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Brain Hemispheric Differences in the Neurochemical Effects of Lead, Prenatal Stress and the Combination and Their Amelioration by Behavioral Experience

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ABSTRACT

Brain lateralization, critical to mediation of cognitive functions and to ‘multi-tasking’, is disrupted in conditions such as attention deficit disorder and schizophrenia. Both low-level lead (Pb) exposure and prenatal stress (PS) have been associated with mesocorticolimbic system-mediated executive-function cognitive and attention deficits. Mesocorticolimbic systems demonstrate significant laterality. Thus, altered brain lateralization could play a role in this behavioral toxicity. This study examined laterality of mesocorticolimbic monoamines (frontal cortex, nucleus accumbens, striatum, midbrain) and amino acids (frontal cortex) in male and female rats subjected to lifetime lead (Pb) exposure (0 or 50 ppm in drinking water), prenatal stress (PS; restraint stress on gestational days 16-17) or the combination, with and without repeated learning behavioral experience. Control males exhibited prominent laterality particularly in midbrain, but also in frontal cortex and striatum; females exhibited less laterality and this was primarily striatal. Lateralized Pb ± PS-induced neurotransmitter changes, assessed only in males given limited sample sizes of Pb+PS females. In males, Pb ± PS changes occurred in left hemisphere of frontal cortex, but right hemisphere of midbrain. Behavioral experience modified the laterality of Pb ± PS-induced neurotransmitter changes in a region-dependent manner. Notably, behavioral experience eliminated Pb ± PS neurotransmitter changes in males. These findings underscore the critical need to evaluate both sexes and brain hemispheres for the mechanistic understanding of sex-dependent differences in neuro- and behavioral toxicity. Further, assessment of CNS mechanisms in the absence of behavioral experience, shown here for males, may constitute less relevant models of human health effects.

Keywords: lead, prenatal stress, laterality, dopamine, glutamate, sex
INTRODUCTION

Brain lateralization has been recognized since the nineteenth century, and more recently highlighted in studies evaluating vocal control in the chaffinch (Nottebohm, 1971; Nottebohm, 1970) and determination of behavioral laterality in rats (Denenberg, 1983; Denenberg, 1984; Denenberg et al., 1980; Rosen et al., 1984; Sherman et al., 1983; Sherman et al., 1980). Laterality is observed across a wide range of species, both vertebrates and invertebrates, and in multiple brain regions (Toga and Thompson, 2003; Vallortigara, 2005). Notable sex differences in asymmetries have been described, being more pronounced in males than in females (Tian et al., 2011), and ascribed, at least in part, to sex hormones including testosterone (Schuepbach et al., 2012; Wisniewski, 1998). These observations may relate to mechanisms of sex differences in central nervous system (CNS) function. Individual differences in laterality as well as which hemisphere dominates are also seen, e.g., paw preference in rodents shows an approximately 50-50 left-right distribution (Sullivan et al., 2012) as well as in opposing hemispheres in an individual capacity.

Asymmetries in multiple different behavioral functions include a left hemispheric dominance for language processing (Broca, 1861), and right hemispheric dominance for visuo-spatial processing and spatial memory (Abrahams et al., 1997; Vogel et al., 2003), although the degree of lateralization for cognitive performance can be task-dependent. Rodents have also been reported to exhibit behavioral laterality for complex functions, with right-hemispheric dominance of spatial memory and left hippocampal bias for information transfer (Klur et al., 2009; Shinohara et al., 2010). The curve relating laterality to cognitive function has been described as an inverted U-shape, wherein performance deteriorates at either extreme ends (Hirnstein et al., 2010).

Laterality has been argued to confer an evolutionary advantage, particularly for cognitive processing (Hirnstein, et al., 2010), minimizing conflict between the two hemispheres and permitting parallel processing or multi-tasking.
capabilities. For example, lateralization in chicks occurred with the ability to simultaneously find food and maintain vigilance against possible predators (Rogers et al., 2004). Notably, altered lateralization has been associated with such conditions as schizophrenia (Oertel-Knochel and Linden, 2011), stroke (Tecchio et al., 2007), dyslexia (Leonard and Eckert, 2008), attention deficit hyperactivity disorder (Keune et al., 2011; Shaw et al., 2009) and autism (Lange et al., 2010; Lo et al., 2011).

Low level environmental lead (Pb) exposure and prenatal stress (PS), co-occurring risk factors for low socioeconomic status communities (Scott and Nguyen, 2011), adversely influence executive functions and cognition (Brockel and Cory-Slechta, 1998; Canfield et al., 2004; Canfield et al., 2003; Cohn et al., 1993; Jett et al., 1997; King and Laplante, 2005; Kuhlmann et al., 1997; Lanphear et al., 2005; Laplante et al., 2004; Lim et al., 2005; Lordi et al., 2000; Lui et al., 2011; Sun et al., 2005; Yang et al., 2007) and attention (Grizenko et al., 2008; Motlagh et al., 2010; Nigg et al., 2008; Nigg et al., 2010) in humans and animal models. Exposures to Pb and to prenatal restraint stress in combination can actually further enhance CNS toxicity, with effects that are frequently sex-dependent (Cory-Slechta et al., 2012a; Cory-Slechta et al., 2010; Cory-Slechta et al., 2012c; Froehlich et al., 2007; Virgolini et al., 2008b). Both Pb and PS also adversely influence the brain mesocorticolimbic systems that mediate these behaviors (Barrot et al., 2000; Cory-Slechta et al., 1999; Cory-Slechta et al., 1998; Diorio et al., 1993; Piazza et al., 1996). Importantly, mesocorticolimbic dopamine/glutamate systems exhibit laterality in neurochemical and biochemical function (Afonso et al., 1993; Belcheva et al., 1990; Carlson et al., 1993; Drew et al., 1986; Rosen, et al., 1984; Sullivan, 2004; Sullivan et al., 2009; Yang, et al., 2007).

