

Lineage- and Sex-Dependent Behavioral and Biochemical Transgenerational Consequences of Developmental Exposure to Lead, Prenatal Stress, and Combined Lead and Prenatal Stress in Mice

Marissa Sobolewski,¹ Kadijah Abston,¹ Katherine Conrad,¹ Elena Marvin,¹ Katherine Harvey,¹ Martha Susiarjo,¹ and Deborah A. Cory-Slechta¹

¹Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, New York, USA

BACKGROUND: Lead (Pb) exposure and prenatal stress (PS) during development are co-occurring risk factors with shared biological substrates. PS has been associated with transgenerational passage of altered behavioral phenotypes, whereas the transgenerational behavioral or biochemical consequences of Pb exposure, and modification of any such effects by PS, is unknown.

OBJECTIVES: The present study sought to determine whether Pb, PS, or combined Pb and PS exposures produced adverse transgenerational consequences on brain and behavior.

METHODS: Maternal Pb and PS exposures were carried out in F0 mice. Outside breeders were used at each subsequent breeding, producing four F1–F2 lineages: [F1 female–F2 female (FF), FM (male), MF, and MM]. F3 offspring were generated from each of these lineages and examined for outcomes previously found to be altered by Pb, PS, or combined Pb and PS in F1 offspring: behavioral performance [fixed-interval (FI) schedule of food reward, locomotor activity, and anxiety-like behavior], dopamine function [striatal expression of tyrosine hydroxylase (Th)], glucocorticoid receptor (GR) and plasma corticosterone, as well as brain-derived neurotrophic factor (BDNF) and total percent DNA methylation of *Th* and *Bdnf* genes in the frontal cortex and hippocampus.

RESULTS: Maternal F0 Pb exposure produced runting in F3 offspring. Considered across lineages, F3 females exhibited Pb-related alterations in behavior, striatal BDNF levels, frontal cortical *Th* total percentage DNA methylation levels and serum corticosterone levels, whereas F3 males showed Pb- and PS-related alterations in behavior and total percent DNA methylation of hippocampal *Bdnf*. However, numerous lineage-specific effects were observed, most of greater magnitude than those observed across lineages, with outcomes differing by F3 sex.

DISCUSSION: These findings support the possibility that exposures of previous generations to Pb or PS may influence the brain and behavior of future generations. Observed changes were sex-dependent, with F3 females showing multiple changes through Pb-exposed lineages. Lineage effects may occur through maternal responses to pregnancy, altered maternal behavior, epigenetic modifications, or a combination of mechanisms, but they have significant public health ramifications regardless of mechanism. <https://doi.org/10.1289/EHP4977>

Introduction

Developmental exposures to lead (Pb) and to prenatal stress (PS) remain significant risk factors for subsequent adverse behavioral outcomes in children (Bock et al. 2015; Canfield et al. 2003, 2004; Jusko et al. 2008; Nigg et al. 2008; Pallarés and Antonelli 2017), including cognitive dysfunction, reduced IQ, and attention deficits (Canfield et al. 2004; Geier et al. 2018; Gutteling et al. 2006; Ji et al. 2018; Lamb et al. 2014; Lanphear et al. 2005; Zhu et al. 2015). These consequences can even persist across the life span (Needleman et al. 1990; Schwabe et al. 2012). Furthermore, in combination, Pb and PS can result in enhanced neurotoxicity as has been seen in both human studies (Tamayo y Ortiz et al. 2017) and animal models (Rossi-George et al. 2011; Sobolewski et al. 2018a, 2018b; Virgolini et al. 2006; Weston et al. 2014), an outcome likely attributable to the shared biological targets of these two factors, that is, the hypothalamic–pituitary–adrenal (HPA) axis, as well as the brain mesocorticolimbic (MESO) system, a network that includes the frontal cortex, nucleus accumbens, and hippocampus (Barros et al. 2004; Berger et al. 2002; Jung et al. 2019; Martínez-Telléz et al.

