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Alterations in glucocorticoid negative feedback following maternal Pb, prenatal stress and the combination: A potential biological unifying mechanism for their corresponding disease profiles

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ABSTRACT

Combined exposures to maternal lead (Pb) and prenatal stress (PS) can act synergistically to enhance behavioral and neurochemical toxicity in offspring. Maternal Pb itself causes permanent dysfunction of the body's major stress system, the hypothalamic pituitary adrenal (HPA) axis. The current study sought to determine the potential involvement of altered negative glucocorticoid feedback as a mechanistic basis of the effects in rats of maternal Pb (0, 50 or 150 ppm in drinking water beginning 2 mo prior to breeding), prenatal stress (PS; restraint on gestational days 16–17) and combined maternal Pb+PS in 8 mo old male and female offspring. Corticosterone changes were measured over 24 h following an i.p. injection stress containing vehicle or 100 or 300 μg/kg (females) or 100 or 150 μg/kg (males) dexamethasone (DEX). Both Pb and PS prolonged the time course of corticosterone reduction following vehicle injection stress. Pb effects were non-monotonic, with a greater impact at 50 vs. 150 ppm, particularly in males, where further enhancement occurred with PS. In accord with these findings, the efficacy of DEX in suppressing corticosterone was reduced by Pb and Pb+PS in both genders, with Pb efficacy enhanced by PS in females, over the first 6 h post-administration. A marked prolongation of DEX effects was found in males. Thus, Pb, PS and Pb+PS, sometimes additively, produced hypercortisolism in both genders, followed by hypocortisolism in males, consistent with HPA axis dysfunction. These findings may provide a plausible unifying biological mechanism for the reported links between Pb exposure and stress-associated diseases and disorders mediated via the HPA axis, including obesity, hypertension, diabetes, anxiety, schizophrenia and depression. They also suggest broadening of Pb screening programs to pregnant women in high stress environments.

Introduction

Recent studies demonstrate that exposure to lead (Pb) even below the currently designated 10 μg/dl blood Pb (PbB) level of concern can have detrimental consequences for both children and adults. No obvious threshold has been found for the IQ reductions associated with Pb in children. Indeed, the magnitude of IQ reduction at PbBs <10 μg/dl is actually steeper than corresponding reductions at higher PbBs (Canfield et al., 2003; Lanphear et al., 2005). In studies of adults, links between environmental Pb exposure and hypertension indicate that cumulative exposures to even low levels of Pb can increase risk (Cheng et al., 2001; Gerr et al., 2002) and PbBs <10 μg/dl have been associated with increased all-cause and cardiovascular mortality (Menke et al., 2006). At the same time, the profile of diseases and disorders associated with low level environmental Pb exposures has broadened to include obesity, diabetes, anxiety, schizophrenia and depression (Kim et al., 1995; Cheng et al., 2001; Gerr et al., 2002; Rhodes et al., 2003; Opler et al., 2004; Rajan et al., 2007).

In the U.S., the highest Pb exposures are sustained by low socioeconomic status (SES) communities, i.e., inner city medically-underserved households with income <$25,000 renting homes built before 1950. A recent report noted that 5% of children screened in New York City had PbBs >10 μg/dl. In some upstate cities (Buffalo, Rochester, Syracuse, Albany and Schenectady), corresponding figures were >20% (Haley and Talbot, 2004). The potential for adverse consequences of long-term Pb exposure is unfortunately only further enhanced by the low rate of follow up of such children after their initial identification (Kemper et al., 2005).

In addition to elevated Pb exposures, low SES communities also sustain higher levels of a variety of diseases and disorders (Dohrenwend, 1973; Starfield, 1982; Pappas et al., 1993; Anderson and Armstead, 1995; Stipek and Ryan, 1997), many of which correspond to those associated with Pb exposure (Signorello et al., 2007; Werner

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et al., 2007; Lawes et al., 2008; Margellos-Anast et al., 2008; Stansfeld et al., 2008). The increased incidences of diseases and disorders associated with low SES has been hypothesized to arise from higher levels of stress associated with such environments resulting in a chronic elevation of cortisol (Munck et al., 1984; Lupien et al., 2000; Lupien et al., 2001; Cohen et al., 2006). One study, for example, found that the greater the number of years spent living in poverty, the greater the elevation in overnight cortisol (Evans and Kim, 2007). Thus, Pb and stress show both congruence as risk factors in low SES populations, and commonalities in diseases and disorders with which each has been associated.

This is notable because Pb and stress can also both act on brain mesocorticolimbic dopamine systems (Diorio et al., 1993; Lowy et al., 1993; Piazza et al., 1996; Pokora et al., 1996; Zuch et al., 1998; Moghaddam, 2002), and on the hypothalamic–pituitary–adrenal (HPA) axis, the major stress response system of the body (Vyskocil et al., 1991c; Cory-Slechta et al., 2004a). This co-occurrence of Pb and stress as risk factors, and the commonality of their biological targets served as the rationale for investigations by our laboratory of their interactive and potentially synergistic effects in experimental models (Cory-Slechta et al., 2004a, 2008; Virgolini et al., 2005, 2006, 2008). Among other findings, these have revealed an enhancement by prenatal and offspring stress of maternal Pb-induced behavioral toxicity in females and potentiated changes in frontal cortical and nucleus accumbens catecholamines and serotonin levels in males and females, and permanent effects of Pb itself on HPA axis function at PbBs in dams averaging only 11 μg/dl at the time of weaning (Cory-Slechta et al., 2004a, 2008; Virgolini et al., 2008). Consistent with these findings, PbBs <10 μg/dl have also now been shown to be associated with altered adrenocortical responses to acute stress in children (Gump et al., 2008).

Corticosterone (cortisol in humans) is released from the adrenal cortex in response to stressors. Although the stress response has evolved to protect the organism against potential danger, prolonged exposure to a stressor and/or an inability to rapidly return to basal cortisol levels after the stressor has been removed, can be detrimental (McEwen, 2007). Indeed, elevated cortisol levels are associated with a variety of SES-associated diseases and disorders, including Cushing's disease, diabetes, metabolic syndrome, hypertension, depression and schizophrenia. In addition, chronic elevation of corticosterone can result in hippocampal neuronal loss and impaired cognitive function (McEwen and Sapolsky, 1995).

Release of glucocorticoids is terminated by a classic negative feedback loop, whereby the unbound form inhibits any further adrenocorticotrophic hormone (ACTH) release from the pituitary gland. This inhibition is further facilitated by glucocorticoid binding to specific brain regions rich in glucocorticoid receptors, such as the hippocampus and other structures of the limbic system. This cascade of events is under tight control in a healthy organism, but aberrations of this system may have pathophysiological consequences (McEwen, 2007). The current study sought to evaluate the potential involvement of altered glucocorticoid negative feedback as a mechanistic basis of the observed behavioral and neurochemical consequences of maternal Pb, prenatal stress, and the combination, by measuring the time course of corticosterone changes over 24 h after a saline or dexamethasone injection stressor (DEX suppression test). DEX is a synthetic, high affinity glucocorticoid ligand that induces negative feedback by acting on the pituitary to suppress secretion of ACTH, and consequently, of corticosterone.