Several questions arise from these observations. First, how widespread and sex-dependent is laterality within mesocorticolimbic circuits that are critical to mediation of cognitive functions? Do the effects of Pb ± PS differ by hemisphere in brain mesocorticolimbic systems? If so, how might these relate mechanistically to the behavioral toxicity of Pb ± PS? Can such differences begin to explain, at a
mechanistic level, the sex differences often observed in response to Pb and to PS and to the combination of these two developmental risk factors?

To begin to address these questions, the current study had several aims. The first was to more fully assess the extent of lateralization and associated sex differences in mesocorticolimbic monoamine and glutamatergic neurotransmitter systems by determining levels in frontal cortex, nucleus accumbens, striatum and midbrain. A second aim was to determine whether the neurotransmitter consequences of Pb ± PS exhibited lateralization in mesocorticolimbic systems, and, if so, how this might relate to the effects of Pb ± PS on cognitive behaviors in males (Cory-Slechta, 2012 #6414). Finally, it examined the potential for a repeated learning behavioral experience to differentially influence these lateralized Pb ± PS neurotransmitter changes.
MATERIALS AND METHODS

Dams and Pb Exposure

Three-week-old female Long Evans rats (Charles River, Germantown, NY) were randomly assigned to receive distilled deionized water drinking solutions containing 0 or 50 ppm Pb acetate and housed in same Pb-treatment condition pairs. Pb exposure was initiated two months prior to breeding to ensure elevated bone Pb levels and Pb body burden at the time of conception, and was continued throughout lactation, consistent with human exposure. When females reached 3 mos of age, they were paired with 3 mo old male Long-Evans rats for breeding. Animals were housed in a vivarium room maintained at 22 ± 5 °C with a 12h light-dark cycle (lights on at 0700h). 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 Medical School University Committee on Animal Resources.

Breeding and Prenatal Stress

At pro-estrous, as determined by vaginal smears, female rats were mated with males (2:1) across two estrous cycles. The presence of vaginal plugs or sperm was considered indicative of pregnancy and deemed gestational day 1 (GD1). Pregnant females in the 0 and 50 ppm Pb-treated groups were weighed and further randomly subdivided to a non-stress (NS) or prenatal stress (PS) condition. Beginning on GD1, all pregnant females were individually housed for the remainder of pregnancy and lactation.

On GD 16 and 17, timed to correspond to the development of key brain regions (hypothalamic nuclei, hippocampus, striatum, frontal cortex) associated with the endpoints under study (Diaz et al., 1997; Yi et al., 1994), dams assigned to PS groups were weighed and subjected to a widely employed restraint stress procedure consisting of three 45 min restraint sessions (0900, 1200 and 1500h) in plastic cylindrical devices (Ward and Weisz, 1984), a protocol we have previously verified to elevate corticosterone levels and alter catecholamine levels in frontal cortex and nucleus accumbens of dams (Cory-Slechta et al., 2004). NS
dams were weighed and subsequently left undisturbed in their home cages. This resulted in four Pb-stress conditions with the following numbers of litters: 0-NS (n=12), 0-PS (n=9), 50-NS (n=8), and 50-PS (n=8).

**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. Pups were weighed and weaned at PND21, separated by gender, and littermates of the same gender were housed as pairs.

From weaning, pups were provided with unrestricted access to the same standard rat chow and to the same Pb drinking solutions that their dam had received. At 2 mos of age, behavioral testing was initiated in a subset of pups from each treatment group, using a single offspring/gender per litter (n=8-12/group). A subset of additional pups from these litters were maintained as non-behaviorally tested offspring in sample sizes of n=4, 5, 3 and 4 for the 0-NS, 0-PS, 50-NS and 50-PS groups of males, respectively, and n=5, 4, 3 and 2, respectively, for females, each of which were derived from independent litters. Because sample sizes for the female 50-PS group were limited, only data for the 0-NS sex comparisons are presented; lateralization of Pb ± PS effects and influence of behavioral testing are not presented.

With the initiation of behavioral testing at 2 mos of age, all offspring (behaviorally tested and non-tested) were provided with sufficient food to allow a 2-3 gm weight gain per day until reaching approximately 250 (females) or 300 g (males). At this point, caloric intake was regulated for the duration of the experiment to maintain this weight as required for food-motivated behavioral performance of the behaviorally-tested subgroup. Because animals were pair-housed, individual feeding was accomplished by separating the residents through the introduction of a cage divider at the time of feeding which remained in place for approximately 90-120 min.

At the completion of behavioral testing, when all offspring were approximately 10 mos of age, all animals were sacrificed and brains rapidly
removed and dissected and stored at -80°C until used for determinations of monoamines, glutamate and its metabolite glutamine, and for GABA.

**Behavioral Testing on a Multiple Repeated Learning and Performance Schedule**

Behavioral test sessions were carried out M-F between 1000 and 1600 hr in rat operant chambers (ENV-008, Med Associates, St. Albans, VT) housed in sound-attenuated enclosures ventilated by a fan, equipped with a grid floor, speaker, house light and three standard (non-retractable) response levers (L: Left, C: Center, R: Right) configured horizontally on the front panel.

Reinforcement consisted of the delivery of a 45 mg food pellet (Bioserv, Frenchtown, NJ) and behavioral contingencies and data were controlled by SoftCtrl™ Cumulative Record interface and Med-PC Version IV Research Control and Data Acquisition software.