2009; Rossi-George et al. 2011; Virgolini et al. 2008b). The potential for combined effects of Pb and PS may be further augmented by the fact that HPA axis and brain MESO neurotransmitter circuits have significant interactions critical to the mediation of executive functions and operant behaviors (Bahari et al. 2018; Hernaus et al. 2018). Accordingly, a variety of outcomes along the MESO dopamine and stress pathways have been shown sensitive to Pb, PS, or combined Pb and PS during lifelong exposure and developmental exposures in F1 rat offspring (Barros et al. 2004; Berger et al. 2002; Cory-Slechta et al. 1998, 1999; Martínez-Telléz et al. 2009; Rossi-George et al. 2011; Virgolini et al. 2008b), including alterations in HPA axis function in rats and mice [serum corticosterone and glucocorticoid receptor (GR) concentrations], in dopaminergic function [alterations in tyrosine hydroxylase (Th), the rate-limiting enzyme in dopamine synthesis], in brain-derived neurotrophic factor (BDNF) concentrations [critical to cognitive function and neuronal differentiation during development], and in behavior [learning of fixed-interval (FI) schedule-controlled behavior] (Sobolewski et al. 2018b; Virgolini et al. 2006, 2008b; Weston et al. 2014). Recently, questions have arisen as to the potential for these phenotypic changes to span multiple generations, particularly questioning whether transgenerational inheritance of such developmental neural reprogramming is possible.

Developmental programming occurs when exposure to a parent (F0) results in a change in the offspring (F1). Given that female gametes are exposed during fetal life, multigenerational programming occurs when the second generation (F2) is altered. Full transgenerational inheritance, particularly for females, is defined as the occurrence of a specific phenotype in a generation not directly exposed, that is, into a third (F3) generation (Figure 1). Several mechanisms exist by which transgenerational effects and programming can occur (Bale 2015; Gapp et al. 2014; Skinner 2014). For example, an F0 exposure can result in transmission of information through parental gametes. However, other mechanisms of transgenerational

Address correspondence to Deborah Cory-Slechta, Department of Environmental Medicine, Box EHSC, University of Rochester Medical School, Rochester, NY 14642 USA. Email: deborah_cory-slechta@urmc.rochester.edu

Supplemental Material is available online (<https://doi.org/10.1289/EHP4977>).

The authors declare they have no actual or potential competing financial interests.

Received 3 January 2019; Revised 2 January 2020; Accepted 6 January 2020; Published 5 February 2020.

Note to readers with disabilities: *EHP* strives to ensure that all journal content is accessible to all readers. However, some figures and Supplemental Material published in *EHP* articles may not conform to 508 standards due to the complexity of the information being presented. If you need assistance accessing journal content, please contact ehponline@niehs.nih.gov. Our staff will work with you to assess and meet your accessibility needs within 3 working days.

passage occur as well, for example, via alterations in the gestational environment, maternal behavior, or social learning, which can influence generational passage of brain and behavioral phenotypes (Jablonka and Raz 2009).

Multigenerational effects of PS following maternal parent generation (F0) exposures have been observed in the subsequent first filial (F1) and second filial (F2) offspring, leading to passage of an altered phenotype. Multigenerational effects of unpredictable maternal separation with or without unpredictable maternal stress in mice were seen in F2 offspring as shown by altered approach avoidance behavior (Weiss et al. 2011). In another multigenerational study (Dias and Ressler 2014), the exposure of F0 female mice to fear conditioning based on odor stimuli increased behavioral sensitivity to this odor in F1 and F2 offspring, with F2 inheritance related to parental gamete transmission. Transgenerational studies have also demonstrated that the male F3 offspring of F1 male mice that had been subjected to chronic and unpredictable maternal separation showed depressive-like behavioral deficits similar to those observed in the F1 males, despite the fact that F3 males were reared in a normal environmental setting (Franklin et al. 2010). In a subsequent study, also based on early chronic and unpredictable maternal separation, alterations in social recognition memory were increased in F1 males as well as in F2 and F3 females, findings that could not be attributed to alterations in olfactory recognition (Franklin et al. 2011).