Methods and materials

Pb exposure

Three week old female Long Evans rats (Charles River, Germantown, NY) were randomly assigned to one of the following drinking solutions: 0 ppm (tap water), 50 or 150 ppm Pb acetate dissolved in distilled deionized water. Previous studies from our laboratory show 50 ppm to produce PbBs in our more recent studies of young adult males when exposure is initiated postweaning averaging 7–12 μg/dl (Brockel and Cory-Slechta, 1998; Cory-Slechta et al., 1998), just at and above the CDC's currently designated level of concern for children. These PbBs are also associated with similar behavioral deficits in rodents and children (Cory-Slechta, 1995). The 150 ppm exposure was associated with mean PbBs in dams ranging from 32.6±4.4 to 42.7 ±4.0 μg/dl and were used because our previous study demonstrated synergistic effects of this Pb exposure level with stress (Cory-Slechta et al., 2004b).

Pb exposure of dams was initiated two months prior to breeding, and was continued throughout lactation to ensure elevated bone Pb levels to produce a Pb body burden in dams (Cory-Slechta et al., 1987), consistent with human exposure. When females reached 3 mo of age, they were paired with 3 mo old male Long–Evans rats for breeding over a period of up to 8 days (two estrous cycles). Animals were housed in a vivarium room with a 12 h light–dark cycle (lights on at 0700 h) that was maintained at 22±5 °C. Standard rat chow diet was provided *ad libitum*. Pb exposure of the offspring of these dams ended at weaning. All procedures and treatments were in accord with NIH guidelines and were approved by the University of Medicine and Dentistry of New Jersey's University Committee on Animal Resources.

Breeding and prenatal 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 in each Pb-treated group were weighed and further randomly subdivided to a non-stress (NS) or prenatal stress (PS) sub-group (see below) and individually housed for the remainder of pregnancy and lactation. This resulted in six groups of Pb-stress conditions with the following sample sizes: 0NS (n=20), 0PS (n=23), 50NS (n=21), 50PS (n=31), 150NS (n=19) and 150PS (n=33).

On gestational days 16 and 17, dams assigned to PS groups were weighed and subjected to three 45 min restraint sessions (0900, 1200 and 1500 h) in plastic cylindrical devices using procedures modified from Ward and Weisz (1984). This procedure, or an even more protracted restraint stress, has been widely employed (Igosheva et al., 2007; Mairesse et al., 2007; Pallares et al., 2007; Baker et al., 2008; Darnaudery and Maccari, 2008). NS dams were weighed and subsequently left undisturbed in their home cages. The choice of days 16 and 17 was timed to correspond to the development of key brain regions associated with the endpoints under study. This protocol, as used in our previous study of Pb and stress, elevated corticosterone levels and altered catecholamine levels in frontal cortex and nucleus accumbens of dams (Cory-Slechta et al., 2004b). At the end of the last restraint session on GD16, blood was collected for corticosterone determinations. GD16 rather than GD17 was chosen for corticosterone determination 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 per litter, maintaining equal numbers of males and females wherever possible. Cross-fostering 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/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 male pups reached approximately 300 g and females 220 g. At this point, caloric intake was restricted to maintain the above-

stated body weights. Animals were paired in same gender combinations for the duration of the experiment.

Experimental procedures

At 5–6 mo of age, blood was collected from a tail nick in littermates of these offspring from all NS and PS groups for determination of basal corticosterone levels. Littermates were used to avoid a potential handling of offspring to be used for injection stress challenge.

At 8 mo of age, offspring from each group were randomly assigned to a group designated to receive a single i.p. injection stress challenge of dexamethasone phosphate (DEX; Sigma, St. Louis, MO) dissolved in physiological saline at a dose of 0 (vehicle), 100 or 150 μg/kg (males) or 0 (vehicle), 100 or 300 μg/kg (females). Because these animals had minimal handling during the 8 mo period following birth, the associated handling and DEX injection served as a stressor, as in prior studies (Lukic and Haldar, 1993; Persico et al., 1993; Johnson and Glick, 1994; Persico et al., 1995). The differential dosing by gender and the time points for blood collection were based both on pilot studies carried out prior to this experiment in our laboratory and on a report of the higher dose levels required to suppress corticosterone in females (Osborn et al., 1996). Only one pup/gender was used from each litter for each DEX dose to preclude any litter-specific effects. The DEX injections began at approximately 8 a.m. and were followed approximately 15 min later by a tail phlebotomy designated as 0 h time point. Additional tail phlebotomies were performed at 3 and 6 h after DEX administration. At 24 h post DEX administration, trunk blood was collected after decapitation without anesthesia. To prevent any additional stress, animals to be terminated were kept in a separate room until the time of decapitation. Serum was separated by centrifugation and stored at −20 °C until assayed.

Changes in fixed interval schedule controlled behavior, response to stress challenges, basal and final corticosterone levels and neurochemical changes following the termination of behavioral testing in littermates of the offspring used in the current experiments have been reported previously (Virgolini et al., 2008).

PbB determinations

PbB levels were measured by anodic stripping voltammetry according to methods described previously (Cory-Slechta et al., 1987, 2004a) in dams (n=7–8 for each group) and pups (n=3–9) at the time of offspring weaning (PND21) from each treatment group. For each sample, 100 μl of blood was collected in heparinized microtubes and transferred to tubes containing pre-assembled reagents. The limit of sensitivity of the assay is 5 μg/dl. Pup PbBs decline after weaning following maternal Pb exposure, such that they are below detection limits by 40 days of age (Widzowski and Cory-Slechta, 1994). Since animals in this experiment were 8 mo of age at the beginning of the procedures, pup PbBs were not followed after weaning.

Radioimmunoassay

Serum corticosterone concentrations were measured by radioimmunoassay (RIA) kits from MP Biomedical (Irvine, CA) with I^{125} as a tracer. The minimal detectable level of corticosterone was 7.7 ng/ml.

Statistical analyses

PbB levels

Overall analysis of PbBs of dams and pups, from blood collected at the time of weaning, was analyzed using ANOVAs with Pb, stress and group (dam, male offspring, female offspring) as between group factors. Subsequent lower order ANOVAs, based on the outcome of the overall ANOVA, were done separately comparing male offspring to dams and female offspring to dams, as well as ANOVAs that included all groups but a single Pb exposure concentration.

Basal corticosterone levels

For comparisons of basal corticosterone levels, three way ANOVAs (Pb, stress, gender) were initially carried out, followed, for each gender, by lower order ANOVAs with appropriate post-hoc tests depending upon outcomes as initially reported (Virgolini et al., 2008).

Time course of corticosterone changes

Given the differences in basal corticosterone (Fig. 1) in relation to Pb (males, females) and PS (females), all corticosterone determinations were calculated as a percent of basal values, as specified below for each analysis to address specific effects as listed.

Effects of Pb exposure on the corticosterone response following injection stress. Effects of maternal Pb per se on time course of corticosterone changes following stress challenge were evaluated using data obtained following the 0 μg/kg DEX dose. Data were first calculated as percent of mean basal corticosterone levels (Fig. 1) determined from littermates (Virgolini et al., 2008). For this purpose, values in

