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Research report

Experimental manipulations blunt time-induced changes in brain monoamine levels and completely reverse stress, but not Pb+/−stress-related modifications to these trajectories

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ABSTRACT

This study sought to further understand how environmental conditions influence the outcomes of early developmental insults. It compared changes in monoamine levels in frontal cortex, nucleus accumbens and striatum of male and female Long-Evans rat offspring subjected to maternal Pb exposure (0, 50 or 150 ppm in drinking water from 2 months pre-breeding until pup weaning)+/−prenatal (PS) (restraint on GD16-17) or PS + offspring stress (OS; three variable stress challenges to young adults) determined at 2 months of age and at 6 months of age in littermates subsequently exposed either to experimental manipulations (EM: daily handling and performance on an operant fixed interval (FI) schedule of food reward), or to no experience (NEM; time alone). Time alone (NEM conditions), even in normal (control) animals, modified the trajectory of neurochemical changes between 2 and 6 months across brain regions and monoamines. EM significantly modified the NEM trajectories, and except NE and striatal DA, which increased, blunted the changes in monoamine levels that occurred over time alone. Pb+/−stress modified the trajectory of monoamine changes in both EM and NEM conditions, but these predominated under NEM conditions. Stress-associated modifications, occurring mainly with NEM OS groups, were fully reversed by EM procedures, while reversals of Pb+/−stress-associated modifications occurred primarily in nucleus accumbens, a region critical to mediation of FI response rates. These results extend the known environmental conditions that modify developmental Pb+/−stress insults, which is critical to ultimately understanding whether early insults lead to adaptive or maladaptive behavior and to devising behavioral therapeutic strategies. That time alone and a set of EM conditions typically used as outcome measures in intervention studies can themselves invoke neurochemical changes, moreover, has significant implications for experimental design of such studies.

1. Introduction

Early life insults, such as prenatal stress and lead (Pb) exposure, have been shown to incur virtually lifelong effects in experimental studies. Prenatal restraint stress in rodents, for example, is associated with enhanced anxiety and depression-like behaviors and, under some conditions, with cognitive impairments, increased propensity to drug abuse, and alterations in function of the HPA axis and response to stress challenges in adult offspring [1–6]. Developmental Pb exposure also exerts long-term effects on cognitive functions as well as on HPA axis reactivity [1,4,6–11]. In addition to their common effects on brain mesocorticolimbic dopamine and glutamate systems [12–18], elevated Pb burden and stress are also co-occurring risk factors, particularly for low socioeconomic status communities [19,20]. Correspondingly, our studies in rats have demonstrated that Pb exposure and stress can produce protracted synergistic behavioral and neurochemical effects at blood Pb levels (11 μg/dl) just above those deemed to be of concern for children by the Centers for Disease Control. For example, marked elevations of response rates on Fixed Interval schedules of reinforcement were

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observed at low levels of Pb exposure that were markedly further enhanced under conditions of Pb exposure combined with prenatal (PS) and offspring stress (OS), raising FI response rates to levels associated with higher Pb exposure levels. Similar Pb+ stress additive effects were noted for a broad range of neurochemical changes [8,21,22].

Whether such developmental insults ultimately resolve (adaptation), exhibit recovery, or lead to further dysfunction or even psychopathology is likely to reflect the course of subsequent environmental influences and events. To determine the subsequent evolution of adaptive vs. maladaptive behavior will require evaluation of the influence of various environmental conditions on the altered CNS milieu arising from such early developmental insults. Only with such an understanding can more precise and refined behavioral therapeutic strategies for these developmental insults be devised.

In that context, procedures such as handling and various other forms of what is referred to as 'environmental enrichment' have been reported in experimental animal studies to have an ameliorating influence on the adverse consequences of prenatal and early postnatal stressors, as well as more broadly in models of drug addiction and psychiatric and neurodegenerative diseases [23–26]. Environmental enrichment comprised of complex housing has also been reported to reverse effects of early Pb exposures in rodents [27–29].

Often in such studies, the enrichment intervention is imposed, or not, over some period of time, and is then followed by measurement of specific outcomes, with differences in that outcome generally presumed to reflect the environmental enrichment intervention. In the case of neurochemical and behavioral outcomes, however, it is also possible that: (1) time itself can result in changes in neurotransmitter levels/function, and (2) the outcome being measured after the intervention (e.g., handling, weighing, behavioral paradigm and reinforcer in the case of behavioral outcomes) can alter neurotransmitter levels/function independently of the intervention.

With respect to the first possibility, age-related changes in all aspects of dopamine systems have been reported that differ by brain region and gender [30–36]. While most such studies have compared early to late stages of the life span, more recent reports demonstrate changes in neurochemical function even between 2 and 6 months of age in mice [34].

In relation to the second possibility, the effects of behavioral history/training on subsequent behavioral performance, pharmacological drug response and neurochemical and biochemical functions have long been recognized. For example, cocaine self-administration is influenced by behavioral history in both rhesus monkeys [37–39] and rats [40]. Prior undrugged test experience has been shown to significantly reduce or even eliminate the efficacy of benzodiazepines in the elevated plus maze anxiety paradigm [41,42]. Thus, behavioral performance itself can directly influence neurochemical function, and differences in behavioral history are likely to be associated with different underlying neurochemical profiles.

Evaluation of the possibilities posed above also has direct relevance to the design of experiments aimed at resolving questions related to efficacious intervention strategies. The current study sought to determine whether such possibilities influenced the trajectory of neurochemical changes occurring following maternal Pb exposure alone or combined with prenatal stress (PS) or PS followed by offspring stress (OS) challenges. For this purpose, changes in monoamine levels in frontal cortex, nucleus accumbens and striatum of male and female Long-Evans rat offspring that had been subjected to maternal Pb alone+/−PS and/or OS were determined in offspring taken at 2 months of age and in littermates at 6 months of age that had subsequently been subjected to experimental manipulations (EM) consisting of daily handling and weighing and performance on an operant fixed interval (FI) schedule of food reward, or that had no such experimental experiences (NEM; time alone manipulation) and remained in their home cages. This permitted a determination of the trajectory of neurochemical changes between 2 and 6 months of age in relation to time alone (NEM conditions) and alterations to time associated changes produced by assessment of the behavioral outcome of interest (EM conditions). It also examined the extent to which the trajectories of neurochemical changes under EM vs. NEM conditions were modified by stress and Pb+/−stress, and whether the specific EM conditions used here could reverse any such modifications.

2. Materials and methods

2.1. Animals and Pb exposure

Three-weeks old female Long-Evans rats (Charles River, Germantown, NY) were randomly assigned to one of the following drinking solutions: 0, 50 or 150 ppm Pb acetate dissolved in distilled deionized water. More recent studies of young adult males in our laboratory show that 50 ppm exposure initiated postweaning results in blood Pb values averaging 7–12 μg/dl [13,43], 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 [44–46]. 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 was used because our previous studies had demonstrated synergistic effects of this Pb exposure level with stress [1,8].

As shown in Fig. 1, Pb exposure of dams was initiated 2 months prior to breeding and was continued throughout lactation to ensure elevated bone Pb levels and Pb body burden [47], consistent with human exposure. When females reached 3 months of age, they were paired with 3 months old male Long-Evans rats for breeding. Animals were housed in a vivarium room maintained at 22 ± 5 °C with a 12 h light–dark cycle (lights on at 07:00 h). Standard rat chow diet was provided *ad libitum*. All experiments were carried out according to NIH Guidelines and were approved by the University of Rochester's University Committee on Animal Resources.

2.2. 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-groups and individually housed for the remainder of pregnancy and lactation.

On gestational days 16 and 17, dams assigned to PS groups were weighed and subjected to three 45 min restraint sessions (09:00, 12:00 and 15:00 h) in plastic cylindrical devices using procedures modified from Ward and Weisz [48] (Fig. 1). This procedure, or an even more protracted restraint stress paradigm, has been widely employed [49–53]. NS dams were weighed and subsequently left undisturbed in their home cages. 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 [1]. 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. This resulted in six Pb-stress conditions with the following numbers of dams: 0NS (n = 20), 0PS (n = 23), 50NS (n = 21), 50PS (n = 31), 150NS (n = 19), and 150PS (n = 33) as shown in Fig. 1. Differences in sample sizes reflect both differences in pregnancy rates and initial assignment of greater number of dams to Pb+PS groups.

2.3. Offspring procedures

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, when Pb exposure effectively ended, separated by gender, and housed in same litter/gender pairs for the duration of the experiment.

From weaning, pups were provided with unrestricted access to tap water (0 ppm) and food (Laboratory Rodent Diet 5001, POMI Foods Inc.) until approximately 2 months of age. At this point, brains from a subset of male and female offspring from each of the six Pb-stress treatment groups defined above were sacrificed to provide basal determinations of corticosterone and levels of monoamines in frontal cortex, nucleus accumbens and striatum.