Assessment of learning relied on performance under a multiple schedule comprised of repeated learning (RL) and performance (P) components, a paradigm we have previously shown to be sensitive to postweaning Pb exposure in male rats (Cohn, et al., 1993) with outcomes for males and females from this study already reported (Cory-Slechta, et al., 2010; Cory-Slechta, et al., 2012c).

In the RL components, the subject was required to learn a sequence of three lever press responses, with each correct sequence resulting in food reinforcement; the correct three response sequence changed with each successive experimental session. In the P components, the subject was required to complete a sequence of 3 responses, but the specific sequence was constant across sessions (see below).

Assessment of behavior under the multiple schedule was preceded by lever press training (1 session on fixed ratio 1 to 100 reinforcers carried out separately for each lever) followed by two- and then three-response sequence RL training, as previously detailed (Cohn, et al., 1993). Advancement from two- to three-response sequence training occurred after 22 behavioral test sessions, and from three-response sequence training to the multiple schedule after 23
behavioral test sessions. Subsequently, the multiple schedule was implemented, where each behavioral test session was comprised of two presentations each of the RL and P components in the order RL1, P1, RL2, P2, with each component lasting for 15 min or until 25 reinforcer deliveries, whichever occurred first. Different discriminative stimuli (lights above the three levers) were used to signal whether the RL or P component of the multiple schedule was currently in effect on the multiple schedule.

Sequences used for three-response RL training included CRL, RCL, CLR, RLC, LRC and LCR; sequences with duplicative responses, e.g., LLR, were excluded to minimize reinforcement of perseverative responding. Based on the high accuracy levels it sustained during three-response sequence training, the sequence LCR was assigned as the correct sequence for the P components on the multiple schedule, and the remaining 5 sequences were used for the RL components, with each of these sequences occurring every 5th behavioral test session. Any error during the completion of the sequence in either the RL or P components initiated a 2 sec timeout and turned off the houselight. Responses during the timeout extended the timeout until 2 seconds had elapsed without a response. Subjects were then required to begin the sequence anew. All correct responses produced a tone. Correct completion of a sequence resulted in food delivery, a tone stimulus and the auditory click of the pellet delivery device. Performance measures included accuracy (correct responses divided by total responses), response rate (responses/time), total responses per completed sequence (total correct and incorrect responses /total completed sequences), session length (total session time), reinforcement density (total reinforcers/ total responses) and total RL reinforcers (number of reinforcers earned in RL1 and RL2).

**Blood Pb Determinations**

Blood was collected from tail nicks in a subset of dams at three time points prior to the initiation of breeding (n=10 at 3 weeks, n=5 at 2 weeks, and n=5 at 1 week prior to breeding) and at weaning (n=2). Trunk blood was collected from
offspring sacrificed at 5-6 days of age (n=3 and 6, NS and PS, respectively), and from tail nicks in non-behaviorally tested female offspring at 2.5 mos of age (n=4 each, NS and PS offspring), and in behaviorally-tested offspring at the completion of behavioral testing, at approximately 10 mos of age (0-NS=12, 0-PS=9, 50-NS=8 and 50-PS=8). Blood Pb was analyzed by anodic stripping voltammetry using the Lead Care II system with a detection limit of 3.3 µg/dl. All 0-NS and 0-PS values were below detection limits. Results have been reported previously (Cory-Slechta, et al., 2010).

**Neurochemical Determinations**

**Sample and stock preparation** - After rapid decapitation, regions were dissected out and placed in 0.1N perchloric acid. Tissues were sonicated and centrifuged for 20 min at 10,000xg. The supernatants were stored at -80°C until analyzed by HPLC. The pellets were digested in 1 ml 0.1N NaOH for determination of protein concentration. Obtained values for all neurotransmitters are reported at ng analyte/mg protein.

**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, striatum, midbrain (ventral tegmental area and substantia nigra) and hypothalamus were analyzed using HPLC with electrochemical detection as previously detailed (Cory-Slechta, et al., 2004; Virgolini et al., 2008a). Commercially prepared MD-TM mobile phase (Environmental Science Associates, San Francisco, CA) was utilized. Standards of DA, DOPAC, HVA, 5-HT and 5-HIAA were assayed at the beginning of each HPLC run. DA turnover (DA TO) was calculated as the DOPAC/DA ratio.

**Glutamate and GABA** - Levels of glutamate (GLu), glutamine (GLn) and GABA in frontal cortex were also assayed using HPLC with a method based on de Freitas Silva et al. (de Freitas Silva et al., 2009). GLn/GLu/GABA standards were prepared in 0.1N perchloric acid at concentrations of 6µg/mL GLn, 12 µg/mL GLu, and 1.2 µg/mL GABA. A precolumn derivatization was performed by
mixing 100 µL sample or standard solution, 20 µL methanolic OPA (5 mg/mL), 75 µL borate buffer (pH 9.9), and 5 µL MPA. The sample/standard solution was vortexed and injected onto the chromatographic column at a volume of 10 µL after 1 minute to allow the derivatization reaction to proceed.

The HPLC system for detection of glutamate and GABA consisted of a Waters 2695 Separation Module with a 100 µL sample loop and a Waters 2475 multi-λ Fluorescence Detector. The Waters XBridge C18 3.5µm 4.6 x 150mm analytical column was used for chromatographic analysis. Mobile phase consisted of 0.05M sodium acetate, tetrahydrofuran, and methanol (50:1:49, v/v), pH 4.0 and was filtered through 0.2 µm nylon filter prior to use. Chromatographic analyses were performed at 25 ±2°C. Analytes were isocratically eluted over a 22 minute run time at a flow rate of 0.8ml/min. Excitation and emission wavelengths were set at 337nm and 454nm, respectively. GLN/GLu/GABA were identified by their retention times (approximately 4.5, 7.6, 18.6 min, respectively) as determined by standard injections. Standards for GLn, GLu and GABA were assayed at the beginning of each HPLC run. GLu turnover was defined by the GLn/GLu ratio previously shown to be sensitive to be altered both in schizophrenia and in epilepsy (Hashimoto et al., 2005; Petroff et al., 2002).