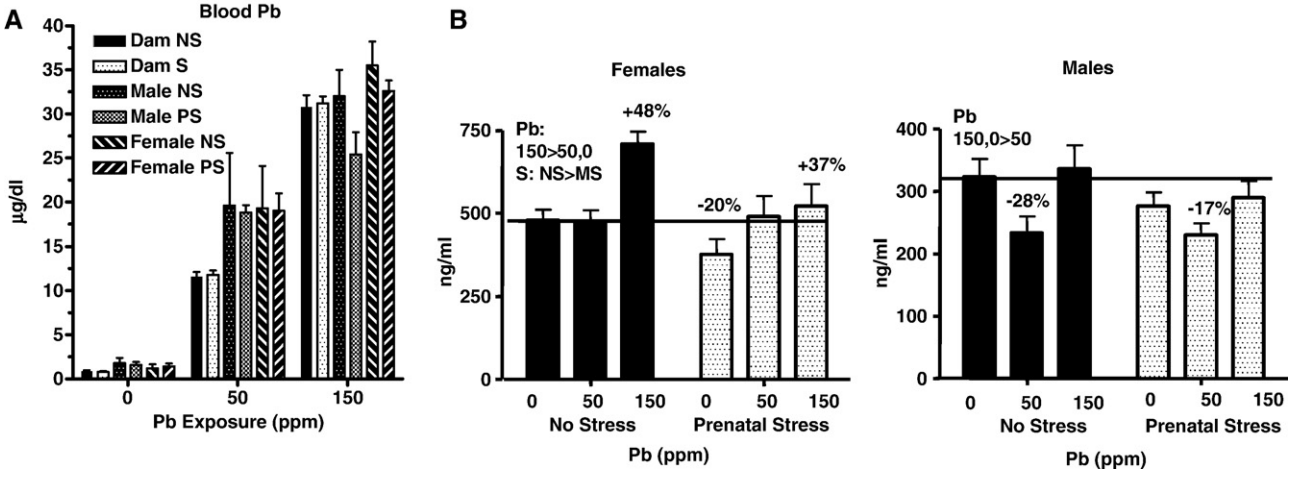

Fig. 1. Panel A: PbBs (μg/dl) of dams and male and female offspring in relation to Pb exposure concentration and stress condition (NS and PS). Each value represents a group mean ± S.E. Sample sizes ranged from 7 to 9 per treatment group in dams and from 2 to 8 in male offspring and 3–9 in female offspring (modified from Virgolini et al., in press). Panel B: Basal corticosterone values (ng/ml) of littermates of rats used in the current study as reported previously (Virgolini et al., 2008). Each bar represents a group mean ± S.E. value for the groups as indicated. ■ = no stress groups, ▒ = prenatal stress groups. Pb = main effect of Pb; S = main effect of stress; Pb × S = interaction of Pb and stress in statistical analyses. The horizontal line across values extends the 0NS control group mean values for comparative purposes. Sample sizes ranged from 8 to 11 for male and female groups.

each PbNS group at each time point were calculated as a percent of the basal PbNS values for each gender (i.e., 0NS values at 0, 3, 6 and 24 h as a percent of the group mean 0NS basal value, 50NS values at 0, 3, 6 and 24 h as a percent of the group mean 50NS basal value, etc.). An overall RMANOVA was then carried out with Pb and gender as between-group variables and time as a within-group variable. This analysis confirmed significant main effects of Pb and gender, as well as a Pb × gender and all time and Pb and gender interactions (including Pb × gender × time). RMANOVAs were then carried out separately for each gender. Significant interactions of Pb × time were confirmed for both genders. Subsequent analyses included lower order RMANOVAs for each Pb group or ANOVAs at each time point and post-hoc tests as appropriate.

Effects of PS on the corticosterone response following injection stress. Effects of PS alone and in conjunction with Pb exposure on time course of corticosterone changes following injection stress were evaluated using data obtained following the 0 μg/kg DEX dose. Data were first calculated as percent of the corresponding group mean basal corticosterone levels determined from littermates (Fig. 1; e.g., 50NS at 0, 3, 6 and 24 h as a percent of the group mean 50NS basal value; 50PS at 0, 3, 6 and 24 h as a percent of 50PS group mean basal value). An overall RMANOVA was then carried out that included Pb, gender and stress as between-group variables and time as a within-group variable. These confirmed significant main effects of Pb, stress, gender as well as lower and higher order interactions that included Pb × stress × gender × time. Subsequent RMANOVAs were then carried out separately for each gender that included Pb and stress as between-group variables and time as a within-group variable. These resulted in significant main effects of Pb and stress for both genders, and a Pb × stress interaction in females. Subsequently, lower order RMANOVAs were carried out for each Pb group with subsequent ANOVAs and post hoc tests as appropriate.

Effects of Pb and PS on corticosterone levels following DEX administration. Two approaches were used to evaluate the efficacy of DEX to suppress corticosterone following Pb, PS and combined Pb+PS. First, an overall RMANOVA was carried out using ng/ml corticosterone values that included Pb, stress, gender, and DEX as between-group variables and time as a within-group variable. Absolute ng/ml corticosterone values were used for these analyses as they focused on changes resulting from DEX doses relative to saline vehicle. Based on the interactions confirmed by that analysis, RMANOVAs that included Pb, stress and DEX as between-group variables and time as a within-group variable were carried out separately for each gender. Based on the confirmed interactions, lower order RMANOVAs were then carried out for each Pb-stress group separately with post-hoc tests as appropriate.

An additional analysis was run to compare the percent changes at each DEX dose in Pb-PS groups to the corresponding percent change observed in the 0NS control group at each DEX dose/time point. This allowed an assessment of how Pb, PS and Pb+PS altered the efficacy of DEX in relation to its effects in 0NS controls. For this purpose data for each DEX dose/time point were first calculated as a percent of the corresponding Pb-stress 0 μg/kg DEX value (e.g., 50NS 100 μg/kg at 0 h was calculated as a percent of 50NS 0 μg/kg 0 h; 150PS 100 μg/kg at 3 h as a percent of 150PS 0 μg/kg at 3 h) to provide a percent change from 0 μg/kg vehicle-associated corticosterone levels for each Pb-stress group. Post-hoc tests were then used to compare the resulting percent changes at a given DEX dose in the 50 and 150 ppm NS and PS groups and the 0PS group to the corresponding 0NS DEX dose change (e.g., percent change at 50NS, 50PS, 150NS and 150PS 100 μg/kg values to percent change at 0NS 100 μg/kg). These analyses were done separately for each time point (0, 3, 6 and 24 h) and provided a statistical comparison of DEX efficacy across experimental groups.

Potentiated effects were defined as an effect in a Pb+PS group in the absence of significant effects in the corresponding PbNS and 0PS groups alone. Synergistic effects were defined as an effect level in a Pb+PS group that was greater than that of the corresponding PbNS and 0PS groups. A p value of ≤0.05 was considered statistically significant for all analyses.

Results

Pb-related increases in PbB levels

PbB levels of dams and offspring measured at the time of pup weaning (n=7–9 dams per group and 3–9 offspring per group) increased in a concentration-related fashion, as previously reported (Virgolini et al., 2008; Virgolini et al., in press) are shown in Fig. 1A. Group mean PbBs (μg/dl) of all groups increased with increasing Pb exposure concentration. Overall ANOVA revealed significant main effects of Pb and group (both p values<0.0001) as well as a group × Pb interaction (p<0.0001), but no main effects or interactions that included stress. Subsequent lower-order ANOVAs confirmed that these effects were due to significantly higher PbBs of male and female offspring at 50 ppm, where levels of dams were approximately 12 μg/dl and those of pups approximately 19 μg/dl. At 150 ppm, PbBs of neither males nor female pups differed from offspring, but those of female offspring were higher than those of male offspring, an effect largely due to the slightly lower PbBs of the 150PS males relative to other groups, although there was no significant interaction with stress. Our prior studies demonstrate that pup PbBs decline after weaning following maternal Pb exposure, such that they are below detection limits by 40 days of age (Widzowski et al., 1994).