Remaining pups from each of the six Pb-stress groups per gender were then allocated to either an experimental manipulation (EM) condition or a non experimental manipulation (NEM) condition. In addition, a subset of offspring from each PS group (0PS, 50PS and 150PS) was allocated to receive offspring stress (OS) treatment as

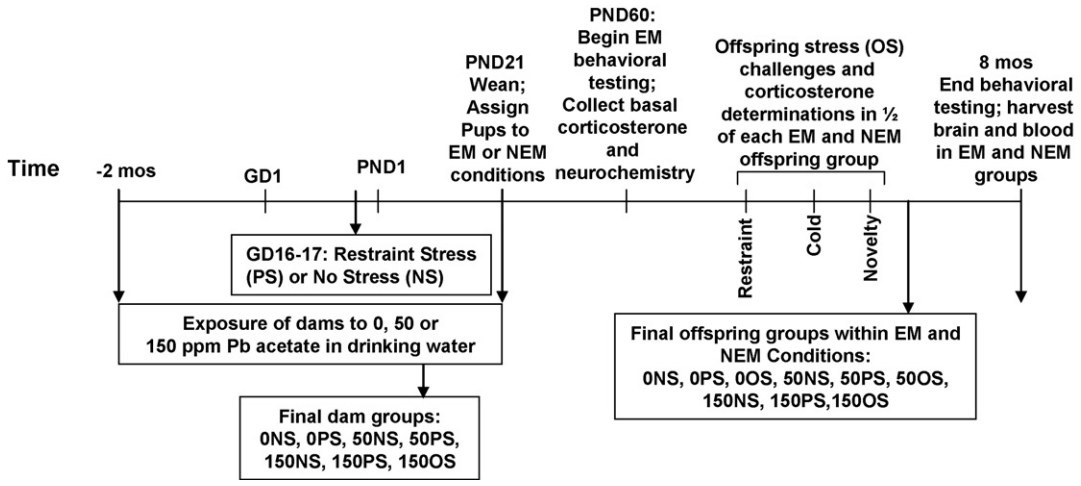

Fig. 1. Schematic of experimental design.

adults that consisted of a series of three additional stressor exposures. Thus final Pb-stress conditions included nine groups for each gender in each of the EM and NEM conditions: 0NS, 0PS, 0OS, 50NS, 50PS, 50OS, 150NS, 150PS and 150OS (Fig. 1).

2.3.1. EM conditions

Rats in EM groups were provided with sufficient food to allow a 2–3 g weight gain per day 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 as required for behavioral evaluation for the duration of the experiment. Because animals were pair-housed, a cage divider was placed in the cage at the time of feeding each day to separate residents and remained there for approximately 90 min.

At approximately 2–3 months of age, EM groups began behavioral testing which involved daily weighing and handling, transport to and from the room housing the behavioral test chambers, and testing on a fixed interval (FI) 1 min schedule of food reinforcement. The FI schedule of reinforcement was used because of its documented sensitivity to Pb exposure across species and developmental periods of exposure [54], and which would therefore facilitate interpretation of Pb-stress interactions. This schedule provided a 45 mg food pellet (Bioserv, Frenchtown, NJ) for the first occurrence of a designated response after a 1 min interval had elapsed over the course of 20 min behavioral test sessions that were conducted 5 days a week (monday–friday) between 10:00 and 15:00 h as described in detail previously [1,8].

Offspring stress challenges for EM OS groups were imposed immediately prior to an FI session to measure impact on subsequent FI performance. A single 45 min restraint stress was imposed prior to session 13 in both males and females, using the same procedure described above for the dams. 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) at 4 °C in a temperature-controlled room for 30 min 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 motor activity in locomotor activity cages as a novel environment stressor. The period of stress challenge in FI animals covered the 14–18 weeks of age range. Blood from tail nicks was collected at the end of the FI session following the stress challenge for the determination of corticosterone levels in FI tested offspring.

2.3.2. NEM conditions

NEM groups remained in home cages throughout the period of time occupied by EM behavioral testing, were weight restricted (although not as precisely as EM groups because of the number of animals involved in the experiment) at the same time as EM groups, and minimally handled (early weighing and culling of litters). NEM offspring allocated to the OS groups, however, were subjected to the same schedule of stressors and post-session determination of corticosterone levels as EM littermates. Given the numbers of animals involved in the study, stress challenges were carried out according to the same schedule in EM and NEM rats, but began approximately 4 weeks later in NEM offspring and covered the 18–22 weeks of age range. Corticosterone determinations were carried out at the same time points post stress challenge in NEM rats as in EM rats. NEM rats remained in transport cages between the stress challenge and corticosterone determination and were then returned to home cages.

Multiple variable stressors rather than a single homotypical stress challenge were used for OS conditions to more closely mimic the human experience across the life span and to preclude any habituation effects. Stress challenges were carried out during the acquisition phase of FI training so that the ability to elicit group differences was maximized. Stress challenges were imposed in the order of restraint, followed by cold, followed by novelty. The use of the most intense stressor, restraint, before other stress challenges was based on its potential to increase sensitivity of the animals to subsequent stressors [55], thereby potentially maximizing the ability to detect treatment-related differences.

In all cases, only a single male and female from each litter were included in each Pb-stress EM and NEM group to preclude litter-specific effects.

Within 24–48 h after FI behavioral testing was completed, brain regions were rapidly harvested following decapitation from all EM and NEM groups for the determination of brain monoamine levels, and blood collected for corticosterone determinations. Results of the FI performance, neurochemical changes following behavioral testing, as well as the corticosterone and behavioral responses to stress challenges are described elsewhere [4,8,56].

2.4. Blood Pb analysis

PbB levels were measured by anodic stripping voltammetry according to methods described previously [21,47] in dams (n = 7–8 for each group) and pups (n = 3–9) at the time of offspring weaning (PND21) from each treatment group.

2.5. Neurochemical determinations of monoamines

Levels of (DA (dopamine), DOPAC (dihydroxyphenylacetic acid), HVA (homovanillic acid), NE (norepinephrine), 5-HT (serotonin) and 5-HIAA (5 hydroxyindoleacetic acid) from frontal cortex, nucleus accumbens and striatum were analyzed using HPLC as described in detail previously [8]. Concentrations of neurotransmitters were expressed in terms of ng/mg protein. DA turnover was calculated as the DOPAC/DA ratio.

2.6. Statistical analysis

2.6.1. Blood Pb analyses

Blood Pb levels were analyzed using ANOVAs that included group (dams, male offspring, female offspring), Pb and stress as between group factors. In the event of main effects or interactions, lower order ANOVAs and post-hoc tests as appropriate were carried out.

2.7. Neurotransmitter level analysis

At the 6 months time point, values for frontal cortex DOPAC and HVA of NEM females were generally below detection limits, and brains from 50 ppm NEM females were lost due to a refrigeration problem. Thus data shown for NEM female frontal cortex from 6 months time points include only DA and NE, and for all brain regions, NEM female data include only 0 and 150 ppm data. To depict the trajectory of change in neurotransmitter levels from the 2 months time point, all data from the 6 months time point were calculated as a percent change from corresponding gender group mean 2 months 0NS values.

The specific purpose of the statistical analyses carried out and the factors analyzed are summarized in Table 1. For the evaluation of basal neurotransmitter levels at 2 months of age, and the determination of the effects of time alone and of stress on the trajectory of neurotransmitter changes between 2 and 6 months, overall ANOVAs including gender were first carried out. These were followed by lower order ANOVAs and post-hoc tests as appropriate based on main effects and interactions. For the determination of the modification by Pb or Pb+stress on the trajectory of neurochemical changes between 2 and 6 months, overall ANOVAs that included gender were not carried out for three reasons: (1) missing data from the female 50 ppm NEM groups would have meant elimination of all 50 ppm data from any analysis representing a significant omission; (2) pronounced gender differences in neurotransmitter levels were already found at 2 months of age, and (3) in analysis of the

Table 1
Summary of methods of statistical analyses by ANOVA.

Purpose of assessment and comparisons	Factors and analyses
Comparison of basal neurotransmitter levels in relation to Pb exposure (0, 50 or 150 ppm) and prenatal stress condition (NS or PS)	1. Gender (male, female) × Pb (0, 50, 150 ppm) × stress (NS, PS) for each neurotransmitter in each region 2. Pb × stress for each neurotransmitter in each region
Trajectory of Neurochemical changes between 2 and 6 months with Time Alone (NS) or following prenatal stress (PS) in normal non-Pb treated (0 ppm) conditions in relation to experimental manipulation condition (EM or NEM).	1. Gender (male, female) × stress (NS, PS, OS) × condition (EM, NEM) for each neurotransmitter in each region 2. Stress (NS, PS, OS) × condition (EM, NEM) for each neurotransmitter in each region
Modification by Pb or Pb+stress of the trajectory of neurochemical changes between 2 and 6 months in relation to experimental manipulation condition (EM vs. NEM)	1. Pb (0, 150 (females) or 0, 50, 150 ppm (males)) × Stress (NS, PS, OS) × condition (EM, NEM) for each neurotransmitter in each region

Abbreviations: Pb = lead; NS = no stress; PS = prenatal stress; OS = maternal and offspring stress; EM = experimental manipulation; NEM = no experimental manipulation.

effects of time and stress (based on 0 ppm data from 6 months of age), gender differences were confirmed for two of the four neurotransmitters in frontal cortex, all six comparisons in nucleus accumbens and all five comparisons in striatum; the latter two are already indicative of broad gender differences. Consequently, these analyses were carried out separately for males and females. In all analyses, a p value of ≤ 0.05 was considered statistically significant. Because of the number of analyses, only p values for specific effects from the ANOVAS are reported for comparisons described.