In the human environment, PS may be particularly impactful in low socioeconomic status communities (Farah 2017), where such stress can occur across generations, consistent with the cyclical nature of poverty. A series of studies in rats (Kiss et al. 2016; McCreary et al. 2016) mimicked this feature of the human environment by imposing PS consisting of restraint stress and forced swimming in a semi-random sequence in an F0, F1, and F2 generation and subsequently examined multiple consequences in female F3 offspring. Under these conditions, increases in basal corticosterone levels were seen across generations, with almost 2-fold increases between the F2 and F3 generations. In addition, F3 females exhibited increases in distance traveled in an open field as well as increases in time spent in the margins of the open field, interpreted by some as an anxiety-emotional phenotype. Magnetic resonance imaging analyses indicated reductions in both whole-brain and hippocampal volumes. Based on an absolute gray volume index, F3 females also exhibited reductions in both cortical and hippocampal neuronal density. In a comparison of paw use as a measure of brain laterality, multigenerationally exposed, but not transgenerationally exposed, F4 males exhibited increases in left paw preference, indicative of altered brain laterality (Ambeskovic et al. 2017).

In contrast, the extent to which multigenerational or transgenerational consequences occur in response to Pb exposure is unknown. One study suggested multigenerational consequences of Pb based on an observed correspondence between DNA methylation changes in dried neonatal blood spots from mother–infant pairs to the grandmother’s blood lead levels (Sen et al. 2015). Given that the cycle of poverty and associated stressors, as well as the cycle of Pb exposure continues, transmission across generations of adverse effects of these environmental factors is of public health relevance, regardless of the mechanism by which it occurs.

The present study sought to determine whether Pb, PS, or combined Pb and PS could produce adverse transgenerational consequences on brain and behavior using a mouse model. The breeding scheme employed Pb, PS, or combined Pb and PS treatment of F0 female mice followed by observation of F3 offspring using an experimental design that allowed tracking of effects by specific lineages across the generations. A primary question to be addressed in a transgenerational study on Pb and PS exposure is whether the

same neural networks, chemical, and behavioral targets shown to be sensitive to Pb, PS, or combined Pb and PS in F1 offspring (developmental exposures) are still impacted into the F3 generation. As such, our initial outcome measures include alterations in the function of key regions in the MESO network (frontal cortex, hippocampus and striatum), of BDNF and Th protein concentrations, as well as changes in HPA axis function including serum corticosterone and GR concentrations and functional behavioral outcomes such as reward-mediated behaviors. In addition, locomotor assessments and elevated plus maze were examined to inform behavioral mechanisms and domains potentially altered. Finally, given human data suggesting that Pb altered DNA methylation patterns multigenerationally (Sen et al. 2015), alterations in DNA methylation of genes of these noted targets, *Bdnf*, *Th*, and *Gr* were assessed, in addition to *Igf2/H19* and *Esr1*, as potential markers of alterations in methylation patterns.