Lack of Pb or stress effects on litter parameters

No differences in numbers of pregnancies or length of pregnancies were found in relation to Pb and/or PS. Neither litter size nor sex ratios differed in relation to Pb, PS or combined Pb+PS. Given the numbers of litters generated, only a subset of litters, randomly chosen across

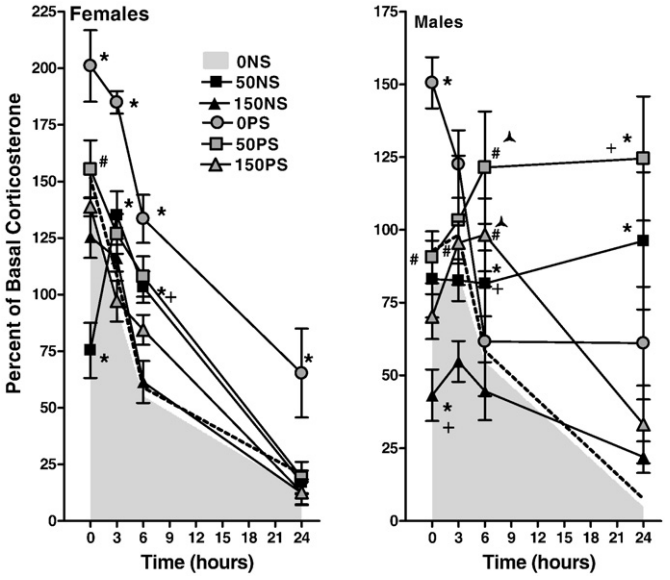

Fig. 2. Corticosterone levels (percent of basal levels) at 0, 3, 6 and 24 h post injection stress in females (left panel) and males (right panel) treated with 0, 50 or 150 ppm Pb exposure alone (NS, no stress) and/or with prenatal stress (PS). Each data point represents a group mean±S.E. value. Sample sizes were n=7–8 for female groups and n=8 for male groups. The shaded area in each plot represents the area under the 0NS mean values, such that the upper boundary is group mean 0NS value; the dashed line indicates the upper 0NS standard error value. * indicates difference from corresponding 0 ppm value; + indicates difference from corresponding 150NS value; # indicates a difference from corresponding PbNS value.

treatment conditions, was systematically weighed. No differences in body weights were detected (Virgolini et al., 2008).

Basal corticosterone levels

Basal corticosterone levels, used to evaluate responses to injection stress in the current study, were determined in littermates of these offspring, as previously reported (Virgolini et al., 2008). Given their use in calculation of percent changes here, basal values are presented in Fig. 1B. ANOVAs confirmed a main effect of Pb in females, with 150 ppm>than either 50 or 0 ppm. This outcome reflects the 48% and 37% increases in the 150NS and 150PS groups, respectively relative to their 0 ppm counterparts. In addition, a main effect of PS derived from the lower corticosterone levels in PS offspring (collapsed across Pb groups). Values of the 0PS females were approximately 20% lower than those of 0NS females.

In males, an inverse U-shaped Pb concentration effect curve was found, with 50 ppm corticosterone levels reduced by approximately 20–30% as compared to 0 and 150 ppm levels, with the latter two not differing. These effects were observed in both NS and PS groups. No effects of PS or interaction of Pb × PS was noted in males.

Maternal Pb exposure prolongs the corticosterone response to vehicle injection stress in a non-linear manner in both genders

To evaluate the time course of corticosterone changes following a stressor as a function of maternal Pb and PS per se, data from the 0 μg/kg DEX dose were used, as hereafter referred to as the injection stressor, with results of those analyses presented in Fig. 2 for females and males as percent of basal corticosterone levels.

In control (0NS) females ($F(6,63)=8.86$, $p<0.0001$), corticosterone levels were increased by approximately 35% at 0 h relative to basal values, and declined thereafter over 24 h. The 50 ppm Pb exposure, but not 150 ppm, significantly prolonged the time course of corticosterone reduction. Although 50 ppm initially reduced corticosterone levels by 25%, to values that differed significantly from 0 and 150 ppm groups, levels subsequently increased above basal values at 3 and 6 h, before declining to 0 ppm levels at 24 h. Thus 50 ppm levels were significantly higher than 0 ppm values at 3 h and higher than 0 and 150 ppm values at 6 h, representing increases of 52% and 84%, respectively in absolute corticosterone levels. Corticosterone changes of the 150 ppm group did not differ from 0 ppm levels at any time point.

A more pronounced effect of 50 ppm Pb was also found in males (Pb × time [$F(6,63)=4.29$, $p=.0011$]. A time-related reduction over 24 h was seen in 0NS males without any initial increase above basal levels. In 50NS males, however, virtually no decline in corticosterone occurred over the 24 h period. At 24 h, absolute 50 ppm corticosterone values were elevated 14-fold relative to 0NS values. Initial reductions in corticosterone levels were observed in 150NS males to levels significantly below 0NS and 50NS values, but values were thereafter comparable to 0NS values at all subsequent time points.

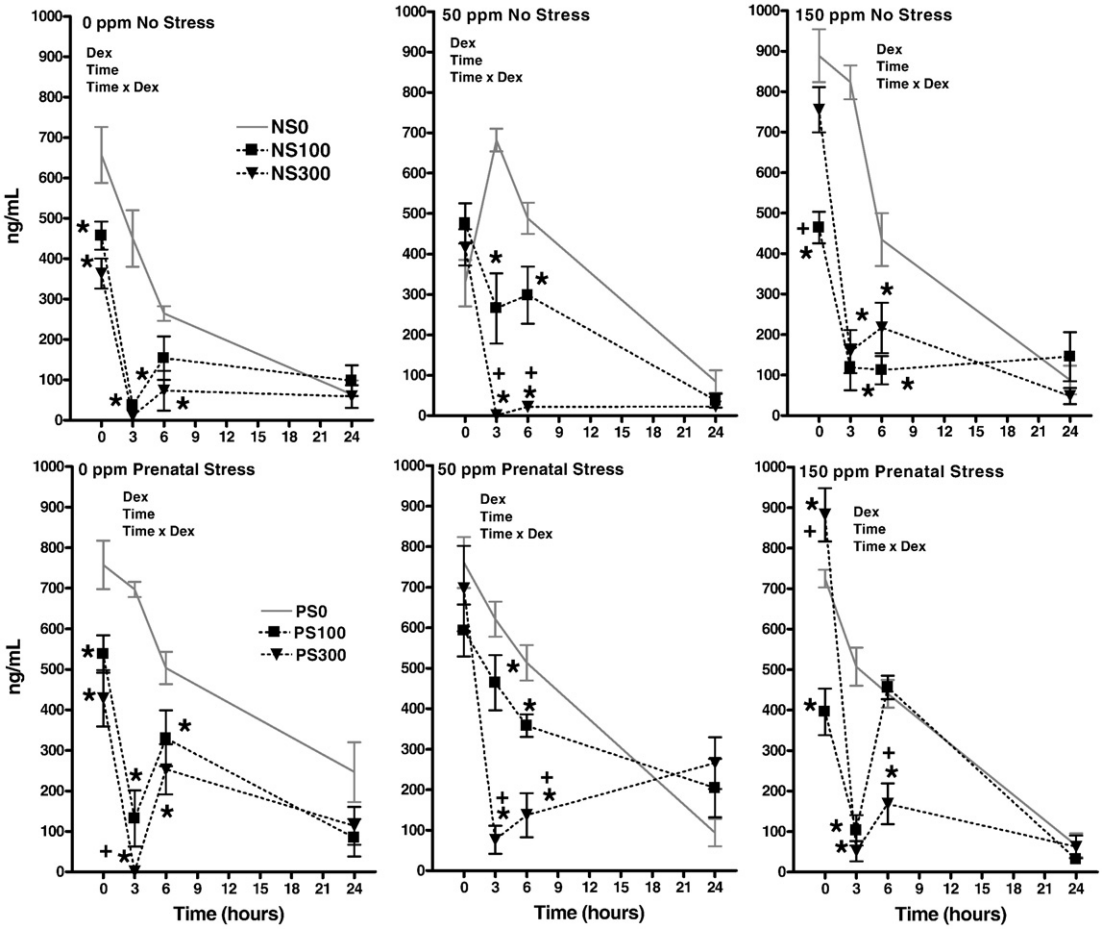

Fig. 3. Corticosterone levels (ng/ml) of female rats at 0, 3, 6 and 24 h post administration of 0, 100 or 300 μg/kg DEX in Pb-treated non-stressed (NS; top row) and prenatally-stressed groups (PS; bottom row). Each data point represents a group mean±S.E. value. Sample sizes were $n=7–8$. * indicates difference from corresponding 0 ppm value; + indicates difference from corresponding 50 ppm value. Dex = significant main effect of DEX; time = significant main effect of time; and Dex × time = indicates significant interaction of Dex × time in corresponding RMANOVA.

