 2003; Canfield et al. 2004; Lanphear et al. 2005). Maternal stress has profound and enduring consequences for multiple systems of offspring, altering endocrine, behavioral, cardiovascular

and metabolic regulation (Seckl et al. 2000; Seckl 2004; Seckl and Meaney 2004; 2006; Seckl and Holmes 2007). For the central nervous system, effects include changes in learning and memory and other behavioral dysfunctions (Lemaire et al. 2000; Lordi et al. 2000; Meek et al. 2000; Szuran et al. 2000; Fumagalli et al. 2007). Importantly, some of these consequences of maternal stress have now been shown to be transgenerational (Kapoor et al. 2006; Seckl and Meaney 2006; Seckl and Holmes 2007).

A prior study of combined Pb and stress from our laboratory demonstrated permanent consequences of Pb on the HPA axis, as well as Pb + stress-engendered neurochemical and behavioral alterations that differed significantly by gender (Cory-Slechta et al. 2004; Virgolini et al. 2006). That study examined a single and relatively high Pb exposure concentration producing PbBs more reminiscent of those associated with occupational exposures. The current study, in examining Pb-stress interactions, utilized PbB levels approximating those designated by the Centers for Disease Control as a level of concern for children (10 ug/dl). In addition, it utilized pair housing of offspring as opposed to individual housing, since isolation housing itself can be a stressor and alter HPA axis function (Cabib et al. 2002; Francolin-Silva and Almeida 2004). A subset of offspring in each treatment group was also subjected to stress in adulthood to determine the extent to which maternal stress produces a silent vulnerability and/or whether cumulative effects were engendered. Behavioral performance was evaluated using a Fixed Interval (FI) schedule of reinforcement, a baseline with demonstrated sensitivity to Pb (Cory-Slechta 1996) and performance on which has been deemed a surrogate for impulsivity, a component of attention deficit disorder (Darcheville et al. 1992; 1993). Measures of catecholamines and serotonin in multiple brain regions as well as multiple corticosterone measures were included to further determine potential mechanisms of effects of combined Pb and stress-associated changes in behavior.

Methods and Materials

Animals and Pb Exposure

Three week old female and 3-month old male Long Evans rats were used for breeding (Charles River, Germantown, NY). Upon arrival, females were randomly assigned to one of three Pb exposure groups: 0 (tap water), 50 or 150 ppm Pb in drinking water. Pb acetate administered in drinking solutions was dissolved in distilled deionized water and prepared fresh on a weekly basis. Previous studies from our laboratory show 50 ppm to produce PbB levels averaging 10-15 ug/dl (Cory-Slechta et al. 1983; 1985), just at and above the Centers for Disease Control's currently designated level of concern for children, and levels associated with similar behavioral deficits in rodents and children (Cory-Slechta et al. 1983; 1985; Cohn et al. 1993; Canfield et al. 2004). The 150 ppm exposure was associated with mean PbB levels in dams ranging from 32.6 ± 4.4 to 42.7 ± 4.0 ug/dl and was used because our previous study demonstrated synergistic effects of this Pb exposure level with stress (Cory-Slechta et al. 2004).

Pb exposure of dams was initiated at 3 weeks of age and was ongoing for 2 months prior to breeding (by which time females were 11 weeks of age) and continued through lactation to ensure elevated bone Pb levels and thus produce a Pb body burden in dams (Cory-Slechta et al. 1987), as consistent with human exposure. Animals were housed in a vivarium room with a 12-h light-dark cycle (lights on at 7:00 a.m.) that was temperature (22 ± 5 °C) controlled. Standard rat chow diet and drinking solutions were provided *ad libitum*.

Breeding and Maternal Stress

At pro-estrus, as determined by vaginal smears, female rats were mated with males (2:1) across two estrous cycles. The presence of vaginal plugs or sperm in vaginal smears collected in the early morning indicative of pregnancy was considered gestational day 1 (GD1). Pregnant

females were weighed and further randomly subdivided to a non-stress (NS) or maternal stress (MS) condition (see below) and individually housed for the remainder of pregnancy and lactation. This resulted in six groups with the following sample sizes: 0NS (n=20); 0MS (n=23); 50NS (n=21); 50MS (n=31); 150NS (n=19); and 150MS (n=33).

On gestational days 16 and 17, dams assigned to MS groups were weighed and subjected to 3 sessions (9:00, 12:00 and 15:00 hr) daily of a 45 min restraint stress using procedures modified from Ward and Weisz (Ward and Weisz 1984). Plastic transparent cylindrical devices (IITC restrainers, model 81; 7 cm diameter, 19 cm long; IITC Life Sciences, Woodland Hills, MA) were used to immobilize the pregnant dams leaving an aperture in the front to allow normal breathing. NS group dams were weighed and subsequently left undisturbed in their home cages. The choice of days 16 and 17 was timed to correspond with development of key brain regions associated with the endpoints under study. This protocol, used in our previous study of Pb and stress, resulted in elevated corticosterone levels and alterations in catecholamine levels in frontal cortex and nucleus accumbens of dams (Cory-Slechta et al. 2004). At the end of the last restraint session on GD16, blood was collected for corticosterone determinations. Corticosterone determination from the first rather than the second day of stress was chosen to prevent potential habituation effects from obscuring any treatment-related differences.

At delivery (postnatal day 1: PND1), litter size was recorded and number of pups culled to 8-9 per litter, maintaining equal numbers of males and females wherever possible. Crossfostering was not performed, as the intent of the study was to model the human environment and culture. Pups were weaned at PND21, separated by gender, and housed in pairs for the duration of the experiment. Only one pup of each gender was used from each litter for each outcome measure to preclude any litter-specific effects.

From weaning, pups were provided with unrestricted access to tap water (0 ppm) and food (Laboratory Rodent Diet 5001, POMI Foods Inc.) until 55-60 days of age when male pups reached approximately 300 g and females 220 g. At this point, behavioral experiments were initiated and caloric intake was restricted to maintain the above-stated body weights.

At weaning, a subset of pups that had undergone MS were assigned to an offspring stress (MS +OS) group, so that the experiment ultimately included 9 different offspring treatment groups: 0NS, 0MS, 0MS+OS, 50NS, 50MS, 50MS+OS, 150NS, 150MS and 150MS+OS. Allocation at this time point rather than after FI behavioral testing began occurred for logistical purposes based on the total number of animals in these experiments. A total of n=10-11 per gender per group (one male and one female per litter) were used for assessment of behavioral performance on the FI schedule of food reward (FI rats) with neurotransmitter levels and final corticosterone levels determined in these groups following the completion of behavioral testing. Littermates of these offspring that were not used in the behavioral evaluation (non-FI rats: NFI) were subjected to the same offspring stress challenges (see below) and sacrificed at various points in the experiment to determine time course assessment of neurochemical and corticosterone changes and to evaluate the impact of behavioral training itself on outcome measures.

Fixed Interval Performance

At 2-3 months of age, offspring began training on a Fixed Interval 1 minute schedule of food reinforcement (FI 1). This schedule reinforced the first occurrence of a designated response after a 1 min interval had elapsed. Reinforcement delivery also initiated the next 1 min FI. Responses during the interval itself had no programmed consequences. This schedule has been demonstrated to be sensitive to Pb exposure across a range of different developmental periods of exposure and concentrations (Cory-Slechta 1996). FI testing was carried out in operant chambers (30.5 cm × 24.5 cm × 21 cm; Med Associates Inc., St. Albans, Vermont) housed in sound-attenuated enclosures ventilated by a fan. They were equipped with a grid floor, speaker,

house light and three response levers configured horizontally on the front panel; only the right lever was active in these experiments. Reinforcement consisted of the delivery of a 45 mg food pellet (BioServ, Frenchtown, NJ) and behavioral contingencies and data were controlled by the SoftCtrl™ Cumulative Record interface and the Med-PC Version IV Research Control and Data Acquisition.