**Statistical Analysis**

**Blood Lead Values**

Because all 0-NS and 0-PS group blood Pb levels were below detection limits, only values from 50-NS and 50-PS groups were analyzed. For dams, these analyses relied on ANOVAs with time point (three time points pre-breeding and weaning) as a between groups factor. Blood Pb values of offspring were analyzed using ANOVAs with stress (NS, PS) and time points as between-groups factors. RMANOVAs were not used for these analyses, since the same pool of rats was not sampled at each time point. Post hoc tests were carried out using Fisher’s protected least significant difference tests as appropriate. These blood Pb values have been reported previously (Cory-Slechta, et al., 2010; Cory-Slechta, et al., 2012c).
Neurotransmitter Changes

Brain Laterality Under Normal Conditions – Given the complexity of the experimental design and the sample sizes, evaluation in the 0-NS only non-behaviorally trained groups was considered definitive confirmation of mesocorticolimbic laterality under conventional conditions. Thus, for assessment of brain laterality under normal (control) conditions, repeated measures ANOVAs were carried out using hemisphere (right or left) as a within group factor and sex as a between-group factor. These were followed appropriately by post hoc tests as determined by main effects or interactions.

Hemispheric Differences in the Effects of Pb, Stress or the Combination on Neurotransmitter Levels - To determine whether Pb ± PS effects differed by hemisphere in males, RMANOVAs were first carried out using Pb and PS as between-group factors and hemisphere (left or right) as a within-group factor in non-behaviorally trained rats. Analyses were carried out separately for each neurotransmitter in each brain region. For those regions in which statistical interactions were confirmed, subsequent ANOVAs with Pb and PS as between group factors were carried out for each hemisphere.

Effects of Behavioral Testing on Laterality and Associated Pb ± PS Neurotransmitter Changes – To evaluate the extent to which behavioral testing modified Pb ± PS-associated neurotransmitter changes in males, ANOVAs were carried out using Pb, PS and behavioral testing condition (behaviorally tested or non-behaviorally tested) as between-group factors. These analyses were carried out separately for each neurotransmitter in each brain region. For those regions in which statistical interactions were identified, subsequent ANOVAs as appropriate with Pb and PS as between group factors were carried out for each behavioral testing condition.

Evaluation of the complete profile of neurotransmitter changes across regions necessarily required multiple statistical tests. For that reason, only findings involving main effects or statistical interactions were subsequently pursued statistically. Further, Because of the large number of comparisons involved in these analyses, the focus was on consistency of patterns of changes.
P values were defined as significant at ≤ 0.05, and given the somewhat restricted sample sizes, near significant effects are also noted where potentially relevant.
RESULTS

Blood Pb and Dam and Pup Outcomes

Blood Pb levels (PbBs) from dams and male and female offspring from these studies have been reported previously (Cory-Slechta et al., 2010; Cory-Slechta et al., 2012c). Briefly, PbB levels of dams measured at 3 time points prior to breeding averaged 5-7 µg/dl and increased to 13 µg/dl at weaning. Offspring exhibited PbBs of 10-13 µg/dl at postnatal days 5-6; these declined to 7 µg/dl at 2.5 mos and increased at 10 mos to 10 µg/dl. Neither litter size (group mean values ranging from 14.25-15.63 pups) nor litter weight (group mean values ranging from 82.13-94.00 gm) were significantly influenced by Pb, PS or the combination.

Brain Laterality Differs by Brain Region and Sex

To determine conditions of hemispheric lateralization under conventional conditions, statistical analyses were carried out using only animals from the control (0-NS) non-behaviorally tested groups. Monoamine neurotransmitters that differed by sex and/or hemisphere in those analyses are shown in Figure 1 by brain region. Increased frontal cortex DA, reduced DA TO and increased 5-HIAA in right hemisphere (DA: p=0.013; DA TO: p=0.01; HIAA: p=0.008) were found in both sexes. Modest increases in right hemisphere striatal DA TO (p=0.006), and in right hemispheric DOPAC (p=0.012) and DA TO in midbrain (p=0.027) were also seen in both sexes.

Sex-dependent differences in neurotransmitters were found primarily in striatum, where females showed lower levels of DA (p=0.006), DOPAC (p=0.006) and HVA (p=0.024) than males.

Sex by hemisphere interactions were most prevalent in midbrain, and were also seen with striatal 5HT and HIAA. Notably, the sex by hemisphere differences in midbrain were due to the absence of hemispheric laterality in females, in contrast to significantly higher levels of DA (p=0.004), HVA (p=0.005), NE (p=0.007), 5HT (p=0.006) and 5-HIAA (p=0.006) in right hemisphere in males. For striatal 5HT (p=0.014) and 5HIAA (p=0.032), similar interactions were
noted, i.e., absence of laterality in females but greater left hemispheric levels in males.

Sex by hemisphere interactions also were seen for all three amino acids assayed in frontal cortex (Figure 1), with higher left hemispheric levels of GLn, GLu and GABA in males, whereas no laterality was evident in females (p=0.0.005, 0.033 and 0.009, respectively). GLu turnover showed no differences.