2.7.1. Effects of Pb and stress on basal neurotransmitter levels

Analyses were carried out to determine the influence of Pb and of stress on neurotransmitter levels prior to the implementation of any EM conditions or OS challenges from brains collected at the 2 months of age time point. For these comparisons, neurotransmitter levels from 2 months old NS and PS groups were analyzed using ANOVAs that included Pb, gender and stress for each neurotransmitter in each brain region. Given main effects or interactions involving gender, subsequent analyses were carried out with Pb and stress as factors separately for males and females. At the 2 months time point, levels of HVA in frontal cortex of females were generally below detection limits.

2.7.2. Changes in the trajectory of neurochemical changes between 2 and 6 months in relation to time alone vs. prenatal stress

To attempt to both simplify and clarify results, data from all normal non-Pb treated groups (i.e., all 0 ppm groups; Figs. 3–5) at the 6 months time point were analyzed by overall ANOVAs including gender (except frontal cortex DOPAC and DATO), condition (EM vs. NEM) and stress (NS, PS, OS) for each neurotransmitter in each region. This allowed a determination as to how neurotransmitter levels had changed under EM vs. NEM conditions in relation to time alone as well as any changes produced specifically by PS. Analyses that revealed main effects or interactions involving gender were followed by subsequent ANOVAs and post-hoc tests to further elaborate the nature of the effects.

2.7.3. Modification by Pb+/−stress of the trajectory of neurochemical changes between 2 and 6 months

To examine whether Pb exposure or Pb+stress altered the trajectory of neurochemical changes between the 2 and 6 months time points in EM vs. NEM conditions, ANOVAs were carried out that included Pb exposure (0, 50 and 150 ppm), stress (NS, PS and OS) and condition (EM and NEM) for all 6 months time point data for each neurotransmitter in each region. For females, this was restricted to 0 and 150 ppm data based on missing 50 ppm NEM values. In the event of main effects or interactions, lower order ANOVAs and post-hoc tests were carried out as appropriate to address specific questions.

3. Results

3.1. Blood Pb levels

Group mean PbBs (μg/dl) of dams (n = 7–9/group) and pups (n = 3–9/gender/group) determined at pup weaning (21 days of age) increased with increasing Pb exposure concentration (Table 2) as previously reported [4,8]. 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. The group × Pb interaction reflected the approximately 7 μg/dl higher PbBs of male and female offspring relative to dams at 50 ppm. At 150 ppm, PbBs 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. Our prior studies have demonstrated that following maternal Pb exposure, pup PbBs are below detection limits by 40 days of age [57].

3.2. Neurochemical profiles at 2 months of age

Monoamine levels in frontal cortex, nucleus accumbens and striatum at 2 months of age exhibited multiple gender-based differences, but only a few occurrences of Pb+/−stress effects (Fig. 2).

3.2.1. Frontal cortex

Significant gender (G) × stress (S) differences in females were found for DOPAC and 5HIAA (169% and 146%; $F(1,75) = 7.68$, $p = 0.007$ and $F(1,104) = 3.94$, $p = 0.0498$, respectively) that reflected, in both cases, elevated levels in 150PS females; no differential changes occurred among male groups. Males exhibited slight but significantly higher levels of NE than females ($F(1,103) = 4.09$, $p = 0.046$). A significant overall Pb × gender interaction for 5HT ($F(2,103) = 3.105$, $p = 0.049$) reflected the higher levels across 150 ppm female NS, PS and OS groups than in either 0 or 50 ppm groups, whereas no differences were found among male groups.

3.2.2. Nucleus accumbens

Significant gender differences were found in nucleus accumbens for DA, DOPAC, NE, 5HT and 5HIAA (DA: $F(1,103) = 109.2$, $p < 0.0001$; DOPAC: $F(1,102) = 27.15$, $p < 0.0001$; NE: $F(1,102) = 13.67$, $p = 0.0004$; 5HT: $F(1,102) = 109.42$, $p < 0.0001$; 5HIAA: $F(1,102) = 15.19$, $p = 0.0002$), where levels in females were significantly greater than in males, and for DATO ($F(1,103) = 51.37$, $p < 0.0001$), where DA turnover rates for females were notably lower than those of males.

Table 2
Blood Pb values at weaning in dams and male and female offspring.

Pb exposure (ppm)	Group					
	Dam NS	Dam PS	Male NS	Male PS	Female NS	Female PS
0	0.89 ± 0.08[a]	0.79 ± 0.10	1.78 ± 0.6	1.58 ± 0.35	1.2 ± 0.45	1.43 ± 0.3
50	11.6 ± 0.51	11.8 ± 0.5	19.6 ± 6.0	18.8 ± 0.85	19.3 ± 4.8	19 ± 2.0
150	30.8 ± 1.34	31.2 ± 0.78	32.00 ± 3.0	25.4 ± 2.5	35.5 ± 2.7	32.6 ± 1.2

NS = no stress; PS = prenatal stress.
[a] Mean ± S.E. values in μg/dl

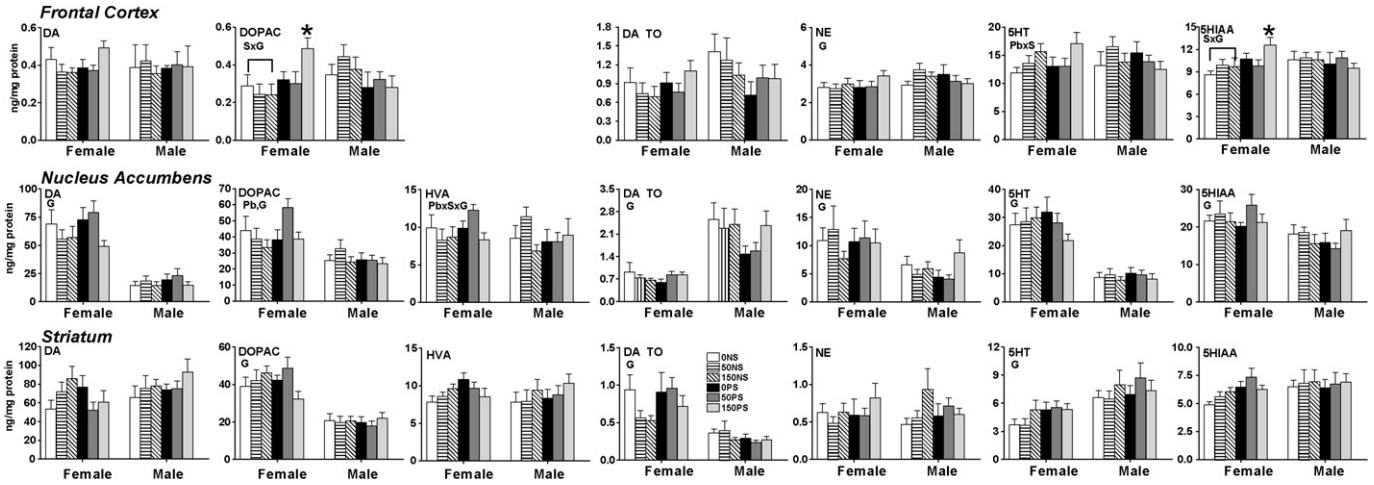

Fig. 2. Group mean ± S.E. levels of neurotransmitters (ng/mg protein) in frontal cortex, nucleus accumbens and striatum of 0NS, 50NS, 150NS, 0PS, 50PS and 150PS female and male offspring determined at 2 months of age. Sample sizes were 4–10 for frontal cortex, 7–12 for nucleus accumbens and 8–10 for striatum. Bars over columns represent stress-related differences. * Signifies greater than all other same gender groups. Outcomes of overall ANOVA: S = stress, G = gender, Pb = lead.

3.2.3. Striatum

DOPAC and DATO values in females were approximately twice those of males (DOPAC: $F(1,104) = 91.78$, $p < 0.0001$; DATO: $F(1,104) = 43.42$, $p < 0.0001$), while 5HT levels of males exceeded those of females ($F(1,106) = 18.68$, $p < 0.0001$).