Lever press response training was first carried out in automated overnight sessions as previously described (Cory-Slechta and Weiss 1985) after which the FI 1 min schedule was implemented. Sessions were initiated by the first response or after 5 min if no response had occurred, and ended following the completion of the 1 min interval that was in progress 20 min after the session began or after a total of 21 min, whichever occurred first. Sessions were conducted 5 days a week (M-F) between 10:00 and 15:00 h. Standard measures of FI performance computed included overall response rate (total number of responses divided by total session time), postreinforcement pause (PRP; time to the first response in the interval) and run rate (number of responses per minutes during the interval, calculated without the PRP).

Daily food allotment was provided after the behavioral test session to maintain body weights. For this purpose, rats were provided with their daily food allotment in their transport cages so as to control food access and maintain body weights of individual animals.

Offspring Stress Procedures

FI Rats—After approximately 10 FI sessions had been conducted, tail blood was collected immediately following a behavioral session in FI rats for determinations of basal corticosterone levels (n=8 of each gender for each group). Offspring stress challenges were then begun and were imposed immediately prior to an FI session to measure its impact on subsequent FI performance. Stress challenges were carried out during the acquisition phase of FI training so that the ability to elicit group differences was maximized. Multiple and variable stressors rather than a single homotypical stress challenge was used to more closely mimic the human experience across the life span. For female offspring, stress challenges were carried out during the diestrus I phase of the sexual cycle to assure constant and low estrogen levels, since fluctuations in hormones can interfere with endogenous and released corticosterone, and stress responsiveness can vary with stage of the estrous cycle (Viau and Meaney 1991; Anderson et al. 1996).

The first such stress challenge was a single 45 min session of restraint stress (prior to session 13 in both males and females), using the same procedure described above for the dams. The use of the most intense stress before other stress challenges was based on its potential to increase sensitivity of the animals to subsequent stressors (Koolhaas et al. 1997), thereby potentially maximizing the ability to detect treatment-related differences. Prior to session 21 for females, and session 20 for males, cold stress, considered a mild physical stress, was imposed. Animals were placed in cages similar to home cages (without the bedding) for 30 min in a temperature-controlled room maintained at 4° C prior to being placed in the operant chambers. Prior to session 30 for females and session 31 for males, animals underwent a 15 min test of locomotor activity in locomotor activity cages as a novel environment stressor. FI rats not assigned to offspring stress (e.g., NS and MS groups) were left undisturbed in their home cages during these periods. Blood samples were collected immediately after the FI session that followed each stress challenge for corticosterone determinations. Behavioral testing continued for an additional three months to allow determination of the time course and stability of any observed group differences.

NFI Rats—Age-matched NFI offspring assigned to MS+OS groups were subjected to the same schedule/timing of stressors, remained in transport cages for the same time as the FI session and were returned to their home cages immediately following the stress challenge.

Detailed results of the stress challenges in both FI and NFI offspring per se will be reported in another communication.

Offspring Tissue Collections

At the termination of FI testing, brains of all FI rats and remaining NFI rats were quickly removed and dissected. For female offspring, this was done during the diestrus stage of the sexual cycle. Brain was dissected for measurement of brain catecholamines and serotonin in nucleus accumbens (NAC), frontal cortex (FC), striatum (STR), and hypothalamus. Hippocampus was stored for future Western blot analysis of the glucocorticoid receptor. Tissue was frozen and stored at -80°C until determinations were carried out.

Neurochemical Determinations of Catecholamines, 5-HT and 5-HIAA

Tissue was placed in 0.1 N perchloric acid, sonicated and centrifuged twice for 20 min at 10,000 rpm. Supernatant was collected to measure catecholamine, 5-HT and 5-HIAA levels using a high-performance liquid chromatography (HPLC) system with electrochemical detector as previously described (Cory-Slechta et al. 2004; Virgolini et al. 2005; Virgolini et al. 2006). The resulting pellets were digested in 1 ml of 0.5 N NaOH for measurements of protein concentration using Bio-Rad assay reagents. The mobile phase consisted of a sodium phosphate buffer with 10-13% methanol, EDTA and sodium octyl sulfate solution added as an ion-pairing agent to 1 liter of mobile phase. Standards of DA (dopamine), DOPAC (dihydroxyphenylacetic acid), HVA (homovanillic acid), 5-HT (serotonin) and 5-HIAA (5 hydroxyindoleacetic acid) were assayed twice daily at the beginning and at the end of the run. Concentrations of neurotransmitters were expressed in terms of ng/mg protein. DA turnover was calculated as the DOPAC/DA ratio.

Blood Pb Analysis

PbB levels were measured in dams ($n=7-8$ for each group) at the time of offspring weaning (PND21). For each sample, 100 μl of blood was collected in heparinized microtubes and transferred to tubes containing pre-assembled reagents to measure PbBs by anodic stripping voltammetry according to methods described previously (Cory-Slechta et al. 1987; Widzowski and Cory-Slechta 1994). The limit of sensitivity of the assay is 5 $\mu\text{g/dl}$.

Corticosterone Determination

Briefly, the tail was soaked in warm water, the tip quickly removed via a straight-blade razor and the tail palpated to collect approximately 200 μl of blood into pre-chilled tubes that were spun at 3500 rpm for 10 min. Sera were separated and stored at -20°C until the time of the assay. Serum corticosterone was measured using the commercially available ImmuChemTM Double Antibody Corticosterone ^{125}I kit according to the manufacturer's instructions (MP Biomedicals, Orangeburg, NY). All standards and samples were run in duplicate, counted using a Cobra II Auto Gamma counter and expressed in ng/ml. The minimum detectible dose in this assay was 7.7ng/ml.

Statistical Analysis

FI Performance—An overall repeated measures ANOVA was first carried out that included all FI test sessions (omitting sessions preceded by stress challenges) for both genders, with data collapsed into blocks of sessions that varied somewhat in terms of number during the stress challenge period due to logistics of conducting stress challenges (females: pre-restraint-6, restraint to cold-4, cold to novelty-5, post novelty-5; males: pre-restraint 5, then 6, restraint to cold-7, cold to novelty-7, post novelty-5). In these analyses, Pb, gender and stress group (NS, MS, MS+OS) were between group factors and session blocks was the within group factor.

These were done separately for each of the three FI performance measures (overall rate, run rate and PFP).

Because all three analyses revealed highly significant main effects of gender and multiple gender, Pb and stress interactions, overall analyses of FI performance measures were then analyzed separately for each gender. For each FI performance measure, these analyses were first carried out across all sessions (omitting sessions preceded by stress challenges with Pb and stress group serving as between group factors. Because the behavioral and physiological effects of various stress challenges clearly differ and could thus differentially impact FI performance (Pacak and Palkovits 2001), additional analyses were then carried out separately across the following blocks of sessions: pre-restraint stress, post-restraint to cold stress, post-cold stress to novelty stress, and post-novelty stress to the termination of the experiment for each gender for each FI performance measure out using Pb and stress as between group factors. When main effects or interactions were found in these analyses, further analyses (ANOVAs, post-hoc tests) were carried out as necessary. A p value ≤ 0.05 was considered statistically significant.

Biochemical Measures—Overall analyses (ANOVAs) that included Pb and stress as independent variables were used to examine changes in biochemical outcomes. Subsequent ANOVAs or post-hoc tests were used as appropriate when main effects or interactions were confirmed. A p value ≤ 0.05 was considered statistically significant.

Principal Component Analysis—Principal component analyses (PCA; SPSS, Inc.) were used to explore relationships among the outcome measures to suggest mechanisms for Pb/stress-induced FI response rate changes. Analyses were done separately for each of the 9 treatment groups, and included FI response rates from a selected session during block 9 or 10 when rate differences among groups of females at 50 ppm were maximal, final corticosterone values, and levels of neurotransmitters for which statistically significant changes were confirmed. Analyses used a varimax rotation in which eigenvalues over 1 were extracted and a maximum iteration for convergence of 100. PCA analyses were only carried out in females based on the results obtained for FI performance measures. Cumulative fits for rotated sums of squared loadings ranged from 85.21-93.01% (Table 1).