**Effects of Pb ± PS Can Differ by Hemisphere**

**Monoamines** - Significant laterality-associated differences in Pb ± PS effects on monoamine neurotransmitter levels (based on Pb x PS x hemisphere, Pb x hemisphere or PS x hemisphere interactions in RMANOVAs) were found only in frontal cortex and midbrain in non-behaviorally tested males (Figure 2). In frontal cortex, Pb ± PS-induced changes were restricted to the left hemisphere. For frontal cortex, Pb+PS selectively increased DA TO (p=0.047), and, correspondingly, marginally reduced DA (p=0.063) in left hemisphere (42%; p=0.01). Pb+PS also selectively reduced left hemisphere NE (21%, p=0.014). No effects of Pb ± PS were found in right hemisphere. Neither right nor left hemispheric frontal cortex glutamine showed any significant influence of Pb ± PS despite the Pb x hemisphere interaction in the overall comparison.

In contrast, Pb ± PS interactions in midbrain were seen only in right hemisphere in non-behaviorally tested males (DOPAC: p=0.047; HVA: p=0.007; NE: p=0.038; 5HT: p=0.045 and HIAA: p=0.014), where the general pattern consisted of Pb or Pb+PS selectively reducing monoamine levels by 12-88% (DOPAC: p=0.049, HVA: p=0.0003 and 0.006 for Pb and PS, respectively; NE: p=0.014; and HIAA: p=0.005).

**Amino Acids** - Significant interactions of Pb ± PS with hemisphere were found for all three amino acids in non-behaviorally tested males (Figure 2; p=0.038, 0.017 and 0.035 for GLn, GLu and GABA, respectively). While no Pb ± PS-related effects on GLn were found in post-hoc ANOVAs in either hemisphere, Pb significantly increased right hemisphere GLu by 43-60% (p=0.021), while left hemisphere levels were not significant. Left hemisphere GABA levels were
significantly reduced by Pb ± PS (p=0.017) by 27-43% while no changes were found in right hemisphere. GLu turnover was not differentially altered.

**Individual Differences in Magnitude and Dominant Hemisphere**

As has previously been reported, individual differences in laterality, including the extent to which it occurs as well as the dominant hemisphere were seen in non-behaviorally tested males (Figure 3). Of the 4 0-NS males, one showed very little laterality, and for all 4 subjects, right hemisphere showed higher levels of neurotransmitter. Albeit based on somewhat limited sample sizes, it can also be seen that while right hemisphere remained dominant for midbrain, the magnitude of laterality tended to be diminished with Pb and Pb+PS. In fact, almost no laterality is evident for midbrain NE in the Pb+PS group.

**Behavioral Testing Influences Pb ± PS-Induced Neurotransmitter Changes in a Hemispheric Manner**

**Monoamines** – In males, behavioral testing differentially influenced absolute neurotransmitter levels (e.g., main effects of BTC) in all virtually all brain regions examined. Interactive effects of Pb ± PS with behavioral testing were most dramatic in frontal cortex and midbrain and were also seen in striatum, and almost invariably consisted of the absence of Pb ± PS effects in behaviorally-tested males relative to non-behaviorally tested male littermates, as shown in Figure 4.

Frontal cortex HVA and DA TO levels were increased particularly by Pb + PS (471% and 201%, and p=0.031 and 0.05, respectively) and DOPAC levels reduced by PS alone (by 35%; p=0.049) with corresponding but non-significant reductions in dopamine in non-behaviorally tested offspring, whereas no Pb ± PS effects were seen in behaviorally-tested males. Pb reduced striatal DA levels by 25-56% (p=0.011), while DA TO levels increased by 58-83% (p=0.001) in non-behaviorally tested males, but no corresponding effects were found in behaviorally-tested males. In midbrain, Pb, and in some cases particularly Pb + PS, markedly reduced midbrain DOPAC, HVA, NE and 5-HIAA levels (p=0.042, 0.027, p<0.0001 and p<0.0001, respectively) by 12-88%, whereas the only effect
in behaviorally-tested littermates consisted of a modest PS-based increase in 5-HIAA (p=0.022).

Data from left hemisphere nucleus accumbens in behaviorally-tested and non-behaviorally tested males indicates that the differential influence of behavioral testing on Pb ± PS induced neurotransmitter levels is not restricted to the right hemisphere (Figure 5). In addition to BTC-associated differences in absolute neurotransmitter levels, differential effects of Pb ± PS were found for DA (p=0.011) and HVA (p=0.029). PS increased DA levels by 53-61% in non-behaviorally tested males, while a modest increase in the 50-PS group relative to 0-PS was found in behaviorally-tested males. PS also increased HVA levels by 45-60% in non-behaviorally tested males, whereas no HVA changes were found in behaviorally tested males.

Amino Acids – Differential effects of Pb ± PS on frontal cortex amino acids in relation to behavioral testing were largely restricted to differences in absolute neurotransmitter levels in males (data not shown).
DISCUSSION

The current study demonstrated that under conventional conditions, brain lateralization is both sex- and region dependent, being more prominent in males particularly in midbrain. Additionally, the effects of Pb ± PS, examined in males, can differ significantly by hemisphere in mesocorticolimbic brain regions, with profiles of changes that differ notably by region. Further, behavioral experience, also examined only in males, and in this case consisting of repeated learning experience, appears to attenuate the effects of Pb ± PS.

Under Conventional Conditions, Hemispheric Laterality of Neurotransmitters is Found Across Mesocorticolimbic Regions in Males, But to a Lesser Extent in Females

Consistent with prior studies, a broad laterality of monoamine/glutamate neurotransmitters was seen in the mesocorticolimbic system (Belcheva, et al., 1990; Capper-Loup et al., 2009; Carlson, et al., 1993; Rodriguez et al., 1990b; Rosen, et al., 1984; Sullivan and Dufresne, 2006), and was more prominent in males (Berrebi et al., 1988; Sullivan, et al., 2009; Toga and Thompson, 2003; Wisniewski, 1998). Comparisons to profiles of laterality reported in prior studies, however, are difficult given the use of mixed or single sexes or identification of regions as high vs. low rather than by side (Rodriguez et al., 1990a; Rosen, et al., 1984).