Methods

Animals and Breeding and Pb and PS Exposures

Eight-week-old C57BL/6J mice were obtained from Jackson Laboratories for F0 exposures and subsequent breeding (Figure 1). Mice were housed in standard mouse caging, pair housed until breeding, under a 12-h light–dark cycle maintained at $22 \pm 2^\circ\text{C}$ fed standard rodent chow (LabDiet Autoclavable Diet 5010) with approximately 3 mm high performance bedding (BioFresh) at the University of Rochester Medical Center. Forty randomly chosen young adult females were assigned to receive distilled deionized drinking water or 100 ppm Pb acetate dissolved in distilled deionized drinking water beginning 2 months prior to mating to ensure a body burden of Pb (e.g., skeletal Pb accumulation) consistent with human Pb exposure. These exposure levels are consistent with our prior studies, allowing direct comparisons, and associated with blood Pb levels in mouse offspring at postnatal day 6–7 (PND6–7) of approximately 10–15 $\mu\text{g}/\text{dL}$ (Sobolewski et al. 2018b; Weston et al. 2014), consistent with the Centers for Disease Control and Prevention definition of a level of concern of 10 $\mu\text{g}/\text{dL}$ prior to 2012 (CDC 2019b) and within the range of human exposures (Pirkle et al. 1994). Relative to the generational time represented in this study, blood leads of 24–36 $\mu\text{g}/\text{dL}$, with the highest exposed children at nearly 60 $\mu\text{g}/\text{dL}$ in 1979, were reported in neurotoxicological studies (Needleman et al. 1979). Following 2 months of Pb exposure, a 1:1 breeding scheme was used, with 40 unexposed male mice. Gestation day 0 (GD0) was designated as the day when a vaginal plug was detected, at which point females were moved to individual housing for the duration of pregnancy and lactation. Half of the females in the 0- and the 100-ppm Pb exposure groups, randomly chosen, underwent prenatal immobilization restraint stress (PS) in a 30-min session on GD15–18, a critical window for glucocorticoid sensitivity (Cintra et al. 1993; Noorlander et al. 2006), whereas the others remained in their home cages. This yielded four groups of F0 dams: 0-NS [control; 0 ppm Pb + no stress (NS), 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS), and 100-PS (100 ppm Pb + PS)].

The F1 females and males were thus developmentally exposed to Pb, PS, or the combination of Pb and PS. At PND60, they were bred to unexposed C57BL/6J mice to generate the F2 offspring. Subsequently, the F2 offspring were bred at PND60 to age-matched unexposed C57BL/6J mice to generate the F3 generation of offspring. At least 12 dams were generated for each of these lineages at each generation (Figure 1). This design allowed us to follow sex-specific differences (F = female; M = male) in each lineage across the experiment from F1 to F3, resulting in four lineages for each F3 sex (female F3 offspring = FFF, FMF, MMF, and MFF; male F3 offspring = MMM, MFM, FFM, FMM). A total of 8–10 dams were used to generate each of the F3 lineage offspring, with a