Results

Effects in Dams

Blood Lead Concentrations—PbB levels of dams were measured at the time of pup weaning. Group mean values (ug/dl; n=7-9 dams per group) were 0.89 ± 0.081 for 0NS dams, 0.79 ± 0.1 for 0MS dams, 11.6 ± 0.52 for 50NS dams, 11.79 ± 0.47 for 50MS dams, 30.84 ± 1.34 for 150NS dams and 31.2 ± 0.78 for 150MS dams. Statistical analyses confirmed a significant main effect of Pb ($p < 0.0001$), with concentration-related effects with 150 ppm > 50 ppm > 0 ppm. No main effect of stress or Pb by stress interaction was found.

Corticosterone Levels—Corticosterone determinations (ng/ml), carried out after the third stress session on GD16 (ng/ml; n=5-8 dams per group), the first day of stress, averaged 52.5 ± 19.4 for 0NS dams, 478.1 ± 21.2 for 0MS dams, 92.1 ± 44.9 for 50NS dams, 385.6 ± 29.8 for 50MS dams, 56.6 ± 29.8 for 150NS dams and 380.5 ± 31.7 for 150MS dams. Statistical analysis confirmed a main effect of stress ($p < 0.0001$), no effect of Pb, and a Pb by stress interaction that approached significance ($p = 0.08$) due to slightly lower levels of corticosterone in the 50 and 150 ppm MS dams relative to 0 ppm MS dams.

Effects in Offspring

Litter sizes did not differ in relation to Pb, stress or combined Pb and stress. Given the numbers of litters generated, only a subset of litters, randomly chosen across treatment conditions, was systematically weighed. No differences were detected.

Fixed Interval (FI) Performance

Overall Response Rates: Figure 1 shows overall response rates across blocks of sessions, with data separated at times of the imposition of offspring stress challenges for female (top row) and male (bottom row) offspring.

Females: Clear significant gender differences in FI performance emerged in response to Pb/stress that preferentially impacted female offspring. In females, a significant $Pb \times stress \times session$ interaction was found across all sessions (omitting those preceded by a stress challenge), indicating differential changes in rates across time among Pb and stress groups ($Pb=0.03$, $stress=0.005$, $Pb \times stress \times session=0.0009$)

Pre-Restraint: Session blocks 1-2 represent behavior prior to the imposition of the first stress challenge (restraint) ($Pb<0.0001$, $Pb \times stress=0.0043$; $Pb \times stress \times session=0.0002$). There was no significant effect of MS in controls, as no significant differences were found among the 0 ppm groups. Concentration-related increases in overall rates were produced by Pb alone; these were approximately 100% for 50NS and 300% for 150NS groups, with post hoc testing showing $150NS>50NS$ and ONS . The 150NS group rates were higher than all three 0 ppm groups. In the case of 50 ppm, MS treatment (no stress challenge had yet occurred, so MS and MS+OS groups are equivalent across this time period) appeared to slightly enhance the rate increases produced by Pb alone, an effect that was significant in the 50 MS+OS group where rates were higher than all 0 ppm groups as well as 50MS and 50NS rates. The increases in the 50MS group relative to 50NS were not significant, and the higher rates of the 50MS group relative to the 50MS+OS group likely reflects the random allocation of subjects to these conditions at weaning. In contrast, at 150 ppm, MS was associated with lower rates than occurred at 150 ppm alone, with lower FI rates in the 150 MS and 150 MS+OS groups, although these were significant only in the 150 MS group, again, likely an allocation effect. Even with these lower rates, the 150 MS+OS group still had rates that exceeded those of all three 0 ppm groups.

Restraint to Cold to Novelty: Following session blocks 1-2, a subset of offspring from each group were subjected to restraint stress (before block 3), followed by cold stress (before block 5) and novelty stress (before block 7). Through the period of stress challenge testing, Pb concentration- and stress condition-related effects emerged (restraint to cold: $Pb<0.0001$, $stress=0.0008$, $stress$ by $session=0.053$; cold to novelty: $Pb=0.002$, $stress=0.0001$, $Pb \times stress \times session=0.045$). Specifically, significant increases in response rate in association with Pb alone continued to be observed only at 150 ppm (150NS), where rates remained approximately 2-2.5 fold greater than ONS and 50NS values, with the latter two not differing from each other. MS treatment was associated with lower rates relative to Pb alone, as was particularly apparent in the 150 ppm group (150 NS vs. 150 MS+OS).

Most notable were the increases in rate associated with MS+OS treatment as opposed to the rate suppression associated with MS only. For example, while the 50NS group did not differ from any 0 ppm group, further marked rate increases were seen across this period in the 50 MS+OS group, with levels that were significantly higher than all 0 ppm groups and the 50NS and 50MS groups. Additionally rates in the 50 MS+OS group corresponded to those of the 150NS and 150MS+OS groups. Rates of the 150 MS+OS group were equivalent to those of the 150NS group, and were significantly higher than all 0 ppm groups and the 150MS group, with these

levels of responding potentially nearing ceiling effects in females. A trend towards an enhanced effect of MS+OS was seen in controls, particularly after cold stress. Although these changes did not produce a significant difference relative to 0NS response rate levels, the magnitude of the increase was large enough to eradicate differences when compared to rates of the 50MS+OS, the 150NS and the 150MS+OS groups over blocks 5-6.

Post Stress Challenge: During the post-stress challenge period (blocks 7-15; stress=0.01; Pb $\times$ stress $\times$ session<0.0001), rates of the 0 ppm groups continued to increase such that residual group differences remained only for the 50MS+OS group across the course of sessions (p=0.015). By the final block of sessions (block 15), rates of the 50MS+OS group remained at 77% above control, while rates of the 150MS+OS group were 37% above 0NS control levels.

Males: In contrast to what was observed in females, no consistent or systematic significant changes in overall response rates on the FI schedule were observed in male offspring (Figure 1, bottom row). Statistical analysis indicated a main effect of Pb (p=0.0223) considered across the entire 13 block of sessions as a result of the 150 ppm exposure collapsed across stress groups.

In the period prior to the imposition of restraint stress (blocks 1 and 2), a concentration-related increase in overall rates was found with Pb (p=0.0163), again as a function of the 150 ppm exposure, with rates approximately 40% higher than control. Over the subsequent stress challenge period (blocks 3 and 4), no group differences in rate were found. While there was some suggestion of Pb/stress effects in the 150 ppm groups post stress challenge (blocks 5-13, main effect of Pb=0.0455, Pb $\times$ session<0.001), these were due to the 150 ppm groups collapsed across stress conditions (150NS, 150MS and 150MS+OS), as no individual treated groups differed significantly from 0NS control.

Run Rates: The pattern of increases in FI run response rates in Pb-stress groups across time generally paralleled those in FI overall rates across these conditions (cf. Figures 1 and 2).

Females: As with overall rates, persistent elevations in run rates were both Pb-concentration and stress-condition-dependent in females (Figure 2, top row).

Pre-Restraint: Statistical analyses failed to identify any significant effect of MS in subjects not exposed to Pb. Run rates, however, were elevated pre restraint in a Pb-concentration-dependent manner (Pb<0.0001; Pb $\times$ stress $\times$ sessions<0.0001). Moreover, as with overall rate, the increases were significant for 150 ppm but not 50 ppm and increased approximately 2.5 fold. Similarly to overall rates, run rates of the 150NS group were higher than all three 0 ppm groups. MS treatment appeared to enhance the effects of 50 ppm, although these effects did not reach acceptable levels of statistical significance. MS was associated with lower rates at 150 ppm, with significant reductions in the 150MS group, and marginal reductions at 150MS+OS relative to 150NS. Run rates of this 150 MS+OS, but not the 150MS group, still exceeded those of all three 0 ppm groups.

Restraint to Cold to Novelty: Again, as with overall rate, Pb concentration- and stress condition-related effects emerged through the period of stress challenge testing (restraint to cold: Pb=0.0001; stress=0.013; cold to novelty: Pb=0.0078, stress=0.0046). Specifically, significant increases in response rate in association with Pb alone continued to be observed only at 150 ppm (150NS), where rates remained approximately 2-2.5 fold greater than 0NS and 50NS values, with the latter two not differing from each other. MS treatment continued to be associated with lower rates as compared to Pb alone in the 150 ppm group (150 NS vs. 150 MS+OS).