3.3. Neurochemical profiles at 6 months of age

3.3.1. Time alone and stress-induced changes

Effects of time alone (EM vs. NEM) and of stress conditions (0NS, 0PS and 0OS groups) on the trajectory of neurotransmitter changes are derived from 0 ppm group values and depicted as percent change from corresponding 2 months group mean gender-based 0NS values in Figs. 3–5 for frontal cortex, nucleus accumbens and striatum, respectively. Results are summarized in Table 3.

3.3.2. Frontal cortex

Gender differences in overall statistical analyses were found for DA (EMxG: $F(1,107) = 4.81$, $p = 0.03$) and HIAA (EMxGxS, $F(2,116) = 3.59$, $p = 0.031$) and thus ANOVAs were subsequently carried out separately by gender for these neurotransmitters. Differential trajectories for EM vs. NEM conditions were found for every neurotransmitter measured in frontal cortex (EM: DA: $F(1,107) = 10.51$, $p = 0.0016$; NE: $F(1,112) = 68.2$, $p < 0.0001$; 5HT: $F(1,116) = 201.3$, $p < 0.0001$; 5HIAA: $F(1,116) = 499.6$, $p < 0.0001$), with an overall highly similar profile in both genders (Fig. 3; Table 3).

NEM-associated increases in DA and DOPAC (measured in males only) of 104–209% at 6 months were reduced by EM procedures to 54–115% or approximately back to, or slightly below, 2 months time point levels (EM: $F(1,58) = 5.44$, $p = 0.023$, $F(1,57) = 4.1$, $p = 0.048$ and $F(1,55) = 26.56$, $p < 0.0001$ for female DA, male DA and male DOPAC, respectively). Similarly, the general increases in 5HT and 5HIAA (EM: $F(1,58) = 309.1$, $p < 0.0001$ and $F(1,60) = 163.9$, $p < 0.0001$ for males and females, respectively) of 65–177% associated with NEM conditions were markedly attenuated by EM procedures to values of 9–24% of 2 months levels. DATO values of males were reduced under NEM conditions, but further reduced by EM conditions (47–74% to 33–37%; $F(1,56) = 19.8$, $p = 0.0001$). In contrast, the modest increases in NE under NEM conditions (118–150%) in both males and females were actually significantly further enhanced to 207–365% by EM conditions.

The only stress-induced modification observed was for 5HIAA levels in females, where OS, under NEM conditions, actually increased 5HIAA levels above 2 months time point values, while values were reduced for NS and PS NEM groups; the absence of corresponding differences under EM conditions indicated that all NEM effects had been reversed (post-hoc EMxS: $p = 0.0001$).

3.3.3. Nucleus accumbens

Gender-related differences were found for every neurotransmitter in the overall analyses (DA: G, $F(1,115) = 4.08$, $p = 0.0475$; DOPAC: GxS, $F(1,115) = 10.42$, $p = 0.0016$; NE: G, $F(1,114) = 8.03$, $p = 0.0054$; 5HT: GxEM, $F(1,113) = 14.45$, $p = 0.0002$; 5HIAA: G, $F(1,115) = 68.47$, $p < 0.0001$), consequently, separate ANOVAs were carried out for each gender and neurotransmitter to further clarify the nature of the effects. Differential trajectories of neurochemical changes under EM vs. NEM conditions at 0 ppm were observed in nucleus accumbens for all neurotransmitters except DA for both genders, and HVA and NE in males (Fig. 4; females: DOPAC: $F(1,59) = 27.39$, $p < 0.0001$; HVA: $F(1,58) = 9.86$, $p = 0.0027$; DATO: $F(1,59) = 33.24$, $p < 0.0001$; NE: $F(1,54) = 4.01$, $p = 0.049$; 5HT: $F(1,54) = 16.71$, $p = 0.001$; 5HIAA: $F(1,59) = 54.75$, $p < 0.0001$; males: DOPAC: $F(1,58) = 96.48$, $p < 0.0001$; DATO: $F(1,56) = 62.03$, $p < 0.0001$; 5HT: $F(1,57) = 49.94$, $p < 0.0001$; 5HIAA: $F(1,57) = 420.46$, $p < 0.0001$).

DA levels were comparably increased by EM and NEM conditions in both males and females (78–215%). The general increases relative to 2 months levels in DOPAC in males (119–164%), and of HVA in females (154–301%) under NEM conditions were significantly attenuated by EM procedures (down to 22–30% for DOPAC: 93–115% for HVA). Under NEM conditions, levels of 5HT and 5HIAA did not change systematically relative to 2 months values in either gender, but all were reduced by EM conditions (5HT: 15–45%; HIAA: 9–63%). In contrast, NE levels of females were reduced to 62–85% under NEM conditions, but increased by EM procedures (108–250%).

Stress-related modifications were found for DOPAC in females (EMxS: $F(2,59) = 5.08$; $p = 0.009$) and DATO for both genders (EMxS: $F(2,59) = 16.63$, $p < 0.0001$ and $F(2,58) = 3.46$, $p = 0.038$ for females and males, respectively) under NEM conditions. OS significantly increased DOPAC levels in NEM females (+114%) and DATO in both genders (+163% and +58% for females and males, respectively) above 2 months time point values, while values in the corresponding 0NS and 0PS groups were generally reduced below 2 months values (all post-hoc p values < 0.0001). Corresponding stress-related differences were not observed under EM conditions, where levels of DOPAC (39–71%) and DATO (8–56%) were similarly attenuated

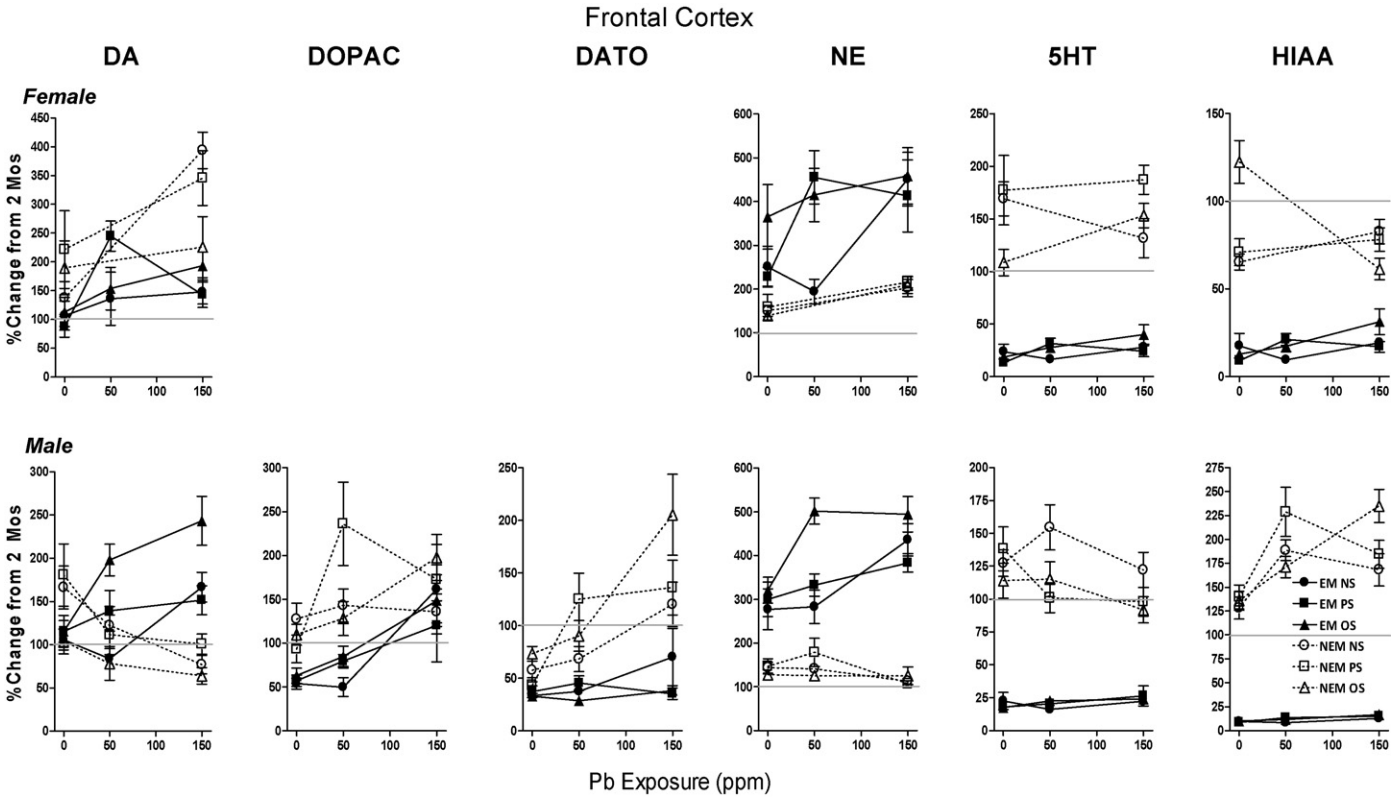

Fig. 3. Group mean ± S.E. levels of DA, DOPAC, DATO, NE, 5HT and 5HIAA in frontal cortex of females (top row) and males (bottom row) at 6 months of age in relation to Pb exposure concentration (ppm) for NS, PS and OS groups in EM and NEM conditions, as indicated. Data are plotted as percent change from corresponding gender group mean 0NS values of littermates determined at 2 months of age (Fig. 1). Sample sizes are as described in Fig. 2 legend. Outcomes from overall ANOVAs: EM = experimental manipulation, Pb = Pb exposure, S = stress. Horizontal line at 100% shown to facilitate visualization of direction and magnitude of change.