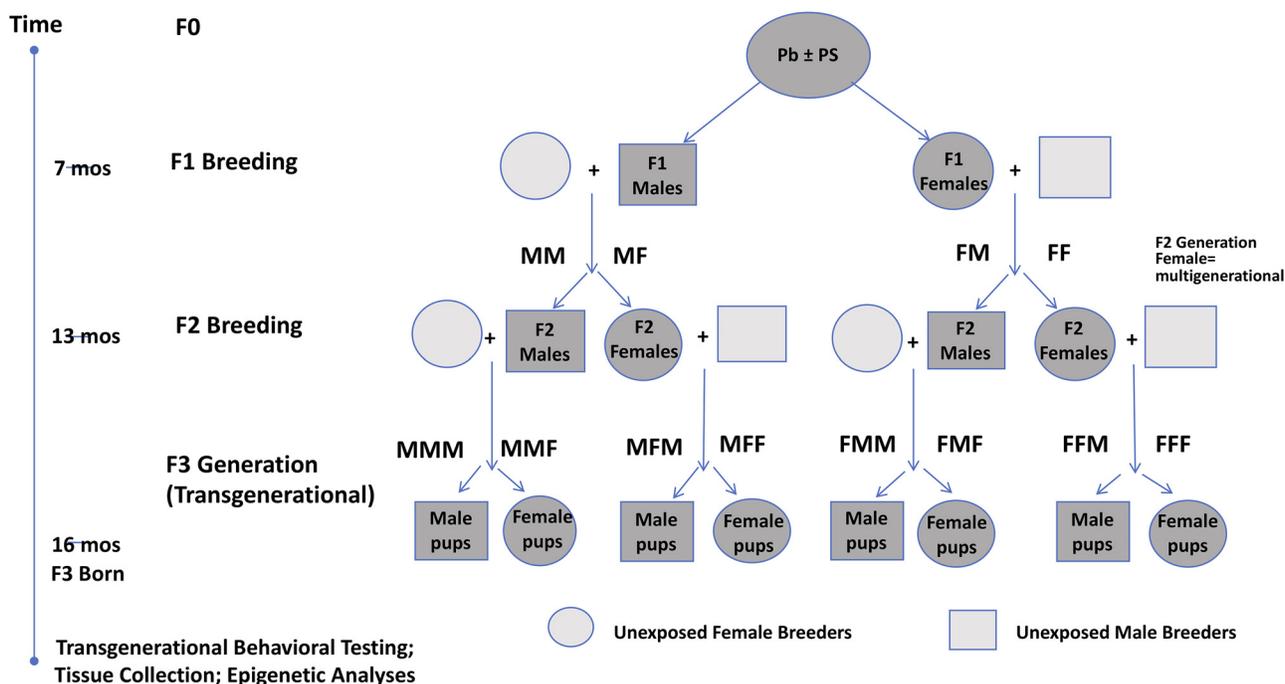


Figure 1. Schematic of breeding scheme. Breeding scheme and experimental design for the transgenerational study. Exposures to lead (Pb), prenatal stress (PS), or combined Pb and PS occurred in the F0 generation. Offspring from each subsequent generation were bred to unexposed age-matched males or females from Jackson Laboratories; this allowed us to follow each specific sex lineage from F1 to F3 separately based on F1 exposure to either dam or sire. Single pups/sex/dam were used for breeding to preclude litter-specific effects. Germ cells were collected from the F1-F2 offspring, as were measures of litter size and weight. Behavioral testing began in a randomly chosen subset of F3 offspring at about 60 d of age. Note: FF, F1 female F2 female; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; FM, F1 female F2 male; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; MF, F1 male F2 female; MFF, F1 male F2 female F3 female; MFM, F1 male F2 female F3 male; MMF, F1 male F2 male F3 female; MM, F1 male F2 male; MMM, F1 male F2 male F3 male.

single pup/sex/dam included in each outcome measure. Germ cells were collected from male and female offspring from F1 and F2 generations and stored at -80°C for future analysis. Mature sperm were taken from the cauda epididymis of 10- to 12-week-old male mice. For oocytes, germinal vesicle (GV)-stage oocytes were retrieved from six female mice per group also at 10–12 weeks of age.

Litter size and total litter weight were measured from PND1 until weaning (PND23–27). Runt counts were defined as pups that weighed less than half the average weight at weaning (F3: F: 14 g and M: 16 g). Any litter that had one runt pup or more was defined as a runt litter, this was a conservative determination to focus on the dam as the independent unit of analysis (see Figure S1). Pups were pair housed by sex/treatment group for the F1, F2, and F3 offspring. F3 offspring groups included subsets that underwent behavioral testing as well as those that did not undergo behavioral assessments (nonbehavioral controls). For each dam in each of the F0-F1-F2 breedings, a single male and female pup were used to preclude litter-specific effects. In the F3 generation, single pups/sex/dam were also used to generate groups for behaviorally tested and nonbehaviorally tested offspring. Sample sizes depend upon the assay, and all animals were randomly chosen in subsets from the full cohort for use in different assays. All experimental activities were approved by the University of Rochester Institutional Animal Care and Use Committee.

Behavioral Outcomes

Behavioral testing on the FI schedule relies on food reinforcement and thus required mice to be food-motivated. For that purpose, caloric restriction was initiated at approximately 60 d of age in all F3 offspring regardless of behavioral testing status to maintain uniform conditions across all mice. Mice were restricted

to approximately 85% of their *ad libitum* feeding weights for the duration of behavioral testing. Animals were tested in a counter-balanced order in all paradigms, and these tests were carried out at a constant time of day.