Most notable, as with overall rate, were the increases in the MS+OS groups as opposed to the rate suppression produced by MS only. For example, the 50NS group did not differ from any 0 ppm group, but further rate increases occurred in the 50 MS+OS group to levels that were significantly higher than all 0 ppm groups and the 50NS and 50MS groups. Rates of the 150 MS+OS group were equivalent to those of the 150NS group, and were significantly higher than all 0 ppm groups and the 150MS group.

Post Stress Challenge: During the post-stress challenge period (blocks 7-15; stress=0.01; Pb $\times$ stress $\times$ sessions<0.0001), rates of the 0 ppm groups continued to increase, such that residual group differences remained only for the 50MS+OS group across the course of sessions. Response rates for subject in this group were higher than all 0 ppm groups and both the 50NS and 50MS groups.

Males: In males (bottom row), Pb effects were confirmed statistically, arising from elevations in the 150 ppm groups collapsed across stress conditions, relative to both 0 and 50 ppm groups. Following the stress challenges (blocks 5-13), rates of both the 150NS and the 150MS+OS groups were significantly greater than ONS control when collapsed across blocks, although these effects were diminished by the final session blocks (e.g., 42% and 58%, respectively, above control at block 8 vs. 3% and 4% at block 13). These effects were similar to those observed with overall rates.

PRP: PRP times across the course of behavioral testing are shown in Figure 3.

Females: Decreases in PRP time also contributed to the increased overall FI response rates observed in female offspring subjected to Pb/stress (Figure 3, top row).

Pre-Restraint: Prior to the imposition of restraint stress (blocks 1 and 2), virtually all treated groups had significantly shorter PRPs than controls (ONS), including the OMS group (main effect of Pb=0.0011, Pb $\times$ stress=0.0013, Pb $\times$ stress $\times$ sessions<0.0001), while none of these groups differed from each other.

Restraint to Cold to Novelty: During the imposition of stress challenges (blocks 3-6), effects were largely Pb-concentration and stress-condition-related, with significantly shorter PRPs found in the 50MS+OS, 150NS and 150MS+OS groups (block 3-4: Pb=0.0045, stress=0.0048; block 5-6: Pb=0.0042, stress=0.0040, Pb $\times$ stress $\times$ sessions=0.0473) relative to the OMS and ONS groups, but comparable to each other. In this period, reductions in PRP ranged from 25-33% relative to ONS controls.

Post Stress Challenge: Following stress challenges (blocks 7-15), a significant shortening (Pb $\times$ stress $\times$ sessions <0.0001) of PRP was evident in the OMS+OS group (p=0.0076) with reductions to 32-39% of ONS controls. Significant reductions were also found in the 150NS and 150MS groups relative to control. Similar but non-significant reductions were observed in the 50MS+OS and the 150MS+OS groups.

Males: Male offspring subjected to Pb, stress or to the combinations thereof showed no systematic significant changes in PRP (bottom row).

Neurotransmitter Levels—Unlike the changes in FI performance, alterations in levels of brain catecholamines, collected after the termination of behavioral testing in FI rats, were not restricted to a single gender. However, the pattern of effects did differ between male and female offspring.

Frontal Cortex Catecholamines: Females: Changes in levels of DA and metabolites in frontal cortex of females (Figure 4 top row) were generally related to Pb exposure alone (main effects of Pb=0.0019, 0.0265 and 0.0434, for DA, DOPAC and HVA, respectively) with 50 and 150 ppm groups showing relatively comparable increases. Increases in DA in the 50 and 150 ppm Pb/stress groups ranged from 134-229% of 0NS control. Corresponding levels for DOPAC and HVA were 129-547% and 147-240%, respectively, with the exception of a decrease in the 50NS group.

Males: For male offspring, changes in DA and the metabolites DOPAC and HVA were largely restricted to 150 ppm Pb, with no difference between the 0 and 50 ppm groups (main effect of Pb=<0.001, 0.001 and 0.0037, respectively). Increases in the 150 ppm Pb/stress groups ranged from 143-230%, 224-300% and 158-173% for DA, DOPAC and HVA, respectively. The one exception was a potentiated increase in DA levels in the 50MS+OS group relative to 0NS controls (187%) as well as to the 0MS+OS and 50NS groups, resulting in a Pb $\times$ stress interaction (p=0.0234). Synergistic increases in DA were found in the 150MS+OS group (>150NS, no effect in 0MS+OS group). DA turnover (DA TO) was not significantly changed in either gender.

NE: Males and Females: Significant interactions of Pb $\times$ stress occurred in both genders for changes in NE levels, in which increases were Pb-concentration and stress-condition related (Pb $\times$ stress: p=0.0317 and 0.0002 for females and males, respectively). In females, significant elevations in NE levels were found in the 50MS, 50MS+OS, 150NS, 150MS and 150 MS+OS groups, with increases ranging from 165-182% relative to 0NS, 0MS and 0MS+OS groups. Potentiated effects were seen in both the 50MS and 50MS+OS groups (i.e., no effects of 0MS, 0MS+OS, 50NS). For male offspring, increases were found in the 50MS+OS, 150NS and 150MS+OS groups that ranged from 140-160%, again consistent with a Pb-concentration and stress-condition related effect. These increases are potentiated effects in the 50MS+OS group in that no effects of 0MS+OS or 50NS were found.

Nucleus Accumbens Catecholamines: Females: Despite what appear to be elevations, there were no statistically significant changes in levels of DA or DOPAC in female offspring (Figure 5, top row). In contrast, a significant Pb $\times$ stress interaction was found for HVA (p=0.050) and NE levels (p=0.0258). In the case of HVA, potentiated increases occurred in the 150MS+OS group where increases of approximately 100% were observed relative to 0NS levels, whereas no effects were found in the 0MS+OS or 150NS groups. Marked increases in NE were observed in the 50MS, 50MS+OS and 150NS groups (ranging from 276 to 278%). Interestingly, in the 150 ppm groups, only Pb exposure alone was associated with increases, while levels in the 150MS and 150MS+OS groups did not differ from 0NS controls.

Males: In the case of male offspring, general increases in levels of DA and metabolites and DA TO were confirmed statistically, but in all cases Pb $\times$ stress interactions were evident (p=0.006, 0.04, 0.019 and 0.0386, for DA, DOPAC, HVA and DA TO, respectively). Increases in DA were generally Pb-concentration and stress condition-related, with increases of 160-185% found in the 50MS, 50MS+OS, 150NS and 150MS+OS groups. Potentiated effects were found in both the 50MS and 50MS+OS groups, with no changes in the corresponding stress and Pb only groups. Similar potentiated effects of 50MS were found for DOPAC and HVA, while changes in DA TO groups were not systematic. In the case of NE, a Pb-related effect was confirmed (p=0.0006), with increases observed only in response to 150 ppm (collapsed across stress groups).

Striatal Catecholamines: Females: The impact of Pb and stress on striatal levels of DA and metabolites and DA TO in females was not systematic (Figure 6, top row). No significant changes in levels of DA or HVA were found. An interaction of Pb $\times$ stress (p=0.0081) for

DOPAC reflected an increase in control levels that was not observed in the 50MS+OS or 150MS+OS groups. The effects on DA TO reflected Pb exposure ($p=0.0048$), with elevations in response to 50 ppm (collapsed across stress groups).

Males: For male offspring, levels of DA, DOPAC, HVA and DA TO were Pb exposure and stress condition-related, with marked elevations in levels observed in the 50MS, 50MS+OS, 150NS, 150MS and 150MS+OS groups (Pb $\times$ stress: $p=0.039$, <0.0001 , <0.0001 , and $p=0.0166$, respectively). Increases in levels of DA across these groups ranged from 149-229%, with corresponding changes of 154-264% and 287-572%, respectively, for DOPAC and HVA. Potentiated changes were observed for 50MS+OS DA, 50MS DOPAC, 50MS and 50MS+OS HVA and 50MS DA TO groups.