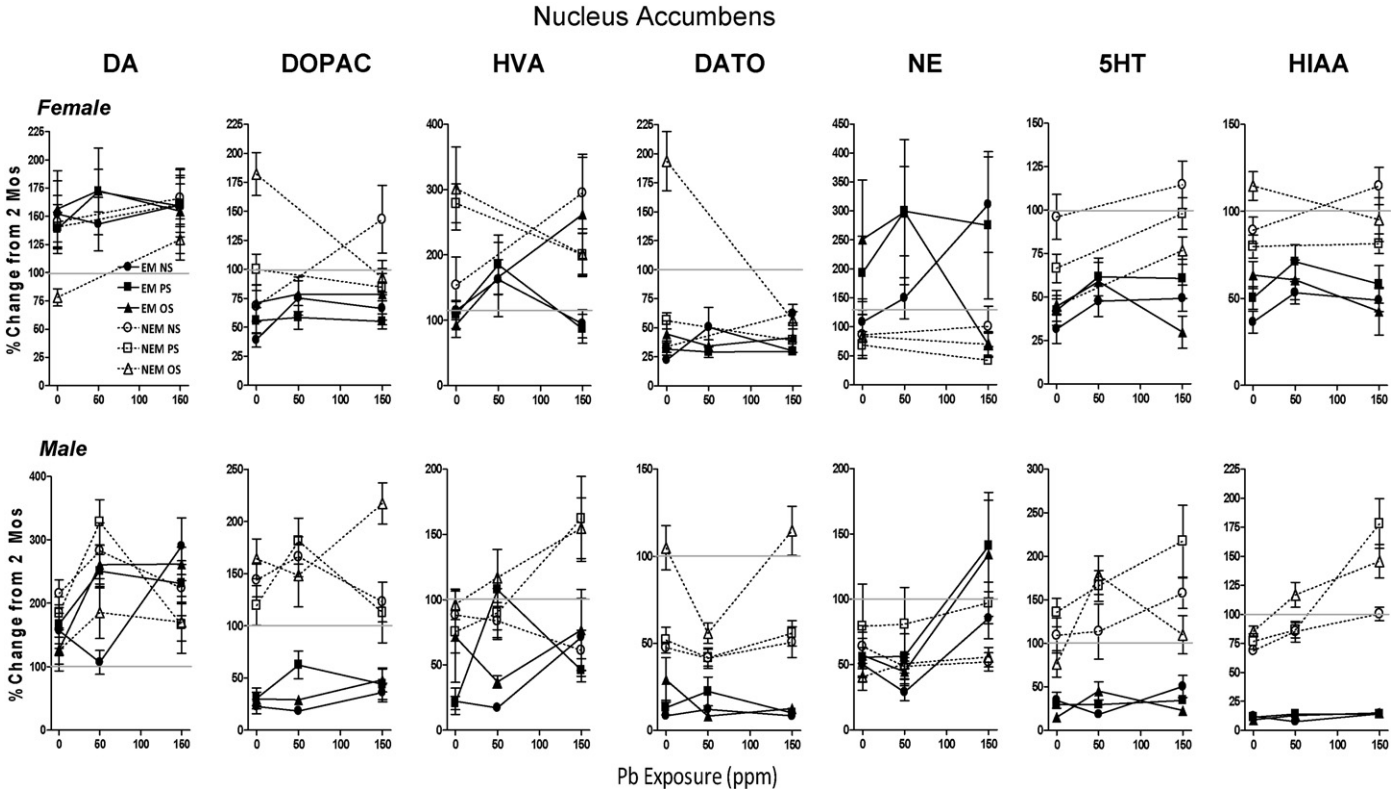

Fig. 4. Group mean ± S.E. levels of DA, DOPAC, HVA, DATO, NE, 5HT and 5HIAA in nucleus of females (top row) and males (bottom row) at 6 months of age in relation to Pb exposure concentration (ppm) for NS, PS and OS groups in EM and NEM conditions, as indicated. Data are plotted as percent change from corresponding gender group mean 0NS values of littermates determined at 2 months of age (Fig. 1). Sample sizes are as described in Fig. 3 legend. Outcomes from ANOVAs: EM = experimental manipulation, Pb = Pb exposure, S = stress. Horizontal line at 100% shown to facilitate visualization of direction and magnitude of change.

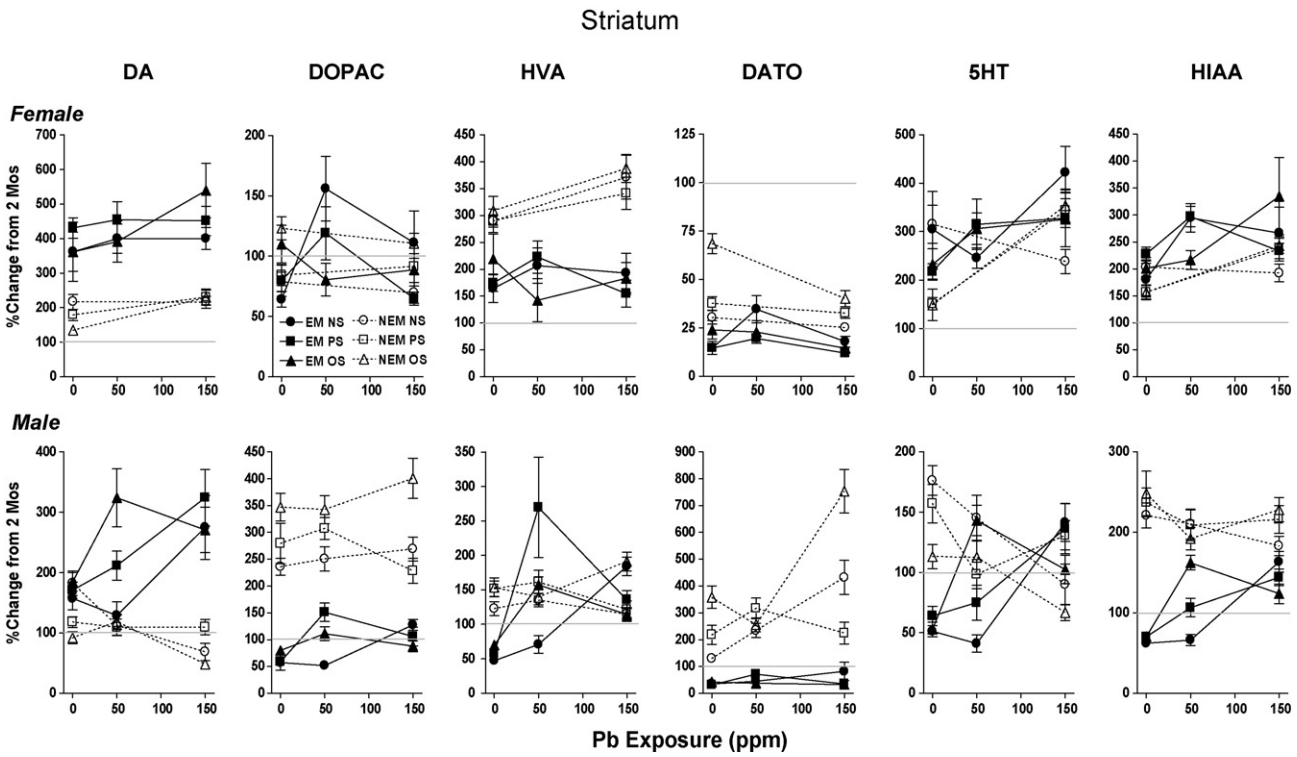

Fig. 5. Group mean ± S.E. levels of DA, DOPAC, DATO, NE, 5HT and 5HIAA in striatum of females (top row) and males (bottom row) at 6 months of age in relation to Pb exposure concentration (ppm) for NS, PS and OS groups in EM and NEM conditions, as indicated. Data are plotted as percent change from corresponding gender group mean 0NS values of littermates determined at 2 months of age (Fig. 1). Sample sizes are as described in Fig. 4 legend. Outcomes from ANOVAs: EM = experimental manipulation, Pb = Pb exposure, S = stress. Horizontal line at 100% shown to facilitate visualization of direction and magnitude of change.

across stress conditions, indicating the reversal of these effects by EM procedures.