Of note, midbrain monoamine laterality was striking in males, characterized by increased levels and turnover in the right hemisphere, but absent in females. Similarly, frontal cortex amino acids exhibited laterality only in males. While sex differences in absolute neurotransmitter levels have been reported for both substantia nigra and ventral tegmental area (Rodriguez, et al., 1990a), this appears to be the first report of major sex-related difference in midbrain laterality. Its consistency in males suggests its contribution to laterality in projection regions, including frontal cortex, striatum and nucleus accumbens. Its absence in females, even with evidence of frontal cortex monoaminergic laterality (albeit of lesser magnitude than in males), argues that other factors
must contribute to cortical lateralization, e.g., sex hormones, which have been shown to influence laterality (Geschwind and Galaburda, 1985; Hausmann and Gunturkun, 2000; Toga and Thompson, 2003; Wisniewski, 1998).

The pronounced sex difference in midbrain laterality may have behavioral and disease and disorder-related mechanistic implications. Midbrain is the site of dopamine cell bodies of both the nigrostriatal and mesocorticolimbic dopamine tracts. Differences in laterality may assist in explaining sex-related differences in behaviors mediated by these systems that can include spatial working and memory and motor activity (Simpson and Kelly, 2012) and diseases and disorders associated with these dopamine tracts that show sex-related differences, e.g., Parkinson’s disease and attention deficit disorder.

**Neurochemical Changes Produced by Pb ± PS Can Differ Significantly by Hemisphere In Mesocorticolimbic Regions**

The potential for differential effects of an environmental neurotoxicant by brain hemisphere does not appear to have been previously assessed. Given limited sample sizes of Pb+PS treated females, examination of the effects of Pb ± PS on brain neurotransmitter levels was carried out only in males. Neurotransmitter changes produced by Pb ± PS in males differed by hemisphere in a brain region-dependent manner. Pb ± PS effects in males in left hemisphere were found in frontal cortex but were prominent in right hemisphere of midbrain. Frontal cortex Pb ± PS-related changes in amino acids were less consistent, with Pb-induced increases in GLu in right hemisphere and both PS and Pb-associated decreases in GABA in left hemisphere (Figure 2). Importantly, there was also suggestive evidence that these hemispheric-dependent Pb effects could be exacerbated by PS, although not necessarily framed in Pb x PS interactions, as noted for left frontal cortex DA turnover, and particularly for midbrain (HVA, DOPAC, NE, and 5HT).

Considered collectively, the highly consistent effects of Pb in right midbrain suggest it may be a primary mechanism of mesocorticolimbic system disruption by Pb (Cohn and Cory-Slechta, 1994a; Cohn and Cory-Slechta,
1994b; Cohn and Cory-Slechta, 1993; Cory-Slechta, 1995; Cory-Slechta et al., 1997; Cory-Slechta et al., 1996; Pokora et al., 1996). Further, it may contribute to the wide range of effects of Pb on CNS function. Midbrain dopaminergic neurons project to multiple regions, including frontal cortex, nucleus accumbens, striatum, hypothalamus and hippocampus (Gasbarri et al., 1991; Oades and Halliday, 1987; Sesack and Grace, 2010). Additionally, midbrain also receives afferent projections from multiple regions including areas of prefrontal cortex, nucleus accumbens, hypothalamus, dorsal raphe and other catecholaminergic nuclei. Many of these regions, and particularly their network/circuitry functioning, are critical to mediation of cognitive functions and to mediation of appetitive behavior and of reward (Sesack and Grace, 2010).

While future studies will be required to define the relative importance of ventral tegmental area (VTA) as compared to substantia nigra to the lateralized effects of Pb ± PS effects in midbrain, the potential analogy of lateralized midbrain dopaminergic dysfunction to the consequences of unilateral lesions of ventral tegmental area is intriguing in that augmented function of an intact hemisphere can be found after unilateral injury, suggesting a compensatory mechanism. Specifically, unilateral lesions of VTA, and thus presumably reduced dopaminergic function, in rat have been shown to enhance contralateral VTA stimulated feeding and exploration and locomotion (Maliszewska-Scislo and Trojniar, 1999; Trojniar and Klejbor, 1999). Several mechanisms have been suggested to account for such findings. One postulates increased dopamine levels in the terminal dopaminergic structures of the other hemisphere (Maliszewska-Scislo and Trojniar, 1999); in that regard Pb ± PS-induced increases in left hemisphere frontal cortex dopamine turnover were observed, but differential Pb ± PS-induced neurotransmitter changes in nucleus accumbens were not notable. A second potential mechanism involves the VTA lesion sensitizing the mesolimbic system by suppressing inhibitory afferents to the contralateral VTA (Majkutewicz et al., 2010); data from the current study do not allow an assessment of this hypothesis.

Numerous other studies already document that brain lateralities extend
from morphological to biochemical outcomes, including such diverse measures of CNS function as D2 receptor density (Drew, et al., 1986), hippocampal gene expression (Klur, et al., 2009) and cell proliferation (Czeh et al., 2008) indicating that laterality is characteristic of brain functioning. These findings raise critical mechanistic questions. For example, which hemisphere should be used to mechanistically relate the effects of Pb ± PS to behavioral function? Should it differ by region? Or would it rely on an understanding of the consequences as integrated across the two hemispheres? Clearly, assessment in only one hemisphere may be misrepresentative, i.e., assessment of only right frontal cortex or left midbrain would have led to the conclusion of no Pb ± PS effects.