5-HT and 5-HIAA Levels Across Regions: Females: Increases in levels of 5-HT and/or its metabolite 5-HIAA in females were found in all brain regions (Figure 7). In frontal cortex, significant increases in 5-HT (Pb $\times$ stress: $p=0.0448$) were produced by 50MS and 150MS +OS, with marginal increases in the 50MS+OS, 150NS and 150MS groups. These increases ranged from 164-181% and were potentiated effects in the 50MS group, given the absence of effects seen in the 0MS and 50NS groups. A highly similar pattern of changes was seen with 5-HIAA (although statistically this was confirmed only as a main effect of Pb ($p=0.0015$) with increases on the order of 158-196% across the 50 and 150 ppm groups. Elevations in 5-HT and 5-HIAA ranging from 151-167% were also seen in nucleus accumbens in females, but these effects achieved statistical significance only for 5-HIAA (Pb by stress: $p=0.0486$) with increases seen in the 0MS+OS, 50MS and 50MS+OS groups. Elevations in levels of 5-HT and 5-HIAA in striatum ranging from 121-208% and 120-163%, respectively, were produced by 50 and 150 ppm Pb exposure per se ($p=0.00013$ and 0.0072 , respectively), with no difference between the two concentrations.

Males: Male offspring likewise evidenced increases in levels of 5-HT and 5-HIAA with a pattern that differed in various brain regions. Frontal cortex 5-HT was significantly increased by 117-151% in response to 150 ppm (collapsed across stress groups; $p=0.0071$). A more complex pattern was observed for 5-HIAA that included a Pb $\times$ stress interaction ($p=0.0002$), where increases ranging from 134-174% were observed in the 50MS, 150NS and 150MS+OS groups, and potentiated increases were evident for 50MS and 150MS+OS groups. In nucleus accumbens, a Pb $\times$ stress interaction for 5-HT reflected the decrement in levels in the 0MS+OS and 150 MS+OS groups relative to their 0NS and 150NS respective controls, while potentiated increases were seen in the 50MS+OS group as compared to 50NS and 0MS+OS. 5-HIAA levels in nucleus accumbens were significantly increased by 150 ppm (collapsed across stress groups; main effect of Pb= 0.0412) as a result of modest increases of 112-123%. Very marked increases in both 5-HT and 5-HIAA were found in striatum in male offspring that were Pb-concentration and stress-condition-related. Specifically, increases in 5-HT of 200-279% of control were found across the 50MS+OS, 150NS, 150MS and 150MS+OS groups, with changes in the 50MS +OS group representing potentiated effects. For 5-HIAA, increases of 172-264% of control were found across the 50MS, 50MS+OS, 150NS, 150MS and 150MS+OS groups, with these effects representing potentiated effects in the 50MS and 50MS+OS groups.

Corticosterone Levels

Basal Corticosterone and the Impact of Behavioral Testing: Basal corticosterone levels were determined in FI and NFI rats at approximately 4-5 mos of age, prior to the imposition of the first stress challenge to offspring, and resulting values presented in Figure 8 for female (left column) and male (right column) FI (top) and NFI (bottom) offspring.

Females: FI Rats: Basal corticosterone levels of female FI rats were not altered by Pb exposure, stress, or the combination; the slightly higher values of the MS offspring were not significantly different from ONS controls.

NFI Rats: Littermates of females not used in behavioral testing showed a different pattern of basal corticosterone levels, with increases due to Pb exposure ($p=0.0007$) and reductions in response to MS ($p=0.0254$). Basal corticosterone values of the 150NS group were elevated 48% above those of the ONS group. In the MS groups, the increase in the 150MS group relative to OMS control was only 37% and values of the OMS group were reduced approximately 20% relative to ONS controls.

Males: FI Rats: Pb and stress did alter corticosterone values of male FI offspring (top right: $Pb=0.0174$, $Pb \times stress=0.0252$). This was primarily the result of increased basal corticosterone in the 150MS group relative to 150NS (49%), whereas decreases in basal corticosterone levels were seen in both the OMS (32%) and the 50MS (26%) groups relative to their ONS and 50NS counterparts.

NFI Rats: Male NFI rats exhibited a pattern somewhat similar to that exhibited by behaviorally-tested counterparts but that was actually more pronounced in magnitude (cf. top right vs. bottom right). Specifically, a U-shaped concentration-effect curve was confirmed statistically ($Pb: p=0.0077$) reflecting lower values of the 50 ppm groups relative to 0 and 150 ppm groups. In this case, however, there was neither an influence of MS, nor an interaction of Pb by MS as seen with FI males.

Final Corticosterone Values: Final corticosterone levels were determined following the completion of behavioral testing, at approximately 8-9 mos of age, and resulting values presented in Figure 9 for female (left) and male (right) FI rats (top row). For comparative purposes, corresponding values from NFI littermates without experience on the FI schedule are also depicted (bottom row).

Females: FI Rats: Final corticosterone values for female FI rats (top left) showed a Pb exposure-concentration relationship that was not impacted by MS or by MS+OS (main effect of $Pb=0.03$). Specifically, levels in the 150 ppm groups (collapsed across stress condition) exceeded those of both the 0 and 50 ppm groups, with the latter two groups differing marginally from each other ($p=0.08$). Values of the 150NS group exceeded those of controls by 220%. Corresponding values for the 150MS and the 150MS+OS groups were 186% and 134%.

NFI Rats: The pattern of Pb- and stress-related differences on corticosterone levels among NFI females differed markedly from those among FI females (bottom left). A $Pb \times stress$ interaction ($p=0.0036$) largely reflected the decrease in corticosterone values in the 50MS+OS and 150MS+OS groups relative to OMS+OS, reductions that were not seen in either the NS or MS Pb-treated groups. In addition, notable reductions were found in the 50MS+OS group relative to both the 50NS and 50MS groups.

Males: FI Rats: Male FI rats (top right) showed no statistically significant differences in final corticosterone values related to Pb or stress conditions.

NFI Rats: A similar effect to that observed with NFI females was found in NFI males in the reductions in corticosterone levels of the 50MS+OS and 150MS+OS groups relative to OMS+OS. In addition, a marked increase in corticosterone values was observed among males in the 50MS group relative to OMS, 50NS and to 150MS groups.

Relationships Among Outcome Measures—Principal component analyses (PCA) were used to explore relationships among the outcome measures to suggest mechanisms for Pb/stress-induced FI response rate changes. Analyses were done separately for each of the 9 treatment groups that included FI response rates from a selected session, final corticosterone values, and levels of neurotransmitters for which statistically significant changes were confirmed. Table 1 summarizes the resultant factor loadings for female groups and the cumulative percent loading for each fit.

For controls (ONS), the primary component in the analysis was based totally on frontal cortex neurotransmitters, and included DA, DOPAC, NE, 5HT and 5HIAA. A second component was based on nucleus accumbens neurotransmitter levels, and included DOPAC, HVA and 5HIAA. A third component was based on nucleus accumbens NE and striatal 5-HT levels. The fourth component included final corticosterone levels, and frontal cortex HVA levels. A final explanatory component involved FI overall response rates.

All treatments, including Pb, stress, or Pb + stress altered this profile of components. One commonality in all of the Pb/stress-treated groups was the involvement of STR transmitters as components accounting for variance in the data. In all groups, a significant involvement of striatal (STR) DOPAC, STR DATO and/or STR 5HIAA was found, whereas none of these contributed to ONS outcomes. For the 50MS+OS group, for example, the group showing the most protracted changes, these striatal neurotransmitter contributed to the third and 6th component of the PCA. For both the 150NS and 150MS+OS groups, these variables were even more significant, contributing to the second component and displacing nucleus accumbens neurotransmitter systems which were the dominant feature of the second component in the ONS group.