Prenatal stress also prolongs the corticosterone response to vehicle injection stress and enhances maternal Pb effects in males but not females

In 0 ppm females, PS produced a significant right-shift of the corticosterone time course curve (Fig. 2; main effect of Pb(1,14)=41.54, $p<0.0001$) due to initially higher corticosterone levels (approximately 60%) at 0 h, but the subsequent rate of decline in corticosterone was similar in 0NS and 0PS females. For that reason, corticosterone levels remained higher (by almost 4-fold in absolute values) in 0PS females even at 24 h. In 50 ppm females, PS increased corticosterone levels at 0 h, but resulted in reductions equivalent to those seen with 50NS thereafter. Corticosterone levels of 150NS and 150PS females were similar at all time points.

In males, main effect of Pb ($F(2,42)=16.2$, $p<0.0001$) and of stress ($F(1,42)=33.4$, $p<0.0001$) and an interaction of Pb × time ($F(6,126)=7.82$, $p<0.0001$), PS significantly increased corticosterone levels across all Pb groups ($p=0.001$, 0.0498 and 0.0009 for the 0, 50 and 150 ppm groups, respectively). Further, when collapsed across NS and PS conditions, higher corticosterone levels were evident at 50 ppm as compared to either 0 or 150 ppm, and at 0 ppm relative to 150 ppm.

Summarized across Pb and PS, Fig. 2 shows that in females, the greatest prolongation of corticosterone reduction post injection stress occurred in 0PS females, with smaller reductions in both the 50NS and 50PS groups, indicating that the increase in the 50NS group is not further enhanced by PS. No changes relative to control were found in 150NS or 150PS groups. In males, delays in corticosterone reduction are apparent for all but the 150NS group, and are particularly striking in the 50PS and 50NS groups. Moreover, evidence of synergistic effects were evident at 50 ppm at 6 h, where 50PS levels were elevated by approximately 75%, while 50NS values were only 15% greater than 0NS, and 0PS values did not differ from controls. Potentiated effects were noted at 150 ppm at 6 h, with 150PS values increased approximately 40% above 0NS controls, while neither the values of the 0PS or the 150NS groups differed from controls.

Maternal Pb and combined maternal Pb and prenatal stress initially reduce DEX efficacy in both genders, then markedly prolong corticosterone suppression in males

Dose–effect curves showing changes in corticosterone levels (ng/ml) over the 24 h period following DEX administration are shown for each Pb-stress group in Fig. 3 for females and Fig. 4 for males.

In females, RMANOVAs confirmed significant main effects of DEX and time, as well as DEX × time interactions in all Pb-stress groups (0NS: $F(6,63)=6.84$, $p<0.0001$; 50NS: 19.26, $p<0.0001$, 150NS: 13.7, $p<0.0001$; 0PS: 7.16, $p<0.0001$; 50PS: 9.13, $p<0.0001$; 150PS: 25.8, $p<0.0001$. In 0NS females (top left), similar reductions in corticosterone were produced by both 100 and 300 μg/kg DEX. These reductions

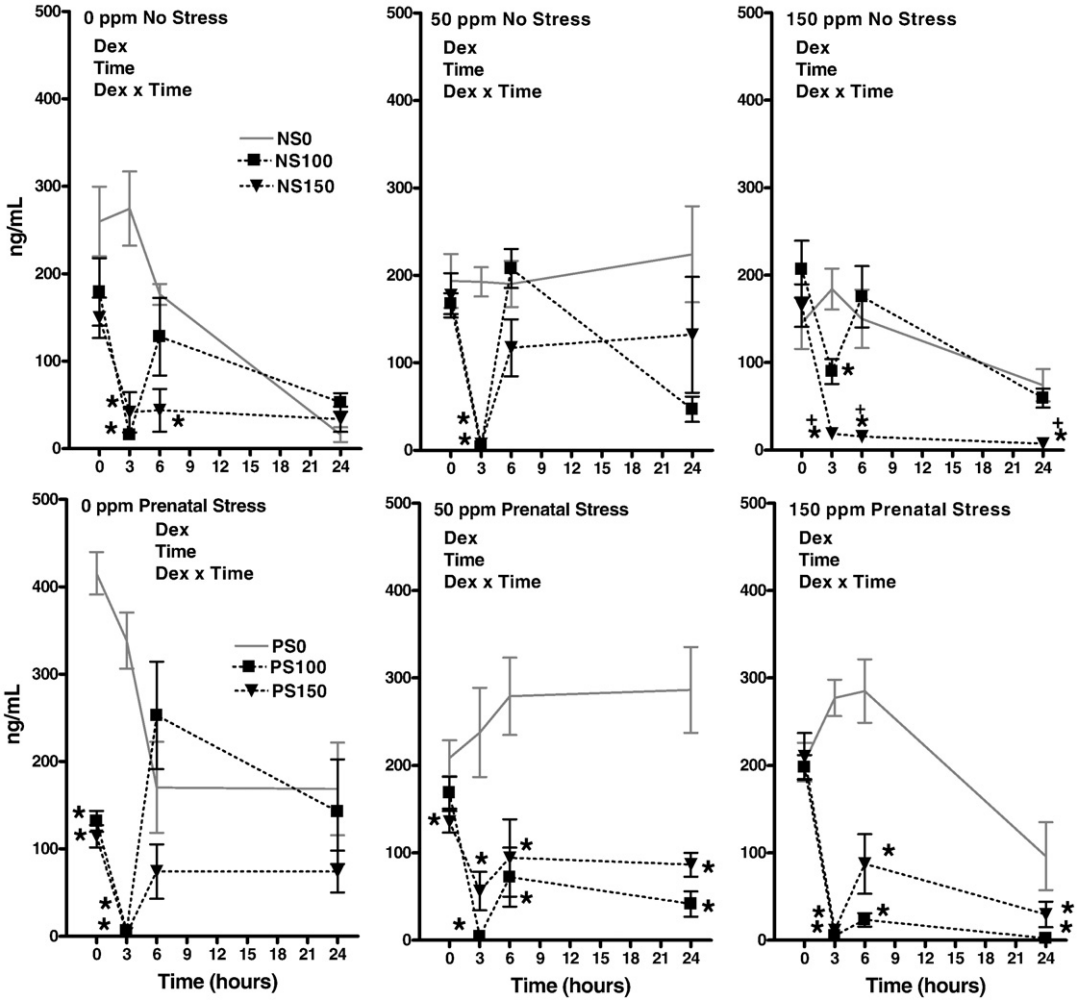

Fig. 4. Corticosterone levels (ng/ml) of male rats at 0, 3, 6 and 24 h post administration of 0, 100 or 300 μg/kg DEX in Pb-treated non-stressed (NS; top row) and prenatally stressed groups (PS; bottom row). Each data point represents a group mean ± S.E. value. Sample sizes were $n=8$. * indicates difference from corresponding 0 ppm value; + indicates difference from corresponding 50 ppm value. Dex = significant main effect of DEX; time = significant main effect of time; and Dex × time = indicates significant interaction of Dex × time in corresponding RMANOVA.