3.3.4. Striatum

Gender-related differences were found for every neurotransmitter in the overall analyses, consequently, separate ANOVAs were carried out for each gender and neurotransmitter to further clarify the nature of the effects (DA: $F(1,117) = 69.02$, $p < 0.0001$; DOPAC: $F(1,117) = 77.72$, $p < 0.0001$; HVA: $F(1,114) = 188.06$, $p < 0.0001$; DATO: $F(1,119) = 53.05$, $p < 0.0001$; 5HT: $F(1,119) = 52.62$, $p < 0.0001$; 5HIAA: $F(1,119) = 10.99$, $p < 0.012$). EM vs. NEM conditions differentially affected the trajectory of all neurotransmitters at 0 ppm in striatum except DOPAC, 5HT and 5HIAA in females (Fig. 5; FEMALE: DA: $F(1,60) = 38.69$, $p < 0.0001$; HVA: $F(1,60) = 9.36$, $p = 0.033$; DATO $F(1,59) = 53.76$, $p < 0.0001$; MALE: DA: $F(1,60) = 6.71$, $p = 0.012$; DOPAC: $F(1,60) = 172.44$, $p < 0.0001$; HVA: $F(1,58) = 120.08$, $p < 0.0001$; DATO: $F(1,59) = 112.9$, $p < 0.0001$; 5HT: $F(1,60) = 114.9$, $p < 0.0001$; 5HIAA: $F(1,60) = 183.5$, $p < 0.0001$).

Table 3
Changes in Levels of neurotransmitters in 0 ppm EM conditions compared to 0 ppm NEM conditions in frontal cortex, nucleus accumbens and striatum and associated stress-induced modifications.

Region	Neurotransmitter						
	DA	DOPAC	HVA	DATO	NE	5HT	5HIAA
Frontal cortex							
Female[a]	↓58%	–	–	–	↑57%	↓45%	↓54%
Interactions[b]							NEM: >OS
Male[a]	↓45%	↓77%	–	↓27%	↑138%	↓100%	↓119%
Interactions[b]							
Nucleus accumbens							
Female	NC	↓29%	↓39%	↓12%	↑22%	↓65%	↓53%
Interactions		NEM: >OS		NEM: >OS			
Male	NC	↓121%	↓116%	↓40%	NC	↓77%	↓56%
Interactions				NEM:>OS			
Striatum							
Female	↓207%	NC	↓129%	↓15%	–	NC	↑EMxS
Interactions							NEM: <PS, OS
Male	↑EMxS	↓179%	↓76%	↓94%	–	↓125%	↓167%
Interactions	↓NEM: PS, OS			NEM: >OS > PS		NEM: < OS	

Symbols and abbreviations: ↓ = decrease; ↑ = increase; DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; DATO = dopamine turnover; NE = norepinephrine; 5HT = serotonin; 5HIAA = 5hydroxyindole acetic acid; NC = no change; gray shading = comparable effects in males and females. Lower order ANOVA outcomes: EM = experimental manipulation; S = stress.

[a] Changes in 0 ppm NS EM compared to 0 ppm NS NEM.

[b] Changes reflecting EMxS interactions.

EM procedures further elevated the increases in DA in NEM conditions in both females and males from 92–217% to 157–430%. NEM conditions increased DOPAC levels in males (236–347%) and HVA levels of both genders (123–308%), all of which were reduced by EM procedures (47–218%). Relative to 2 months values, DATO declined under NEM conditions in females (30–69%), but increased in males (129–358%); both were reduced by EM procedures (15–42%. Levels of 5HT were comparably increased by EM and NEM conditions in females (148–315%), whereas NEM-induced increases in 5HT in males (113–176%) that were reduced by EM (51–64%). The increase in 5HIAA levels under NEM conditions in males (221–248%) was markedly attenuated by EM procedures (62–70%).

Stress-related modifications of these trajectories were observed for DA (EMxS: $F(2,60) = 8.71$, $p = 0.0005$), DATO (EMxS: $F(2,59) = 11.65$, $p < 0.0001$), and 5HT (EMxS: $F(2,60) = 4.67$, $p = 0.013$) in males. Specifically, DA levels were significantly lower in the NEM PS and OS groups than in EM counterparts, whereas no significant differences were found with EM vs. NEM NS groups. NEM conditions significantly increased DATO levels in PS and OS groups, but not in the NS group, resulting in a stress-related change OS > PS > NS. 5HT levels were increased in a reverse manner by NEM relative to EM values, with increases of 345%, 245% and 192% for the NS, PS and OS groups, respectively. In all three cases, stress-related differences were not found in the corresponding EM conditions, indicating reversal of these effects. For females, increases in 5HIAA in the 0NS NEM group were suppressed by both PS and OS (EMxS: $F(2,58) = 3.96$, $p = 0.024$; reduction to 46%;), but these effects were not found under EM conditions, consistent with their reversal.

Table 3 summarizes changes produced by time alone (0NS EM vs. 0NS NEM) and any stress-related interactions (EMxS) across brain regions for each gender. As it shows, EM generally attenuated levels of DA and metabolites and of 5HT and its metabolite 5HIAA across brain regions with similar effects in both genders, with a few exceptions (DA in striatum; striatal 5HIAA in females). Although less data was available, measurements of NE levels, in contrast, increased under the influence of EM procedures. Stress-related modifications of these effects were primarily related to OS, and observed only under NEM conditions, i.e., were reversed by EM procedures. Further, stress-induced modifications exhibited no systematic pattern, with the exception of the corresponding enhancements of DATO in nucleus accumbens of both genders.

3.4. Pb and Pb-stress-induced changes

Pb concentration effect curves for all NS, PS and OS EM and NEM groups are depicted as a percent change from corresponding gender group mean 2 months 0NS values for frontal cortex, nucleus accumbens and striatum, respectively, in Figs. 3–5; corresponding results are summarized in Table 4. For all female groups, analyses are based only on 0 and 150 ppm data due to lost samples at 50 ppm.

3.4.1. Frontal cortex

Pb exposure influenced the trajectories of all neurotransmitters in frontal cortex except 5HT in both genders. However, the profiles of effects differed in males vs. females. In females, significant Pb-associated increases in DA (Pb: $F(1,110) = 11.63$, $p = 0.0009$) and NE levels (Pb: $F(1,112) = 18.73$, $p < 0.0001$) from 87–221 at 0 ppm to 143–405% at 150 ppm, and, correspondingly, from 139–365% to 202–459%, for DA and NE, respectively, were of comparable magnitude in EM and NEM conditions. Elevated levels of 5HIAA in the 0 ppm OS NEM group were not seen in the 150 ppm OS NEM group (PbxEMxS: $F(2,113) = 14.39$, $p < 0.0001$), whereas slight, but significant, Pb-related increases occurred in the EM condition (9–13% to 17–31%; post-hoc $p = 0.022$;).

Table 4
Pb and Pb+stress modifications of the trajectory of neurotransmitter changes between 2 and 6 months.

	Neurotransmitter						
	DA	DOPAC	HVA	DATO	NE	5HT	5HIAA
Female							
EM-FC	↑				↑	–	↑
-NAC	–	–	PbxS	–	–	↑	–
-STR	↑	↑	PbxS	↑+		↑	↑
NEM-FC	↑				↑	–	–
-NAC	–	PbxS	PbxS	PbxS	–	↑	PbxS
-STR	↑	–	↑	↓		↑	↑
Male							
EM-FC	↑+	↑*		–	↑+	–	↑
-NAC	↑	–	–	–	–	–	–
-STR	↑+	↑	↑*	–		↑*	↑*
NEM-FC	↓	↑		↑	↓	–	–
-NAC	↑	PbxS	PbxS	PbxS	–	↑	↑
-STR	↓	PbxS	–	↑		↓	↓

Symbols and abbreviations: ↓ = decrease; ↑ = increase. Lower order ANOVA outcomes: PbxS = Pb × stress interaction; FC = frontal cortex; NAC = nucleus accumbens; STR = striatum; DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; DATO = dopamine turnover; NE = norepinephrine; 5HT = serotonin; 5HIAA = 5hydroxyindole acetic acid; NC = no change. EM = experimental manipulation; NEM = no experimental manipulation; black shading = not measured; gray shading: reversal of NEM Pb effect by EM conditions; * = effect of Pb over-rides attenuating influence of EM; + = opposite effect of Pb in EM vs. NEM.

In males, Pb exposure decreased DA (PbxEM: $F(2,172) = 15.94$, $p < 0,0001$; 104–181 to 84–101%) and NE (PbxEM: $F(2,178) = 11.95$, $p < 0.0001$; 128–147% to 107–125%) under NEM conditions, but increased levels (106–115% to 151–243% and from 277–320% to 383–495%, respectively) under EM conditions, with EM elevations even further enhanced by OS at 50 ppm for DA (+93%) and NE (+181%), and at 150 ppm for DA (+128%). Pb increased DOPAC levels (PbxEM: $F(2,182) = 3.49$, $p = 0.032$) in both EM (53–63% to 121–162%) and NEM (93–127% to 135–197%) conditions, with a more pronounced effect in the 50PS NEM group (+143%) relative to the 0PS NEM group (post-hoc PbxS: $p = 0.009$). Notably, while DOPAC levels were lower at 0 ppm under EM than NEM conditions, the increases at 150 ppm in EM groups reached levels comparable to those of NEM groups; thus, Pb exposure overrode the attenuating influence of EM. Pb-induced increases in DATO (PbxEM: $F(2,175) = 8.62$, $p = 0.0003$; 47–73% to 120–205%) and 5HIAA (PbxEM: $F(2,185) = 11.34$, $p < 0.0001$; 128–140% to 168–235%) under NEM conditions were generally reversed by EM procedures.