Fixed-interval schedule-controlled behavior. Assessment of FI schedule-controlled behavior was conducted in operant chambers (Med Associates) housed in sound-attenuating cabinets equipped with white noise for attenuation of distracting sounds and fans for ventilation. Three response levers were located horizontally across the back wall of the chamber (left, center, right), with a liquid dipper and dual pellet dispenser for reinforcer delivery on the front (opposite wall). Mice were initially trained to press the left lever via an overnight autoshaping program previously developed in our laboratory (Cory-Slechta and Weiss 1985). It began with a 20-min period during which food rewards were delivered independently of behavior on a random time basis, whereas a response on the left lever could also produce a food reward. Following the 20-min period, free food deliveries ceased and only a response on the left lever produced a sucrose pellet reward (20 mg; Bio-Serv) and this schedule remained in effect until 50 reinforcers were earned. Subsequently, an FI 60-s schedule of reinforcement (FI60) was imposed, which was programmed to reward the first left lever response that occurred after a fixed interval of time (60-s) had elapsed. Reward delivery also initiated the next 60-s interval. Responses during the 60-s interval had no programmed consequences; responses on either the center or right lever never provided reward. A total of 30 FI schedule behavioral test sessions, each of which were 30 min in duration, were carried out 5 d/week (Monday–Friday). Behavioral measures of FI performance included overall response rates (total responses/total session time), postreinforcement pause time (PRP; time to the first response in the interval), run rate (total responses/total time–postreinforcement pause time), and inter-response times (IRTs),

that is, time between each successive response (Rossi-George et al. 2011).

Locomotor activity. A single 60-min test session was carried out to examine locomotor activity in automated chambers equipped with 48-channel infrared photobeams (Med Associates). Photobeam breaks were recorded in 5-min bins to assess horizontal, vertical, and ambulatory movements across time (resulting in 12 bins for a 1-h locomotor activity session). Ambulatory counts were defined as the number of beam breaks, whereas in ambulatory movement; ambulatory episodes were defined by a minimum number of ambulatory counts (at least three successive photobeam breaks). The total ambulatory time refers to the time in ambulatory movement status, whereas distance represents the Euclidean distance of all ambulatory episodes, which together can also be used to determine speed (average velocity). Vertical activity was defined as movement that broke photobeams placed in the *z*-axis with breaks in the *x* and *y*-axes as well. Resting time was defined as time spent with no new photobeam breaks. Stereotypic counts and stereotypic time measure activity within a defined space in the arena. Jump counts are measured as the number of time periods that no *x* or *y*-axis beam breaks were detected, with a break in the *z*-axis, whereas jump time reflects the total duration in seconds of the jump counts.

Elevated plus maze. The elevated plus maze (EPM) measured time spent in enclosed versus open (nonpreferred) arms (Hogg 1996). Mice were initially placed in a center arena of the apparatus. Behavior was recorded as either time spent in the closed arms (with sides) or time spent in the attached open arm and center (no sides) platform over a 5-min period. Performance was reported in total duration of time (in seconds) spent in either condition. Sessions were videorecorded and subsequently scored by an observer blinded to treatment status using Observer XT (version 13.0; Noldus).

Tissue collection. Tissue was collected from both behaviorally tested and nonbehaviorally tested F3 offspring after the conclusion of behavioral testing at approximately 120 d of age. Mice were weighed and then sacrificed by cervical dislocation without the use of sedatives and fresh brains removed and hemisected. Randomization across groups was used during sacrifice to prohibit circadian variation occurring in a single group. The striatum, frontal cortex, and hippocampus were dissected from each brain after which they were flash-frozen. Trunk (whole) blood was collected into pre-chilled centrifuge tubes and centrifuged for 20 min at 3,500 × *g* to obtain serum. Serum samples were stored at -20°C until day of assay.