In addition, the primary component for each of these groups (that explaining the largest amount of variance) differed from the ONS control. Further, in the three groups that exhibited the highest rates (50MS+OS, 150NS and 150MS+OS), the reduction in explanatory power of FC NE was the greatest. For the 50MS+OS group, frontal cortex (FC) NE and FC 5HT assumed less explanatory power, moving from component 1 to components two and four. Moreover, frontal cortex 5HIAA had no contribution, with FC DOPAC and FC HVA instead assuming primary explanatory roles. For the 150NS group, FC DA moved from the 1st to the 5th component, while FC HVA is a member of the 1st rather than the 4th component, as was the case in the ONS group. In the 150MS+OS group, FC NE drops from the 1st to the 3rd component, and FC DOPAC moved from no explanatory power to the 1st component.

Discussion

These studies demonstrate that a history of stress exposure, specifically maternal followed by offspring stress, as would typify human environments, can enhance the adverse effects of maternal Pb exposure on central nervous system function. The nature of the changes across groups represents a type of 'dose'-related phenomenon, where dose includes Pb concentration and stress condition. This enhancement was particularly evident in behavioral performance of female offspring on a FI schedule of reinforcement, but was also evident in neurochemical changes in both genders. For some measures, 50 ppm Pb exposure, associated with a mean blood Pb value of only 11 ug/dl, was associated with adverse effects only when coupled with an MS+OS history. Indeed, 50MS+OS generally produced effects of comparable magnitude to those associated with 150NS. Increases in FI response rates in females followed this pattern. Increases in FC NE in female offspring were not found in the 50NS group, but were seen in both the 50MS and 50MS+OS groups as well as in the 150NS, 150MS and 150MS+OS groups. Similarly, increases in frontal cortex 5-HT levels were observed only in combined Pb+stress groups. Potentiated effects were observed for HVA in nucleus accumbens, such as the

observation that only 150MS+OS produced significant changes. These types of Pb concentration+stress condition dependent effects were also reflected in changes in FC NE, NAC DA and DOPAC, STR DA, and FC and STR 5-HT and 5-HIAA among male offspring. Potentiated effects are particularly notable given that they can be obscured by underlying differences in the slopes and efficacy of the dose-effect curves for each variable alone. For example, a steep and highly efficacious dose-effect curve for Pb combined with a shallower, lower efficacy dose-effect curve for stress can preclude the ability to observe synergistic activity or even appear as a reduction of effects.

This phenomenon of Pb/stress synergism could be interpreted either as an unlocking by stress experienced later in life of 'silent' changes induced by maternal stress, or as a potential 'cumulative' effect of maternal Pb/ stress combined with offspring stress. Since the design of the experiment did not include an offspring stress only group, it is difficult to ascertain the specific contribution to this vulnerability. While inclusion of such a group was initially considered in the experimental design, its relevance to human scenarios seemed more remote and would have increased experimental scope beyond what was logistically feasible. Nevertheless, the current findings clearly demonstrate that the interaction of stress with 50 ppm Pb exacerbates adverse effects. Importantly, PbB levels of 11ug/dl are consistent with the CDC's designated level of concern for children of 10 ug/dl. Moreover, it is important to point out that the effects at these levels do not represent thresholds for such interactions, but simply the lowest levels yet examined in these animal models.

The use of variable stress challenges in this study was designed to prevent habituation of any stress impact and maximize the likelihood of observing group differences. Different stressors activate different systems. For example, immobilization stress is thought to activate the HPA axis through both paraventricular noradrenergic and extra-paraventricular noradrenergic mechanisms, whereas cold stress acts through the hypothalamo-pituitary-thyroid axis to re-establish homeostasis (Pacak and Palkovits 2001). It was therefore possible that differential effects on FI would emerge with different stressors. However, while such differences in FI performance did occur on the day of the stress challenge (to be reported elsewhere), they were not persistent as reflected in any differential changes in group performance between stressors.

Differential vulnerability by offspring gender to effects of prenatal stress has been reported in many studies (Weinstock 2007). Several possibilities could contribute to the preferential effects of Pb and stress on FI performance of female offspring. One includes gender-based differences observed in the neurochemical changes in mesocorticolimbic dopamine/glutamate systems. Using regional microinjections, we have shown NAC and FC to play a critical role in mediating FI response rates (Cory-Slechta et al. 1996; Cory-Slechta et al. 1997b; Cory-Slechta et al. 1998; Cory-Slechta et al. 2002) wherein an inverse U-shaped dose effect curve relates NAC DA levels to FI response rates, with both low and high DA concentrations inducing rate suppression and intermediate DA levels increasing FI rates. Further, microinjections of DA1 and DA2 receptor antagonists into NAC concurrently with lidocaine or saline into either agranular insular or prelimbic areas of FC showed that prelimbic cortex exerted a tonic influence on FI performance through its interactions with NAC, whereas the agranular insular cortex modified NAC control of FI performance (Evans and Cory-Slechta 2000). Consistent with these findings, PCA used here to explore relationships between patterns of neurochemical and FI performance changes found that the primary component explaining variance for normal non-treated (ONS) female offspring was FC DA, DOPAC, NE, 5-HT and 5-HIAA. FC catecholamine changes were also associated with FI response rates in similar PCA analyses in our previous study (Cory-Slechta et al. 2004).

Females did appear to exhibit a greater sensitivity to Pb/ stress-induced changes in FC catecholamines. Pb-associated increases in levels of DA, DOPAC and HVA were found in

both genders, but occurred at lower Pb/stress levels in females. Specifically, increased FC DA levels in females occurred in all three 50 and 150 ppm groups, but only in the 50MS+OS group for males. Similarly increased FC DOPAC and HVA levels were found in females in all three 50 and 150 ppm groups, but only in the 150 ppm groups in males. Thus, even though the magnitude of increases was similar in both genders, females may sustain a lower threshold for such changes to induce behavioral dysfunction.

Interestingly, in PCA analyses, FC catecholamines in the highly affected 50 MS+OS, 150NS and 150 MS+OS groups assumed a less prominent role in explaining variance of data relative to the 0NS group, and suggested as well an important role for STR catecholamines in the FI performance changes of females, with STR levels of DOPAC and 5-HIAA and of DA turnover contributing to factor loading in all groups other than controls (Table 1). STR catecholamine changes contributed to the 3rd and 5th components of the 50MS+OS group, and to the 2nd component for the 150NS and 150MS+OS groups. Given this, it might seem inconsistent that no systematic STR changes were found in female FI rats when measured at the termination of behavioral testing. However, neurochemical determinations made from 2-3 mos of age females, i.e., prior to the initiation of behavioral testing and offspring stress challenges, did reveal STR catecholamines and 5-HT and 5-HIAA changes consisting of Pb-related increases in levels of these neurotransmitters, in conjunction with an MS-induced increase in controls (OMS) that was blunted in Pb+MS treated offspring (Pb $\times$ stress interactions for DA, DOPAC and HVA). The absence of changes following behavioral testing could indicate that such effects had adapted and that alterations in system dynamics might be observed in other functional indices such as autoradiographic assessment of receptors. Future studies will be required to address these issues.

Data from this study do not suggest, however, a simple relationship between neurochemical and behavioral changes. In fact, males showed what may be interpreted as a broader (and presumably permanent) array of changes in brain neurochemistry than females, with significant changes in FC, NAC and STR. Significant changes in brain catecholamines in both NAC and STR, the terminal projection regions of the mesolimbic and nigrostriatal dopamine pathways, respectively, were generally restricted to males. Both male and female FI rats exhibited increased FC catecholamine levels, and even though females tended to show greater sensitivity to such changes, not all female groups that showed changes in neurotransmitter levels also showed elevations in FI response rates. Moreover, females showed virtually no changes in NAC catecholamines, unlike males.

Another potential basis to consider for gender differences in FI groups is ratedependency. Numerous studies over the years have demonstrated that low rates of FI responding are increased, while higher rates are unaffected or decreased in response to a many different psychoactive compounds (Dews 1958). FI response rates of female 0NS rats were approximately half of those of male 0NS rats (Figure 1). Thus, if Pb+stress resulted in rate-dependent effects, it might be expected that increased rates would occur in females, with either no effects, or even decreased rates in males. In support of this possibility, we reported (in a study examining only male rats) that administration of DA itself into STR decreased FI response rates of high baseline rate subjects, and increased response rates of low baseline rate subjects (Cory-Slechta et al. 2002). One mechanism to address this question would have been to adjust FI schedule parameters in males vs. females to produce comparable absolute overall response rates. However, this would induce other difficulties of interpretation as well. It is also possible that potential FI rate increases in males could have been offset by behaviors incompatible with lever pressing that may have been evoked by the marked increases in STR catecholamines that occurred in males.