3.4.2. Nucleus accumbens

Pb influences were found for all neurotransmitters in nucleus accumbens with the exception of DA and 5HT in females and NE in both genders. Effects in females were variable and generally not remarkable. They included variable but significant Pb-related increases in 5HT (Pb: $F(1,111) = 10.09$, $p = 0.0019$), as well as markedly elevated DOPAC (PbxSxEM: $F(2,114) = 5.32$, $p = 0.0062$) and DATO (PbxSxEM: $F(2,114) = 14.08$, $p < 0.0001$) levels at 0 ppm in the OS NEM group but no corresponding stress interaction at 150 ppm. PS resulted in modest but significantly lower 5HIAA levels in female NEM groups (PbxS: $F(2,115) = 5.54$, $p = 0.005$). Pb+stress differentially affected HVA levels in females (PbxSxEM: $F(2,110) = 6.02$, $p = 0.003$), due to increases in the 150 ppm OS EM group (post-hoc PbxS: $p = 0.034$), whereas the increases in NEM PS and OS groups at 0 ppm were not seen at 150 ppm (post-hoc PbxS: $p = 0.042$).

In males, Pb exposure increased DA levels (PbxEM: $F(2,185) = 5.24$, $p = 0.006$) in both EM (127–211% to 231–290%) and NEM (125–215% to 166–224%) conditions. In the EM condition,

more pronounced increases with PS (+143%) and OS (+154%) were found than in NS at 50 ppm (PbxS: $F(4,185) = 3.08$, $p = 0.0047$). DOPAC levels (EM: $F(1,185) = 177.65$, $p < 0.0001$) were increased under NEM conditions only for 150 ppm combined with OS (+64%; PbxS: $F(4,185) = 3.23$, $p = 0.0137$), a potentiated effect reversed by EM procedures. Similarly, potentiated Pb-induced increases in HVA (PbxSxEM: $F(4,157) = 4.06$, $p = 0.0037$) under NEM conditions were found at 150 ppm in PS (+66%) and OS (+59%) groups (post-hoc PbxS: $p = 0.032$), a Pb+stress additive effect reversed by EM procedures. Under NEM conditions, DATO (PbxS: $F(4,183) = 4.29$, $p = 0.0024$; PbxEM: $F(2,183) = 6.77$, $p = 0.0015$) was markedly elevated by OS at 0 and 150 ppm, but not at 50 ppm (post-hoc PbxS: $p = 0.010$); these stress modifications were also eliminated by EM procedures. Pb exposure increased 5HT comparably in EM and NEM conditions (Pb: $F(2,184) = 4.04$, $p = 0.019$; 77–109% to 114–210%). Pb-associated increases in 5HIAA levels were seen only in NEM conditions (PbxEM: $F(2,184) = 18.66$, $p < 0.0001$; 69–85% to 85–191%). Potentiated increases in 5HIAA were produced in the NEM condition (post-hoc PbxS: $p = 0.0005$) by OS at 50 ppm (+32%) and at 150 ppm by PS (+93%) and OS (+60%), effects reversed by EM in both cases.

3.4.3. Striatum

Pb exposure influenced the trajectory of changes in striatum of all neurotransmitters measured in both genders. In females, Pb-induced increases in DA, 5HT and 5HIAA levels were of comparable magnitude under NEM and EM conditions (DA: $F(1,113) = 8.78$, $p = 0.0037$; 5HT: $F(1,113) = 14.4$, $p = 0.0002$; 5HIAA: $F(1,110) = 15.53$, $p = 0.0001$. In contrast, Pb-associated increases in HVA (PbxEM: $F(1,113) = 6.76$, $p = 0.0106$) under NEM conditions (290–309% to 341–387%) were reversed by EM procedures. The influence of Pb on DOPAC occurred under EM conditions (PbxSxEM: $F(2,113) = 3.33$, $p = 0.0394$), where increased levels at 0 ppm were conferred by OS, and higher levels at 50 ppm produced by NS (post-hoc PbxS: $p = 0.008$). For DATO (PbxEM: $F(1,110) = 6.46$, $p = 0.012$; PbxS, $F(2,110) = 7.83$, $p = 0.0007$), OS elevated levels at both 0 and 150 ppm only under NEM conditions (post-hoc PbxS; $p = 0.01019$).

In males, Pb reduced levels of DA (PbxEM: $F(2,186) = 19.75$, $p < 0.0001$; 92–182% to 49–110%), 5HT (PbxEM: $F(2,189) = 29.55$, $p < 0.0001$; 113–176% to 67–136%) and 5HIAA (PbxEM: $F(2,188) = 18.7$, $p < 0.0001$; 221–248% to 183–228%) under NEM conditions, but increased levels under EM conditions (142–185% to 271–324%, 51–64% to 102–141% and 62–70% to 124–163%, respectively). For all three neurotransmitters, EM effects were enhanced by OS, where peak levels were already attained at 50 ppm (post-hoc PbxS: $p = 0.03$, 0.0001 and <0.0001; +208%, +85% and +96%, respectively). Furthermore, for both 5HT and 5HIAA, the increases at 150 ppm in EM groups essentially produced values consistent with NEM levels, indicating that 150 ppm Pb exposure over-rode the EM-associated attenuation of NEM effects. Similarly, Pb-associated increases in HVA (PbxEM: ($F(2,184) = 10.79$, $p < 0.0001$) in 50 ppm OS and 50 ppm PS EM groups, and in all 150 ppm EM groups reached levels associated with NEM conditions, consistent with a Pb exposure associated over-ride of the EM influence (47–70% to 115–246%). Pb-associated increases in DATO (PbxSxEM: $F(4,186) = 5.05$, $p = 0.0007$) under NEM conditions (129–358% to 225–753%), were markedly enhanced (potentiated) by OS, but fully reversed by EM procedures. Modest Pb-associated increases in DOPAC occurred in both EM and NEM conditions (Pb: $F(2,189) = 3.02$, $p = 0.05$; SxEM: $F(2,189) = 7.4$, $p = 0.0008$; 57–80% to 52–151%), although 150 ppm OS further increased levels under NEM conditions (post-hoc PbxS: $p < 0.0001$), while 50 ppm levels were stress-related under EM conditions, with PS > OS > NS (post-hoc PbxS: $p = 0.004$).

Table 4 summarizes Pb and Pb+/−stress-associated modifications of EM vs. NEM trajectories of neurotransmitter changes between 2 and 6 months for each brain region for females and males. As it shows, Pb or Pb+stress modulation of 2 to 6 months trajectories were found under both EM and NEM conditions in both genders, although a greater percent change was found under NEM conditions in both cases, especially for males (65 vs. 71% and 53 vs. 84%, for females and males, respectively). In females, Pb exposure generally increased neurotransmitter levels (except striatal DATO under NEM conditions), whereas in males, increases occurred under EM conditions and both increases and decreases were found under NEM conditions.

EM attenuated some but not all effects of Pb and or Pb+stress, with reversals predominating in nucleus accumbens in both genders (gray shading Table 4; females: DOPAC, HVA and DATO; males: DOPAC, HVA, DATO, 5HT and 5HIAA; indicated by Pb or PbxS effect in NEM condition but no corresponding change in the EM condition) and NEM DATO changes in all regions in males. Pb exposure over-rides of the attenuating influence of EM, i.e., where EM values reached NEM levels, were also found, but only in males, where they occurred with frontal cortex DOPAC, and striatal HVA, 5HT and 5HIAA as Pb exposure concentration increased. In some cases (specifically striatal HVA of females, frontal cortex DA and NE and striatal DA of males), Pb-induced changes were in opposite directions in EM vs. NEM conditions. Although not shown, conditions in which stress modified Pb effects were always driven by OS in females, but examples of both OS and PS modulation were observed for males.

4. Discussion

Results from this study confirmed that neurotransmitter levels change over time alone (NEM conditions), even in normal (control: 0 ppm NS) animals, between 2 and 6 months of age and did so across multiple brain regions and monoamines. Moreover, EM conditions significantly modified the trajectories seen under NEM conditions, and with the exception of increases in NE and DA in striatum, EM conditions typically blunted the changes in monoamine levels that occurred with time alone. Pb+/−stress modified the trajectory of monoamine changes in both EM and NEM conditions, but predominated under NEM conditions. While modifications induced by stress alone over time under NEM conditions were fully reversed by EM procedures, Pb+/−stress-associated modifications in NEM were far less responsive to reversal, and occurred primarily in nucleus accumbens. Indeed in some cases, Pb exposure effects over-rode the attenuating influence of EM, resulting in neurotransmitter levels associated with NEM conditions.