Pb determinations in F1 and F3 blood and F3 bone. Blood Pb (PbB) measurements were determined from trunk blood of F1 PND5–6 offspring and F3 120-d offspring using a PerkinElmer PinAAcle™ 900Z atomic absorption spectrophotometer equipped with longitudinal Zeeman background correction and a transverse heated graphite furnace (PerkinElmer Life and Analytical Sciences). Lead absorption was measured at 283.3 nm using a PerkinElmer System 2 electrodeless discharge lamp source. In a 1.5-mL microcentrifuge tube, 50-μL blood samples were diluted 1:10 with chemical modifier solution (450 μL) containing 0.2% wt/vol ammonium phosphate [(NH₄)₃PO₄]; 0.5% (vol/vol) Triton™ X-100; and 0.2% nitric acid (HNO₃). Sample aliquots (15 μL) of the diluted blood were deposited in the graphite furnace with an auto sampler.

Bone was isolated from 120-d-old F3 offspring and digested with HNO₃ and brought to a constant volume for determination of Pb content using a PerkinElmer PinAAcle™ 900Z atomic absorption spectrophotometer equipped with longitudinal Zeeman background correction and a transverse heated graphite furnace (PerkinElmer Life and Analytical Sciences). Lead absorption was measured at 283.3 nm using a PerkinElmer System 2 electrodeless discharge lamp source. A bone matrix modifier of (NH₄)₃PO₄ and calcium nitrate [Ca(NO₃)₂] was used to stabilize lead during the pyrolysis

furnace step. The modifier solution contained 2% HNO₃, 0.4% (wt/vol) ammonium dihydrogen phosphate (NH₄H₂PO₄), and 0.4 g/L calcium as Ca(NO₃)₂.

Serum corticosterone determinations. Serum corticosterone was measured in duplicate using a commercially available enzyme immunoassay kit (Arbor Assays) according to manufacturer's specifications. Sample replicates with coefficient of variation (CVs) higher than 15% were excluded from analysis.

Levels of striatal Th, GR, and BDNF. Protein expression levels of Th, GR, and BDNF were assessed in frontal cortex and hippocampal nuclear and cytosolic brain fractions. Nuclear and cytoplasmic fractions were extracted from samples in 50-mM Tris buffer with 10% sucrose, 0.5 M sodium chloride (NaCl), and 1 Complete™ Mini Protease Inhibitor Cocktail tablet (Roche) per 10 mL buffer, using repeated homogenization and centrifugation on ice. Total protein concentration was quantified by standard bicinchoninic acid assay (Pierce). Sample volume required for 10 μg of protein was diluted 1:1 with loading buffer (Bio-Rad Laemmli Sample Buffer, 5% β-mercaptoethanol), denatured for 5 min in a 100°C water bath, and loaded onto 7–10% Tris-glycine. Sample analyses were counterbalanced by treatment and behavioral group across all gels. Gels were run using Tris/glycine/sodium dodecyl sulfate buffer (Bio-Rad) transferred to a 0.2-μm nitrocellulose membrane using a semi-dry transfer apparatus at 15 V for 30 min. Membranes were then washed in Tris-buffered saline (TBS) containing 1% Tween- 20 (TBST), blocked in TBST with 5% nonfat milk and incubated overnight in primary antibody as follows: GR, 1:500 dilution (catalog no. MA1-510; Thermo Scientific); actin, 1:10,000 dilution (catalog no. A1978; Sigma-Aldrich); BDNF, dilution 1:300 (catalog no. AB1534; Millipore); and Th, dilution 1:2000 (catalog no. AB152; Millipore). All primary and secondary antibodies were validated before use and delivered in TBST with 2% nonfat milk. Blots were developed using 1 mL per membrane of Clarity Western ECL Substrate (Bio-Rad) for 5 min and visualized using a Bio-Rad ChemiDoc MP Imaging System. Images were transferred to ImageJ software (Schneider et al. 2012) and bands for each sample were normalized to β-actin loading controls. Data were then expressed as normalized relative optical density (ROD). The proteins were detected at the following molecular weights: GR: 97kDa, BDNF: 18 kDa with homodimer at ~27–30 kDa (detected at around 30), and Th: 62 kDa.