were evident at 0 h, maximal at 3 h, began to recover at 6 h, and were equivalent to vehicle dose levels at 24 h. Dose–effects were evident only at the 6 h time point, where significant corticosterone suppression was still evident at 300 μg/kg, but not at 100 μg/kg DEX. Highly similar outcomes were observed in the 0PS group, although the 100 μg/kg DEX dose retained its efficacy at 6 h in this group. In both the 50NS and 50PS groups, the onset of corticosterone reduction following DEX was delayed to 3 h. Unlike the changes seen at 0 ppm, dose-dependent effects were seen in both the 50NS and 50PS groups at 3 and 6 h. In both the 150NS and 150PS groups, the 300 μg/kg, but not the 100 μg/kg DEX dose was associated with a delayed suppression of corticosterone, consistent with an inverse U-shaped concentration effect curve for Pb at 0 h. Maximal suppression occurred at 3 h where the two doses were equally effective. By 6 h, 100 μg/kg DEX was no longer effective in 150PS females, whereas it continued to suppress corticosterone at levels comparable to 300 μg/kg DEX in 150NS females. No residual suppression was apparent by 24 h.

Significant main effects of DEX and of time, as well as significant DEX × time interactions were also observed in all male Pb-stress groups (Fig. 4; 0NS: $F(6,63)=6.3$, $p<0.0001$; 50NS: 3.94, $p=0.002$, 150NS: 6.23, $p<0.0001$; 0PS: 7.7, $p<0.0001$; 50PS: 2.38, $p=0.039$; 150PS: 11.51, $p<0.0001$). In 0NS males (top left), both the 100 μg/kg and the 150 μg/kg DEX doses reduced corticosterone levels beginning at 3 h. At 6 h, only the higher dose still suppressed corticosterone. At 24 h, all groups displayed equivalent corticosterone levels. In 0PS males, significant corticosterone suppression occurred at 0 h and was maximal at 3 h; recovery was evident in both doses by 6 h. No dose-related differences in potency were observed in 0PS males. DEX suppression of corticosterone was limited to the 3 h time point in 50NS males, where the two doses produced a comparable and virtually complete suppression. In 50PS males, both DEX doses equally reduced corticosterone levels beginning at 3 h and persisting across the entire 24 h period. At the final time point, reductions of 86 and 70% were still present at the 100 μg/kg and 150 μg/kg doses, respectively. In 150NS males, dose-related suppression of corticosterone was not observed until the 3 h time point, at which time dose-related differences were observed. At the 150 μg/kg dose, reductions were sustained through the 24 h measurement period. DEX effects were particularly notable in 150PS males, where significant reductions of corticosterone levels were present at both doses from 3 to 24 h, with reductions of 30–98% still evident at 24 h.

To provide a comparison of the magnitude of DEX effects in relation to treatment, data were first calculated as a percent of corresponding Pb-stress 0 μg/kg group/time point values (e.g., 50NS 100 μg/kg and 50NS 300 μg/kg at 3 h as a percent of 50NS 0 μg/kg at 3 h; 150PS 100 μg/kg and 150PS 300 μg/kg at 0 h as a percent of 150PS 0 μg/kg at 0 h, etc) based on the assumption that the 0 μg/kg data at each time point represented the 'normal' response to the injection stress. Post hoc tests were then carried out comparing the magnitude of changes at each dose/time point in the Pb-PS conditions to that of the 0NS group, e.g., comparisons of 50NS 100 μg/kg and 150NS 100 μg/kg at 0 h to 0NS 100 μg/kg effect at 0 h, etc., to provide comparison of percent DEX changes in 50 and 150 ppm NS and PS groups and the 0PS group in relation to percent changes in the 0NS control group. Results are shown in Fig. 5 and summarized across all conditions in Table 1.

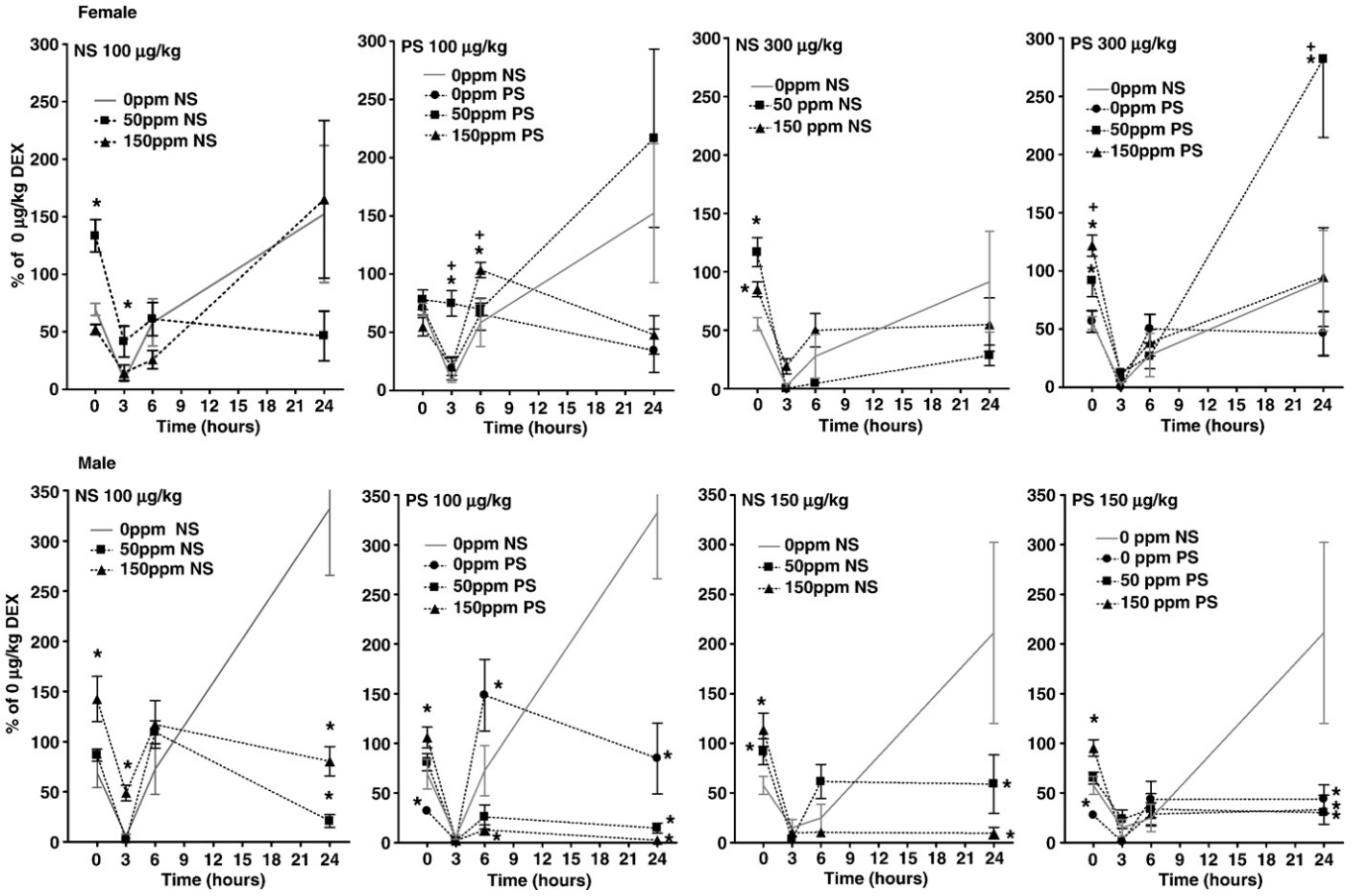

Fig. 5. Corticosterone levels as a percent of corresponding Pb-stress 0 μg/kg group/time point values (e.g., 50NS 100 μg/kg and 50NS 300 μg/kg as a percent of 50NS 0 μg/kg; 150PS 100 μg/kg and 150PS 300 μg/kg as a percent of 150PS 0 μg/kg) of female (top row) and male (bottom row) offspring exposed to no stress (NS, left column) or prenatal stress (PS, right column) in response to DEX administration (100 or 300 μg/kg for females and 100 or 150 μg/kg for males). Sample sizes as defined above. * indicates difference from corresponding 0NS DEX dose/time point value.