Ascending serotonergic systems appear to contribute to glucocorticoid-induced HPA programming prenatally (Slotkin et al. 1996; Dean and Matthews 1999; Meaney et al. 2000; Slotkin et al. 2006). Changes in levels of 5-HT and metabolites have been reported in response to prenatal stress, as have behavioral changes in response to administration of serotonergic compounds (Ishiwata et al. 2005). Maternal Pb exposure per se has also been shown, in some studies, to influence levels of serotonergic compounds (Szuran et al. 2000; Cory-Slechta et al. 2004). Thus, a potential role for 5HT in the effects of Pb/stress on FI response rates is interesting to consider, and changes in levels of 5-HT and 5-HIAA were seen almost uniformly across regions in both genders. While the specific profile of changes differed by gender, the increases in FC and NAC were slightly greater in females when calculated as percent of control. As yet, however, the ability of serotonergic systems to influence FI performance, and thus its ability to account for the changes in FI performance in females, remains unknown.

Our prior study of maternal Pb and maternal and offspring stress involving comparisons by gender at 21 days of age and in behaviorally-tested offspring in adulthood revealed quite distinct gender differences. In these studies, effects of Pb, stress and the combination also differed by brain region in each gender (Cory-Slechta et al. 2004). Of course, comparisons of changes in neurotransmitter levels or any outcome measures across these studies are complicated by differences in Pb/stress parameters. For example, the current study involved pair housing of animals, while subjects in the previous study were housed individually, a paradigm that itself is considered a stressor that can notably influence outcomes (Cabib et al. 2002; Francolin-Silva and Almeida 2004). Another cautionary note is that neurotransmitter changes were measured at only a single time point in FI rats rather than in a dynamic pattern across time. Thus, any conclusions about the nature and extent of such changes is necessarily limited given that changes may only be manifest with age, and/or the pattern of effects may change with time. Sloboda et al. (Sloboda et al. 2002), for example, found elevated plasma cortisol in offspring of sheep following maternal betamethasone injection at 1 year of age, but not at 6 mos of age.

Finally, the preferential FI changes in females may reflect *in utero* exposure to a higher level of corticosterone from maternal stress. Montano et al. (Montano et al. 1993) found 58% higher baseline serum corticosterone concentrations following maternal stress in female fetuses as compared to males at day 18 of pregnancy that was attributable to a greater passage of corticosterone across the placenta of females. Thus, it is possible that a higher level of maternal stress (higher maternal corticosterone levels) would increase FI response rates in males.

A critical role for behavioral history is indicated by the notable differences in both basal and final corticosterone levels between FI and NFI groups (Figures 8-9). Corticosterone levels of NFI offspring of both genders were greater than those of FI littermates. In addition, patterns of Pb/stress effects differed in FI and NFI offspring. It is likely that these differences reflect at least in part the more regular handling of FI groups and the associated experience in multiple environments. These findings have specific implications with respect to experimental design of stress studies. First, they demonstrate that the use of non-behaviorally tested littermates as a source of mechanistic information on time course of changes in biochemical outcomes to relate to behavioral changes is potentially misleading. In our studies, it indicates that Pb, stress or Pb/stress effects determined in non-behaviorally trained animals cannot necessarily be generalized to animals with behavioral histories. Only additional behaviorally tested littermates can be used to provide such information.

Current screening requirements for elevated blood Pb levels focus on children. The data from this study, however, clearly indicate the need to screen pregnant women, particularly those with high-stress lifestyles, given the apparent permanent changes in behavior and in neurochemical and corticosterone function conferred by maternal Pb/stress to offspring.

Results from such studies may have particular significance for understanding the true health risks posed by Pb for human populations, especially since the cycles of poverty and elevated Pb are so congruous. Indeed, it can be asserted even more globally that a full understanding of the true risk posed by all environmental toxicants will ultimately require assessments of their interactions with other environmental and genetic risk factors.

Acknowledgements

Supported in part by ES05017 (D. Cory-Slechta, PI)

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Figure 1.

Overall response rates (responses per minute) in female (top row) and male (bottom row) offspring exposed maternally to 0 (left column), 50 (middle column) or 150 ppm (right column) Pb (NS groups) and/or maternal stress (MS groups) and/or offspring stress (MS+OS groups) as indicated. Each data point represents a mean $\pm$ 1 S.E. value over a block of sessions (females: pre-restraint-6, restraint to cold-4, cold to novelty-5, post novelty-5; males: pre-restraint 5,6, restraint to cold-7, cold to novelty-7, post novelty-5). For comparative purposes, control group (0 ppm NS) performance is shown in each plot as a solid line with dashed lines representing 1 S.E. Interruptions in the x axis represent points where offspring stress challenges were carried out including restraint stress (females and males: post block 2), cold stress (females: post block 4; males: post block 3) and novelty stress (females: post block 6; males: post block 4). Sample sizes ranged from 9-11 for male and female groups. The 3 horizontal lines without symbols in the 50 ppm and 150 ppm plots show 0NS group mean $\pm$ SE values for comparative purposes.

Figure 2.

Run rates (responses per minute after subtraction of PRP values) in female (top row) and male (bottom row) offspring exposed maternally to 0 (left column), 50 (middle column) or 150 ppm (right column) Pb (NS groups) and/or maternal stress (MS groups) and/or offspring stress (MS +OS groups) as indicated. Each data point represents a mean $\pm$ 1 S.E. value over a block of sessions (females: pre-restraint-6, restraint to cold-4, cold to novelty-5, post novelty-5; males: pre-restraint 5,6, restraint to cold-7, cold to novelty-7, post novelty-5). For comparative purposes, control group (0 ppm NS) performance is shown in each plot as a solid line with dashed lines representing 1 S.E. Interruptions in the x axis represent points where offspring stress challenges were carried out including restraint stress (females and males: post block 2), cold stress (females: post block 4; males: post block 3) and novelty stress (females: post block 6; males: post block 4). Sample sizes ranged from 9-11 for male and female groups. The 3 horizontal lines without symbols in the 50 ppm and 150 ppm plots show 0NS group mean $\pm$ SE values for comparative purposes.

Figure 3.

PRP times (seconds) in female (top row) and male (bottom row) offspring exposed maternally to 0 (left column), 50 (middle column) or 150 ppm (right column) Pb (NS groups) and/or maternal stress (MS groups) and/or offspring stress (MS+OS groups) as indicated. Each data point represents a mean $\pm$ 1 S.E. value over a block of sessions (females: pre-restraint-6, restraint to cold-4, cold to novelty-5, post novelty-5; males: pre-restraint 5,6, restraint to cold-7, cold to novelty-7, post novelty-5). For comparative purposes, control group (0 ppm NS) performance is shown in each plot as a solid line with dashed lines representing 1 S.E. Interruptions in the x axis represent points where offspring stress challenges were carried out including restraint stress (females and males: post block 2), cold stress (females: post block 4; males: post block 3) and novelty stress (females: post block 6; males: post block 4). Sample sizes ranged from 9-11 for male and female groups. The 3 horizontal lines without symbols in the 50 ppm and 150 ppm plots show 0NS group mean $\pm$ SE values for comparative purposes.

Frontal Cortex

Figure 4.