From a methodologic point of view, few studies define which hemisphere was used for neurochemical or biochemical assessments, or whether it was the same across all animals. Also clear from these findings is that random use of right or left hemisphere, may result in negative findings if effects differ by hemisphere. Hemispheric differences are unlikely to be restricted to Pb ± PS, but will likely be characteristic of other developmental neurotoxicants as well. Indeed, prenatal alcohol exposure demonstrates lateralized effects (Zimmerberg and Reuter, 1989).

**Behavioral Experience Appears to Reverse Hemisphere-Dependent Effects of Pb ± PS in Males**

The findings also show that neurotransmitter levels and metabolism can differ significantly in relation to behavioral experience in a region-dependent context. Here, behavioral experience appeared to mitigate the effects of Pb ± PS in male offspring. Whereas numerous Pb ± PS-induced neurotransmitter changes were seen in males lacking behavioral experience, only a single PS-induced increase in midbrain HIAA was seen in males with behavioral experience.

It could be presumed that behavioral experience on the multiple schedule of repeated learning and performance in this study functioned much like an ‘environmental enrichment’. Environmental enrichment paradigms have been
reported to attenuate effects of Pb and of PS (Fox et al., 2006; Guilarte et al., 2003; Schneider et al., 2001). As has been shown in other studies, however, the impact of environmental enrichment depends upon numerous different factors, outcome can often differ by gender, and ‘enrichment’, depending upon how formulated, can actually worsen outcome (Simpson and Kelly, 2011).

Importantly, the fact that Pb ± PS can produce neurochemical changes that differ by hemisphere and that behavioral testing can alter the neurochemical consequences of Pb ± PS in a hemispheric capacity, certainly complicates the assessment of neurochemical mechanisms of behavioral toxicity. From the simplest point of view, one might surmise that the enhanced learning seen with Pb ± PS in behaviorally-tested males (Cory-Slechta et al., 2012d) as compared to the enhanced impairments in learning accuracy arising from Pb + PS in behaviorally-tested females (Cory-Slechta, et al., 2010) reflected in part the differential ‘enrichment’ capacities of the behavioral testing, wherein Pb ± PS-associated neurochemical changes were virtually eliminated in males but without apparent effects in females. Increased learning accuracy in males appeared to have been the result of increased response rates with a resulting increase in reinforcement density (Cory-Slechta, et al., 2012d). Understanding the mechanisms of increased response rates in the males may require comparisons of the pattern of associated neurochemical changes in non-behaviorally tested males, such as altered levels of dopamine and turnover seen in frontal cortex, striatum and midbrain, as compared to those in behaviorally-tested littermates, since it is clear that behavioral testing per se can change brain neurochemistry (Cory-Slechta et al., 2009) and can also alter the consequences of Pb ± PS (Cory-Slechta et al., 2012b). It will ultimately require an understanding of how behavioral performance(s) interact with compensatory neurotransmitter changes as seen in non-behaviorally tested males.

In summary, the current findings demonstrate that Pb ± PS can induce neurochemical changes that are hemisphere dependent. Additionally, behavioral testing, whether Fixed Interval schedule controlled performance (Cory-Slechta, et al., 2009) or repeated learning, as shown here, can alter the neurochemical
consequences of Pb ± PS in a hemisphere-dependent manner. These observations have many implications. To the best of our knowledge, this is the first study to demonstrate hemispheric laterality of the effects of an environmental developmental neurotoxicant (Pb) ± PS. However, given that laterality extends to multiple aspects of CNS structure and function (Czeh, et al., 2008; Drew, et al., 1986; Klur, et al., 2009; Toga and Thompson, 2003), and that prior studies have indicated that prenatal ethanol can alter laterality (Zimmerberg and Reuter, 1989), it seems highly likely that other environmental neurotoxicants might also differ in their effects by hemisphere. Based on these findings, it is possible that assessment of behavioral or biochemical outcomes only in behaviorally tested offspring may be misinformative, and that ultimately information from both hemispheres, as well as from behaviorally-tested and non-behaviorally tested brains, is required for such a purpose. It is also evident that the hemisphere used in analyses should be explicitly identified. These findings also underscore the imperative need to examine neurochemical, and likely biochemical CNS effects in both sexes, given the broad sex-dependence of laterality under conventional conditions seen here. Moreover, based on the sex differences, it would seem that different therapeutic interventions, whether behavioral or pharmacological, might be required for Pb ± PS-induced CNS toxicity in males vs. females.

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FIGURE LEGENDS

Figure 1. Group mean ± S.E. neurotransmitter levels (ng/mg protein) as identified for control (0-NS only) non-behaviorally tested males and females in right vs. left hemisphere in frontal cortex, striatum and midbrain. Only those neurotransmitters within a region that exhibited statistically significant differences are shown. H=main effect of hemisphere; S=main effect of sex, SxH = sex by hemisphere interaction. * indicates significantly less than corresponding other sex outcome. DA = dopamine, DOPAC = dihydroxyphenylacetic acid, HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, 5-HT = serotonin, and 5-HIAA = 5 hydroxyindoleacetic acid.

Figure 2. Group mean ± S.E. neurotransmitter levels (ng/mg protein) of non behaviorally tested males as identified for treatment groups in right vs. left hemisphere in frontal cortex and midbrain. Only those neurotransmitters within a region that exhibited statistically significant differences are shown. H=main effect of hemisphere; Pb=main effect of lead, PS = main effect of prenatal stress; PbxB = lead by hemisphere interaction, PSxH = prenatal stress by hemisphere interaction, PbxPSxH = lead by prenatal stress by hemisphere interaction in repeated measures ANOVAs comparing hemispheres. Pb = main effect of Pb, PS = main effect of prenatal stress, PbxBPS = lead by prenatal stress interaction in hemisphere-specific ANOVAs. DOPAC = dihydroxyphenylacetic acid, HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, 5-HT = serotonin, 5-HIAA = 5 hydroxyindoleacetic acid, GLn = glutamine, GLu = glutamate, GABA = gamma amino butyric acid. + differs significantly from corresponding 0-PS group; # differs from corresponding 0-PSS group, # differs from corresponding 50-NS group.