4.1. Time alone (NEM condition) was associated with neurochemical changes between 2 and 6 months of age

Importantly, time alone resulted in often quite dramatic neurochemical changes both in control (0 ppm) and Pb+/−stress challenged offspring between 2 and 6 months time points, with differences as great as 300%. While the dynamic nature of neurochemical function over time is known, most comparisons involve early vs. aged stages of the life span [30,31,35,36]. This study provides evidence of ongoing dynamics post-development of monoamine systems [58,59] but well before age-related changes ensue, indicating their modulation even during the adult period. While a previous study reported reductions in amphetamine-stimulated striatal DA release at 2 vs. 6 months of age in male, but not female mice, a difference attributed to the presence vs. absence of hormonal activities at earlier vs. later ages [34], the profile of changes with time here showed general gender comparability (Table 3), albeit differences in magnitude of change. Differences in findings could easily reflect the species and outcome measure used. The fact that time alone changes neurochemical function at this

stage has particular implications for experimental design of intervention studies. First, the marked differences between the EM and NEM neurotransmitter profiles at the 6 months time point indicate that neurochemical changes, and perhaps other biochemical changes, observed from animals not subjected to environmental manipulations may not be generalizable to, or explanatory for, those with specific environmental histories. Secondly, determinations of basal neurotransmitter changes prior to imposing intervention vs. no intervention conditions may allow a separation of the actual underlying neurochemical changes directly relevant to the intervention procedure per se separate from time-based changes.

4.2. Experimental manipulations modify the trajectory of neurotransmitter changes occurring under NEM conditions

EM conditions modified, sometimes dramatically and broadly across regions (frontal cortex, nucleus accumbens, and striatum) and neurotransmitters, the trajectory of changes observed in NEM conditions between 2 and 6 months (Figs. 3–5 and Table 3). Such behavioral plasticity was first proposed by Hebb [60], with subsequent studies demonstrating that differential experiences with problem solving, and later, differential housing environments could produce significant changes in both brain structure and neurochemical function [61,62]. The differences in trajectories seen here between EM and NEM conditions are indeed consistent with such previous reports. It is notable that EM vs. NEM conditions here also resulted in significantly different trajectories of corticosterone changes in response to stress challenges, as we have previously reported [4,56].

What cannot be ascertained from the current experiment is which component(s) of the EM condition are responsible for the ameliorating influence of EM on neurotransmitter levels. EM here included daily handling, weighing, and transport, behavioral testing, reinforcement availability and physical activity involved in FI performance. Physical exercise as an intervention has been generally shown to be less efficacious as an intervention strategy than either enrichment or formal training, suggesting it might be less influential [62]. Handling during the early postnatal period has been previously reported to attenuate stress effects [63,64]. While early handling (weighing and culling of litters) was comparable in EM and NEM offspring, differences could certainly reflect the more sustained handling of EM animals as a component of behavioral testing.

The role of the specific behavioral paradigm, as well as the use of reinforcement contingencies may also be critical and is a particularly intriguing question. Passive avoidance training of mice was associated with multiple neurochemical changes and increased plasma corticosterone levels, whereas exposure to the apparatus alone increased corticosterone levels but did not produce neurochemical changes, suggesting both that the behavioral contingencies were the critical influence, and that neurochemical changes may be dissociated from changes in corticosterone [65]. However, other studies have also reported increases in nucleus accumbens DOPAC levels of mice of both genders [66] and increases in HVA in nucleus accumbens in male mice [67] simply in response to exposure to a novel environment, suggesting reinforcement contingencies are not critical. Obviously the contradictory nature of these findings leaves open the question of the importance of reinforcement contingencies per se and their potential influence independently of other conditions of behavioral testing. It is likely that both alter neurochemical function, but in ways that differ according to the specific intervention.

If reinforcement contingencies per se are important in determining the nature of the changes in the neurochemical profile, then an additional question is how the nature of the contingency itself influences the expression of neurochemical changes. Dunn et al. [65] utilized passive avoidance, a negative reinforcement-based paradigm considered by them as a form of stress that has indeed been shown to increase corticosterone levels [68]. It will be important to determine the extent to which positive vs. negative reinforcement based behavioral experience influences both the profile of neurochemical changes, and performance in subsequent behavioral situations following early insults. A related question is whether the influence of the behavioral paradigm would differ depending upon its mediation or not by brain regions likewise impacted by Pb and or stress.

Importantly, the EM procedures used here are typical of those used in many experiments evaluating the consequences of developmental insults, and are often carried out in conjunction with measures of changes in neurochemical function. The current findings suggest that the neurochemical changes seen post-behavioral testing may not be the same as those operative before testing, i.e., those presumably associated with the developmental insults per se, and therefore raise the question of which of such changes, or both, are important. That is, the potential confounds associated with behavioral experience leave many residual questions about whether effects attributed to early developmental insults reflect the insult itself, or the subsequent sequence of environmental influences as they interact with the developmental insult [69]. Nevertheless, it is ultimately an understanding of these interactions of developmental insults, such as Pb+/−stress, with different behavioral contexts that will be critical to elaborating the development of dysfunction or psychopathology and for devising effective behavioral therapeutic strategies.

4.3. Stress, but not Pb+/−stress-associated changes in NEM neurotransmitter trajectories were reversed by EM procedures

It is notable that stress-induced modifications of neurochemical changes were actually minimal at the 2 months time point, restricted to frontal cortex DOPAC and 5HIAA in females but, instead, emerged over time in both genders primarily in OS groups, i.e., those that had sustained PS followed by OS challenges. Since corresponding changes were not found with PS alone, OS-induced neurotransmitter changes could reflect cumulative stress, the unmasking of PS effects by later stress challenges, or OS itself. An OS only group was not included in this experiment given the extent to which it would have expanded the effort, so the latter possibility cannot yet be evaluated. But in relation to the former two possibilities, it is interesting to note a report that acoustic startle responding was increased in offspring of dams exposed either to chronic mild stress or to dexamethasone only if they had also experienced immobilization stress for blood collection [70]. Similarly, PS was associated with changes in heart rate, temperature and physical activity not under basal conditions, but only under conditions of stress challenge [71]. Collectively such studies support the potential for cumulative or unmasked stress effects and support the use of stress challenges in adulthood as a useful method to unmask such effects.

Interestingly, all stress-associated changes in neurotransmitter levels under NEM conditions were reversed by EM procedures, findings consistent with other reports describing the ability of environmental interventions to reverse stress-associated effects [23]. Frequently such reversals have been achieved through ‘enriched’ housing [24,25,72] or handling during the postnatal period [5]. While daily handling occurred in the course of behavioral testing and weighing, it did not begin until offspring were already 2–3 months of age, well past the period handling is typically applied in such studies [2] or even later in periadolescence [26]. Thus, the current findings extend the set of conditions under which stress-related effects, at least some of the neurotransmitter changes, can be ameliorated; behavioral differences were not ameliorated [8,56].

In contrast to the reversal of OS-associated changes in neurotransmitter levels under NEM conditions by EM procedures, reversals of Pb+/−stress-associated neurochemical changes were generally restricted to nucleus accumbens where they were seen in both male and female offspring. In prior studies, enriched housing was shown to reverse learning deficits in the water maze, glutamatergic NR1 subunit mRNA deficits and neurotrophic factor gene expression changes in hippocampus [27,29], suggesting a broad efficacy. The restriction of the ameliorative effects of EM to nucleus accumbens in this study is particularly notable given the critical role of this region in mediating response rates on the FI schedule as we have previously demonstrated [13,73,74], and therefore raises the possibility that behavioral procedures evoking sustained activity in a region may contribute to overcoming Pb+/−stress effects in that region. The current findings, which extend the set of conditions under which Pb effects can be reversed, are reminiscent of those suggesting that reversals of Pb-induced toxicity may be both intervention and task dependent [28].

In addition to the fact that reversals of Pb+/−stress-induced neurotransmitter changes were restricted to nucleus accumbens, Pb exposure under EM conditions also actually increased levels of frontal cortex DA, and striatal HVA, 5HT and 5HIAA of males to values associated with NEM conditions, indicating its ability to over-ride the ameliorating influence of EM. In that regard, and related to the potential for effective intervention strategies to be both intervention and task-dependent, it is notable that EM conditions also actually increased frontal cortical NE levels in females relative to NEM conditions, and produced a profile of Pb-stress related NE changes with marked similarity to Pb+stress effects on fixed interval response rates, that were Pb+stress additive [8]. In fact, in corresponding principal component analyses that included all neurotransmitters and fixed interval behavioral performance, frontal cortex NE levels were among the strongest predictors of behavioral outcome. The latter observation again underscores the need to understand how various environmental conditions act on the altered CNS milieu arising from early developmental insults.

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