Levels of catecholamines (ng/mg protein) in frontal cortex of female (top row) and male (bottom row) offspring. Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = 0 ppm, □ = 50 ppm; ▒ = 150 ppm. NS=no stress, MS=maternal stress, MS+OS=maternal and offspring stress. Pb=main effect of Pb, S=main effect of stress; Pb x S=interaction of Pb by stress in statistical analysis. DA=dopamine; DOPAC=dihydroxyphenylacetic acid; HVA=homovanillic acid; DA TO= dopamine turnover, DOPAC/DA; 5-HT=serotonin; 5-HIAA=5 hydroxyindoleacetic acid. *signifies different from 0 ppm NS; # signifies different from corresponding Pb NS group; + signifies different from corresponding 0 ppm stress group. The horizontal line across values represents 0NS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

Nucleus Accumbens

Figure 5.

Levels of catecholamines (ng/mg protein) in nucleus accumbens of female (top row) and male (bottom row) offspring. Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = 0 ppm, □ = 50 ppm; ▒ = 150 ppm. NS=no stress, MS=maternal stress, MS+OS=maternal and offspring stress. Pb=main effect of Pb, S=main effect of stress; PbXS=interaction of Pb by stress in statistical analysis. DA=dopamine; DOPAC= dihydroxyphenylacetic acid; HVA=homovanillic acid; DA TO= dopamine turnover, DOPAC/DA; 5-HT=serotonin; 5-HIAA=5 hydroxyindoleacetic acid. * signifies different from 0 ppm NS; # signifies different from corresponding Pb NS group; + signifies different from corresponding 0 ppm stress group. The horizontal line across values represents 0NS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

Striatum

Figure 6.

Levels of catecholamines (ng/mg protein) in striatum of female (top row) and male (bottom row) offspring. Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = 0 ppm, ▨ = 50 ppm; ▩ = 150 ppm. NS=no stress, MS=maternal stress, MS+OS=maternal and offspring stress. Pb=main effect of Pb, S=main effect of stress; Pb $\times$ S= interaction of Pb by stress in statistical analysis. DA=dopamine; DOPAC= dihydroxyphenylacetic acid; HVA=homovanillic acid; DA TO= dopamine turnover, DOPAC/DA; 5-HT=serotonin; 5-HIAA=5 hydroxyindoleacetic acid. * signifies different from 0 ppm NS; # signifies different from corresponding Pb NS group; + signifies different from corresponding 0 ppm stress group. The horizontal line across values represents ONS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

5HT and 5HIAA

Figure 7.

Levels of 5-HT and 5-HIAA (ng/mg protein) in frontal cortex (left column), nucleus accumbens (middle column) and striatum (right column) of female (top row) and male (bottom row) offspring. Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = 0 ppm, ▨ = 50 ppm; ▩ = 150 ppm. NS=no stress, MS=maternal stress, MS+OS=maternal and offspring stress. Pb=main effect of Pb, S=main effect of stress; Pb $\times$ S= interaction of Pb by stress in statistical analysis. DA=dopamine; DOPAC= dihydroxyphenylacetic acid; HVA=homovanillic acid; DA TO= dopamine turnover, DOPAC/DA; 5-HT=serotonin; 5-HIAA=5 hydroxyindoleacetic acid. * signifies different from 0 ppm NS; # signifies different from corresponding Pb NS group; + signifies different from corresponding 0 ppm stress group. The horizontal line across values represents 0NS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

Baseline Corticosterone

Figure 8.

Basal corticosterone values (ng/ml) of female (left column) and male (right column) offspring. The top row depicts values from offspring used in FI behavioral performance evaluation; the bottom row shows values from littermates with no behavioral history (NFI). Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = no stress groups, ▨ = maternal stress groups. Pb= main effect of Pb; S=main effect of stress; Pb x S=interaction of Pb and stress in statistical analyses. Post-hoc group differences are shown by connecting lines. Baseline corticosterone was collected prior to the first offspring stress challenge and thus no MS+OS groups are shown. The horizontal line across values represents ONS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

Final Corticosterone

Figure 9.

Final corticosterone values (ng/ml) of female (left column) and male (right column) offspring. The top row depicts values from offspring used in FI behavioral performance evaluation; the bottom row shows values from littermates with no behavioral history (NFI). Each bar represents a group mean $\pm$ S.E. value for the groups as indicated. ■ = 0 ppm groups, ▨ = 50 ppm groups, ▩ = 150 ppm groups; C=no stress; MS=maternal stress; OS=maternal stress and offspring stress. Pb= main effect of Pb; S=main effect of stress; Pb x S=interaction of Pb and stress in statistical analyses. Post-hoc group differences are shown by connecting lines. The horizontal line across values represents ONS control group mean values for comparative purposes. Sample sizes ranged from 8-11 for male and female groups.

Table 1

Summary of Principal Component Analysis for Females

	CPT1 ³	CPT2	CPT3	CPT4	CPT5	CPT6	Missing Relative to ONS	Added Relative to ONS
0NS ¹ (88.57%) ²	FC NE ⁴	NA DOPAC	NA NE	FC HVA	RATE			
	FC 5HT	NA HVA	STR 5HT	CORT				
	FC HIAA	NA HIAA						
	FC DA							
0MS (93.01%)	FC DOPAC							
	NA DOPAC	FC 5HT	FC DA	CORT	FC HVA		FC DOPAC	STR DOPAC
	NA HVA	FC HIAA	FC NE	RATE			NA HIAA	STR DATO
	STR DOPAC	NA NE						STR HIAA
0MS+OS (87.67%)	ST DATO							
	FC DA	FC HVA	NA NE	STR DOPAC	RATE		NA HVA	STR DOPAC
	FC DOPAC	NA DOPAC	STR DATO	STR 5HT			NA HIAA	STR DATO
	FC NE	CORT		STR HIAA				STR HIAA
50NS (85.21%)	FC 5HT							
	FC HIAA							
	FC DA	NA DOPAC	STR DOPAC	FC HVA			FC DOPAC	STR DOPAC
	FC NE	RATE	STR DATO	NA HVA			NA HIAA	STR DATO
50MS (92.67%)	FC 5HT		STR HIAA				NA NE	STR HIAA
	FC HIAA						CORT	
	FC DA						STR 5HT	STR DOPAC
	FC NE	FC NE	NA HIAA	NA HVA	NA DOPAC	STR DOPAC	NA NE	STR DATO
50MS+OS (90.04%)	FC HVA	FC 5HT	STR DATO	RATE			STR 5HT	STR DOPAC
	FC DA	FC HIAA	STR HIAA				STR 5HT	STR DATO
	FC DOPAC	FC 5HT	STR 5HT	FC NE	STR DOPAC	STR DATO	FC HIAA	STR DOPAC
	FC HVA	NA DOPAC	STR HIAA	NA HVA	CORT		NA NE	STR DATO
150NS (91.22%)	FC HVA	NA HIAA			RATE		NA HVA	STR HIAA
	FC NE	STR DOPAC	NA DOPAC	NA NE	FC DA		STR 5HT	STR DOPAC
	FC 5HT	STR DATO	NA HIAA	CORT	FC DOPAC			STR DATO
					RATE			
150MS (88.34%)	FC HIAA							
	FC DOPAC	NA HIAA	NA HVA	STR DOPAC	FC DA		NA DOPAC	STR DOPAC
	FC HVA	STR DATO	STR 5HT	STR HIAA	RATE		CORT	STR DATO
	FC NE							STR HIAA
150MS+OS (88.7%)	FC 5HT							
	FC HIAA							
	NA NE							
	FC DA	STR DOPAC	NA NE	STR 5HT	NA HIAA	FC NE	FC 5HT	STR DOPAC
	FC DOPAC	STR DATO	CORT	RATE			NA DOPAC	STR DATO
	FC HIAA	STR HIAA					NA HVA	STR HIAA
							FC HVA	

¹ Groups: Pb(0, 50 or 150 ppm to dams; MS=maternal stress; MS+OS=maternal stress followed by stress to offspring² Rotation Sums of Squared Loadings cumulative percent.³ Components: CPT1= first component, CPT2= second component; CPT3=third component; CPT4=fourth component; CPT5=fifth component⁴ Outcome Measures: FC=frontal cortex, NA=nucleus accumbens; STR=striatum; DA=dopamine; DOPAC=dihydroxyphenylacetic acid, HVA=homovanillic acid, 5HT=serotonin; HIAA=5 hydroxyindoleacetic acid