Figure 3. Group mean ± S.E. neurotransmitter levels (ng/mg protein) of males as identified for treatment groups in right hemisphere of behaviorally tested (BTC) vs non-behaviorally tested offspring (NBTC) in frontal cortex, striatum and
midbrain. Only those neurotransmitters within a region that exhibited statistically significant interactions are shown. BTC=main effect of behavioral testing condition; Pb=main effect of lead, PS = main effect of prenatal stress; PbxBTC = lead by behavioral testing interaction, PSxBTC = prenatal stress by behavioral testing condition interaction, PbxPSxBTC = lead by prenatal stress by behavioral testing interaction in ANOVAs comparing behavioral testing effect. \( Pb \) = main effect of Pb, \( PS \) = main effect of prenatal stress, \( PbxPS \) = lead by prenatal stress interaction in hemisphere-specific ANOVAs. DA = dopamine, DOPAC = dihydroxyphenylacetic acid, HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, 5-HIAA = 5 hydroxyindoleacetic acid, GLn = glutamine, GLu = glutamate, GABA = gamma amino butyric acid.. * differs significantly from corresponding 0-NS control; + differs significantly from corresponding 0-PS group; # differs from corresponding 50-NS group.

**Figure 4.** Levels (ng/mg protein) of midbrain DOPAC (top) and NE (bottom) of individual male non-behaviorally tested subjects from treatment groups as identified. Closed circles represent values from right hemisphere and open circles from left hemisphere.

**Figure 5.** Group mean ± S.E. neurotransmitter levels (ng/mg protein) of males as identified for treatment groups in left hemisphere of behaviorally tested (BTC) vs. non-behaviorally tested offspring (NBTC) in nucleus accumbens. Only those neurotransmitters within this region that exhibited statistically significant interaction are shown. BTC=main effect of behavioral testing condition; PSxBTC = prenatal stress by behavioral testing condition interaction, PbxPSxBTC = lead by prenatal stress by behavioral testing interaction in ANOVAs comparing behavioral testing effect. \( PS \) = main effect of prenatal stress, \( PbxPS \) = lead by prenatal stress interaction in hemisphere-specific ANOVAs. DA = dopamine and HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, and 5-HIAA = 5 hydroxyindoleacetic acid. * differs significantly from corresponding 0-NS control; + differs significantly from corresponding 0-PS group.
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Figure 1. Group mean ± S.E. neurotransmitter levels (ng/mg protein) as identified for control (0-NS only) non-behaviorally tested males and females in right vs. left hemisphere in frontal cortex, striatum and midbrain. Only those neurotransmitters within a region that exhibited statistically significant differences are shown. H=main effect of hemisphere; S=main effect of sex, SxH = sex by hemisphere interaction. * indicates significantly less than corresponding other sex outcome. DA = dopamine, DOPAC = dihydroxyphenylacetic acid, HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, 5-HT = serotonin, and 5-HIAA = 5 hydroxyindoleacetic acid.

86x42mm (300 x 300 DPI)
Figure 2. Group mean ± S.E. neurotransmitter levels (ng/mg protein) of non behaviorally tested males as identified for treatment groups in right vs. left hemisphere in frontal cortex and midbrain. Only those neurotransmitters within a region that exhibited statistically significant differences are shown. H=main effect of hemisphere; Pb=main effect of lead, PS = main effect of prenatal stress; PbxH = lead by hemisphere interaction, PSxH = prenatal stress by hemisphere interaction, PbPSxH = lead by prenatal stress by hemisphere interaction in repeated measures ANOVAs comparing hemispheres. Pb = main effect of Pb, PS = main effect of prenatal stress, PbxPS = lead by prenatal stress interaction in hemisphere-specific ANOVAs.

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DA = dopamine, DOPAC = dihydroxyphenylacetic acid, HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, 5-HIAA = 5 hydroxyindoleacetic acid, GLn = glutamine, GLu = glutamate, GABA = gamma amino butyric acid... * differs significantly from corresponding 0-NS control; + differs significantly from corresponding 0-PS group; # differs from corresponding 50-NS group.

124x81mm (300 x 300 DPI)
Figure 4. Levels (ng/mg protein) of midbrain DOPAC (top) and NE (bottom) of individual male non-behaviorally tested subjects from treatment groups as identified. Closed circles represent values from right hemisphere and open circles from left hemisphere.

Figure 4x42mm (300 x 300 DPI)
Figure 5. Group mean ± S.E. neurotransmitter levels (ng/mg protein) of males as identified for treatment groups in left hemisphere of behaviorally tested (BTC) vs. non-behaviorally tested offspring (NBTC) in nucleus accumbens. Only those neurotransmitters within this region that exhibited statistically significant interaction are shown. BTC = main effect of behavioral testing condition; PSxBTC = prenatal stress by behavioral testing condition interaction, PbxBBTC = lead by prenatal stress by behavioral testing interaction in ANOVAs comparing behavioral testing effect. PS = main effect of prenatal stress, PbPS = lead by prenatal stress interaction in hemisphere-specific ANOVAs. DA = dopamine and HVA = homovanillic acid, DA TO = dopamine turnover, NE = norepinephrine, and 5-HIAA = 5 hydroxyindoleacetic acid. * differs significantly from corresponding 0-NS control; + differs significantly from corresponding 0-PS group.

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