Table 1
Summary of differences in DEX efficacy in Pb and PS groups relative to DEX effects at the corresponding 0NS dose and time point control

Group	Females				Males			
	0 h	3 h	6 h	24 h	0 h	3 h	6 h	24 h
0PS100[a]					↓		↓	↑
0PS300/150[b]					↓			↑
50NS100[a]	↓[c]	↓						↑[d]
150NS100[a]			↓		↓	↓		↑
50NS300/150[b]	↓				↓			↑
150NS300/150[b]	↓				↓			↑
50PS100[a]		↓[e]						↑
150PS100[a]			↓[e]		↓		↓	↑
50PS300/150[b]	↓			↓[e]				↑
150PS300/150[b]	↓[e]				↓			↑

[a] Compared to 0NS 100 μg/kg DEX dose as percent change.
[b] Compared to 0NS 300/150 (female/male) μg/kg DEX dose as percent change.
[c] Indicates reduced efficacy relative to 0NS DEX dose.
[d] Indicates greater efficacy relative to 0NS DEX dose.
[e] Indicates effects greater than corresponding NS group.

These analyses demonstrated examples of significantly reduced DEX efficacy in both genders over the first 6 h post-administration. For females, this was found at 0 h for the 100 μg/kg dose in the 50NS group, and for the 300 μg/kg dose in the 50NS, 150NS, 50PS and 150PS groups, with the magnitude of effect in the 150PS group greater than that in the 150NS group, indicative of enhancement by PS. Reductions in DEX efficacy at 3 h were seen at the 100 μg/kg dose in both the 50NS and 50PS groups, with significantly greater magnitude of effect in the 50PS group, and at 6 h in the 150PS group whereas no effect was seen in the 150NS group, again indicating PS enhancement. The 50PS group showed a markedly greater rebound following 300 μg/kg than did the 0PS group at 24 h, and no such reduction was noted in the 50NS group.

In males, significant reductions in DEX suppression at 0 h were observed at the 100 μg/kg DEX dose in the 150NS and 150PS groups, and at the 150 μg/kg dose in the 50NS, 150NS and 150PS groups. Reduced efficacy was also found at 3 h for the 100 μg/kg dose in the 150NS group. 0PS males, by contrast, showed enhanced DEX suppression at both the 100 μg/kg and 150 μg/kg doses at 0 h, and reduced suppression at the 100 μg/kg dose at 6 h. By 24 h, however, DEX potency was found to be significantly greater in all 50 and 150 ppm NS and PS groups than in corresponding 0NS controls as well as in the 0PS groups. At this time point, corticosterone values of the 0NS males dosed with 100 μg/kg and 150 μg/kg DEX had rebounded to mean levels of approximately 350% and 200% of the 0NS 0 μg/kg value. In contrast, corresponding values of the Pb-stress groups ranged from only 2 to 90% of the 0NS 0 μg/kg values.

Discussion

These studies demonstrate that both maternal Pb and PS alone delay corticosterone reduction following an injection stress challenge, with non-linear changes observed with Pb that were more pronounced at 50 ppm than at 150 ppm in both genders. This non-linearity was particularly striking in males, where PS further enhanced the effects of maternal Pb. In accord with these findings, the efficacy of DEX to suppress corticosterone was attenuated by Pb during the first 6 h post-administration, effects showing evidence of enhancement by PS in females. However, DEX suppression was also markedly prolonged by both Pb and PS, although not additively, in males. Collectively, these findings demonstrate a hypo-responsive HPA axis, and consequently hypercortisolism, in males and females, with evidence of later HPA hyperresponsiveness and hypocortisolism in males, findings that may explain the corresponding disease profiles associated with Pb and elevated stress/cortisol levels.

PS diminishes glucocorticoid negative feedback in females and males

Both maternal Pb and PS alone prolonged the reduction in corticosterone following injection stress. Injection stress challenge increased corticosterone levels to a greater extent in 0PS males and females than in 0NS counterparts, and these groups also sustained higher corticosterone levels over the subsequent 24 h measurement period (Fig. 2). For 0PS males in particular, the decline in corticosterone was virtually negligible between 6 and 24 h. These findings replicate previous reports of delayed reduction of corticosterone following stress challenge in PS offspring (e.g., Szuran et al., 2000; Maccari et al., 2003; Darnaudery and Maccari, 2008). The current study, however, extends those findings, demonstrating a protracted duration of the effect, lasting at least 24 h in males.

Maternal Pb also diminishes negative glucocorticoid feedback in both genders, but produces an inverse U-shaped concentration effect function

Pb exposure also prolonged the post-injection stress reduction in corticosterone over 24 h (Fig. 2), but did so in a non-monotonic fashion in both genders, with a more pronounced impact at 50 than 150 ppm exposure. HPA hypo-responsiveness was particularly striking in 50NS males, where virtually no reduction in corticosterone occurred at any time point over 24 h. While our prior studies have revealed preferential vulnerability of females to behavioral consequences of Pb and stress (Virgolini et al., 2008), the current data suggest a greater impact of Pb and PS and the combination on HPA negative feedback in males.

The greater impact in males is interesting in light of the report that PS actually produces greater in utero corticosterone levels in females than males (Montano et al., 1993), and points to the likelihood of different operative mechanisms by gender. As also appears to be the case for PS (Darnaudery and Maccari, 2008), the HPA axis dysfunction produced by maternal Pb apparently represents a permanent change, since offspring were already 8 mo of age when experiments were initiated and occurred following exposures of dams associated at weaning with PbBs averaging only 11 μg/dl, i.e., just above the CDC's currently designated level of concern for children.

The non-monotonic dose–effect curve for Pb is also particularly notable, given accumulating evidence of greater effects of Pb on IQ in children at lower as compared to higher PbBs (Canfield et al., 2003). Additionally, these findings add to an accumulating literature reporting non-linearity of Pb effects for such varied endpoints as locomotor activity, cerebral cortex growth, operant behavior, maze performance, age at vaginal opening, serum urea and urate and body weight and height (Davis and Svendsgaard, 1990; Leasure et al., 2008). Moreover, inverse U-shaped concentration curves were noted in male offspring for basal corticosterone as well (Fig. 1; Virgolini et al., 2008). The mechanism(s) underlying this non-linearity remain unknown as of yet, but the U-shaped curves point to different operative mechanisms at differing levels of exposure, e.g., engagement of homeostatic mechanisms at lower levels that may be overwhelmed at higher exposure levels.

Pb-associated reduction of glucocorticoid negative feedback was enhanced by PS in males, but attenuated in females

Corticosterone levels were further increased when Pb was combined with PS in males at both 50 and 150 ppm (Fig. 2). Indeed, 50PS male offspring had the highest corticosterone levels of all male groups following vehicle injection; and at 6 h, corticosterone levels of the 50PS and 150PS groups were significantly higher than their PbNS counterparts, consistent with enhanced effects of Pb+PS. In contrast, Pb generally appeared to reduce the effects of PS in females, given the smaller increases in corticosterone in the 50NS and 50PS groups, and the lack of any effects in the 150NS and 150PS groups, relative to OPS. It is not currently possible to ascertain the mechanism(s) underlying these gender differences. At the very least, they suggest that Pb effects

predominate for glucocorticoid negative feedback in females. Therefore, if Pb and PS act by similar or overlapping mechanisms in females, then the peak magnitude of effect that occurs is defined by the Pb concentration effect function. In the case of males, it is conceivable that Pb and PS act through different mechanisms, both of which ultimately block glucocorticoid feedback and thereby additively prevent reductions in corticosterone. Surprisingly, the impact of Pb exposure on different components of HPA axis function is virtually unexplored, with the single exception of an in vitro investigation reporting that Pb exposure reduced ACTH-induced steroid production with a site of action likely at the plasma membrane (Nishiyama et al., 1985) and early reports of corticosterone changes (Vyskocil et al., 1991a, 1991b, 1991c). Clearly, additional experiments will be required to determine associated mechanisms and the impact of Pb on the different components of the HPA axis, but the current findings clearly caution against any generalizations across genders.

Pb exposure initially diminished DEX suppression of corticosterone in both genders with some PS enhancement observed in females

HPA axis hypo-responsivity associated with Pb and PS was concordant with the diminished efficacy of DEX-induced suppression of corticosterone in Pb and Pb+PS treated offspring observed initially following DEX injection (Figs. 3–5). Direct comparisons of the magnitude of suppression produced by DEX doses across Pb-stress groups relative to 0NS controls (Fig. 5 and Table 1) confirmed a reduced efficacy of DEX ranging from 15 to 65% in females and 34 to 73% in males during the first 6 h post DEX administration in Pb and Pb+PS groups of both genders, with Pb effects further enhanced by PS in females.

Altered pharmacokinetics or pharmacodynamics of DEX in response to Pb, PS or Pb+PS is one potential basis for this attenuation, but cannot be directly ascertained in the absence of direct measures of tissue DEX levels. But that potential mechanism may be hard to disentangle from the alternative, but related explanation, that the attenuated DEX efficacy is a function of the higher corticosterone values seen in Pb and Pb+PS groups following stress challenge, since hypercortisolemia itself can actually decrease DEX half-life through the induction of hepatic enzymes by circulating corticosterone levels (Stokes et al., 2002). Such a possibility would be consistent with the higher corticosterone levels seen with PS and Pb, and the fact that the most consistent attenuation of DEX efficacy was observed at the first time point of measurement (Table 1). However, that explanation is inconsistent with our observations that despite the pronounced increases in corticosterone levels in 0PS females at all time points (Figs. 3 and 4), no corresponding attenuation of DEX efficacy was found in this group. Moreover, attenuated DEX efficacy was found in 150NS and 150PS females, despite minimal evidence of increased corticosterone levels in these groups. Finally, at 24 h, as noted above, enhanced, rather than attenuated DEX suppression was observed in all male groups relative to the 0NS controls.

Other potential mechanisms include increases in ACTH secretion and/or in corticotropin releasing hormone, vasopressin and/or oxcytocin that could indirectly lead to increased cortisol release (Cole et al., 2000), or indirect effects mediated through alterations in CNS glucocorticoid receptor function. DEX administration effectively produces a hypocortisolism in brain, and prior studies have described reductions in glucocorticoid receptors in response to PS (Koehl et al., 1999; Szuran et al., 2000; Maccari et al., 2003). In addition, mice under-expressing glucocorticoid receptors sustain higher corticosterone levels in DEX suppression tests (Ridder et al., 2005).

Both maternal Pb and PS markedly prolong the effects of DEX on corticosterone suppression in males but not females

DEX was associated with reduction of corticosterone even out to 24 h in males, whereas females showed full recovery by this time, notable findings given that male rats actually show more rapid DEX clearance than females (Lamiable et al., 1991). Nevertheless, male rats do appear to be more sensitive than females to DEX. A previous study noted a requirement for higher DEX doses to suppress corticosterone in females than in males (Osborn et al., 1996), an observation that served as the rationale for the use of a 300 μg/kg DEX dose for females and a 150 μg/kg DEX dose for males in this study; these did appear to produce comparable levels of suppression here, with the exception of the final 24 h point, when males exhibited a greater rebound effect relative to basal levels than females. It may also be the case that maternal Pb/PS differentially influences corticosterone synthesis in males vs. females. Gender differences in corticosterone synthesis and metabolism have been long known (Glenister and Yates, 1961; Kitay, 1961; Gala and Westphal, 1965). Rats in these experiments were also food restricted to equate conditions with those of littermates undergoing behavioral testing, and the current experiments did not explicitly control for phase of the estrous cycle, parameters that can also affect corticosterone levels (Anderson et al., 1996; Stamp et al., 2008).

HPA alterations as a biological unifying mechanism for commonalities in Pb- and PS-associated diseases and disorders

The hypo-responsivity of the HPA axis shown here is consistent with hypercortisolism, which, if occurring in humans exposed to Pb, would be expected to have significant public health implications, since increased cortisol levels have been implicated in numerous disease states, including Type 2 diabetes (Taniguchi et al., 2008), depression (Carroll et al., 2007), obesity (Dallman et al., 2003) and schizophrenia (Gallagher et al., 2007). Cushing syndrome, a hormonal disorder characterized by prolonged exposure to high levels of cortisol, is characterized by upper body obesity, weakened bones, hypertension (metabolic syndrome) and anxiety and depression. It is notable that Pb exposure has indeed been linked to obesity, hypertension, diabetes, anxiety, schizophrenia and depression in epidemiological studies (Kim et al., 1995; Cheng et al., 2001; Gerr et al., 2002; Rhodes et al., 2003; Opler et al., 2004; Menke et al., 2006; Rajan et al., 2007). In male Pb and PS offspring, a delayed hyper-responsive HPA axis was also found. Hypocortisolism likewise has adverse health consequences. Chronic primary adrenal insufficiency in Addison's disease, for example, is associated with malaise, anorexia, diarrhea, weight loss and joint and back pain (Nieman and Chanco Turner, 2006).

Our experimental studies demonstrating that Pb, PS and combined Pb and PS produce permanent, sometimes additive HPA axis dysfunction may, therefore, provide a biologically plausible unifying mechanism for the overlapping disease profiles associated with Pb and with stress in humans. Moreover, while current screening programs for elevated Pb burden focus on children, the fact that the permanent HPA axis effects arose from maternal only Pb exposure in this study suggests the need to screen pregnant women, particularly those with high-stress lifestyles. Indeed, PbBs measured in children are not necessarily reflective of the greater exposure of the fetus prenatally, given the increases in PbB during pregnancy (Rothenberg et al., 1994; Hertz-Picciotto et al., 2000; Manton et al., 2003; Gulson et al., 2004), exposures which have been linked more strongly than postnatal PbBs to poorer infant mental development (Hu et al., 2006). Similar increases during pregnancy are seen in rats (Dearth et al., 2002, 2004) which may in part explain the increased PbBs of the 50 ppm offspring at weaning, although the lack of a similar effect in 150 ppm offspring suggests the potential for additional operative mechanism. 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.

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