DNA methylation. Targeted DNA methylation analysis focused on the *Bdnf* and *Th* genes in the frontal cortex and *Nr3C1*, *Bdnf*, *Th*, *Esr1* and insulin-like growth factor 2 *Igf2/H19* in the hippocampus. Hippocampal genomic DNA was extracted using the DNeasy Blood and Tissue DNA extraction kit (Qiagen) according to manufacturer's instruction and quantified using the NanoDrop spectrophotometer. In addition, frontal cortex genomic DNA and total RNA were simultaneously extracted using the AllPrep DNA/RNA extraction kit (Qiagen) according to manufacturer's instruction and nucleic acids quantified as described for hippocampal DNA. One microgram genomic DNA was bisulfite-treated using the EpiTect Bisulfite Conversion kit (Qiagen) and 20 ng of bisulfite-converted DNA used for polymerase chain reaction (PCR) amplification. We designed PCR and sequencing primers using the Pyromark Q24 Assay Design Software. PCR primer information is described in Table S1. Amplified PCR products were sequenced using a Pyromark Q24 Advanced Pyrosequencer and analyzed using the PyroMark Q24. Four CpG sites within the exon IV gene body of *Bdnf* were assessed; CpG site 1 of 4 is a cAMP-response element binding protein (CREB) binding site, a transcription factor essential for *Bdnf* transcription (Kundakovic et al. 2015). Six CpG sites within the imprinting control region of the imprinted gene locus, *Igf2/H19* were analyzed because the sites are sensitive to environmental perturbation and also considered a site for DNA methylation alterations (Rivera et al. 2008;

Susiarjo et al. 2013). For *Nr3c1*, two CpG sites within the promoter that are established nerve growth factor-inducible protein (NGFI-A) binding sites were examined as sites critical for *Nr3c1* transcription (Lieberman et al. 2012). Six CpG sites within the promoter of *Th* were assessed; these sites were altered by isolation stress, resulting in altered *Th* gene expression in a model of isolation stress (Niwa et al. 2013).

Statistical Analyses

Two approaches to data analyses were included given that few studies include both sex and lineage as factors in transgenerational inheritance. To determine whether transgenerational F3 effects were present, analyses of all outcome measures were undertaken using F3 sex as a factor, that is, collapsed across lineages. In addition, to examine potential lineage-based effects, analyses of all outcome measures were done by specific lineage (F1-F2-F3). Repeated measures analyses of variance (ANOVA) were used to evaluate measures of FI performance and locomotor activity, with Pb and PS as between group factors; and time (FI: across sessions; locomotor activity: across 5-min bins within the 60-min session) as the within group factor. Based upon our innumerable prior observations of differential effects of both Pb and PS by sex (Cory-Slechta et al. 2017, 2010, 2013a; Schneider et al. 2016; Varma et al. 2017; Virgolini et al. 2006, 2008a), these analyses were carried out separately by sex. Significant main effects or interactions were followed by post hoc *t*-tests or two-factor ANOVA as appropriate. Elevated plus maze data, as well as striatal protein expression levels and DNA methylation changes, were analyzed separately by sex using two-factor ANOVA with Pb and PS as between group factors. Significant main effects or interactions were followed by post hoc *t*-tests. Cases where

Pb + PS (100-PS) were solely significantly different from control in post hoc tests are indicated on graphs.

Statistical analyses of potential lineage-based differences across 0-NS control groups were also examined for all behavioral measures to determine whether control group differences might underlie any apparent F3 transgenerational effect. Assessment of changes in numbers of runted litters in F3 offspring was determined using chi-square analyses. A $p \leq 0.05$ was considered to confirm statistical significance; trends (i.e., $p > 0.05$), where relevant, are indicated.

Results

Clinical Outcomes

No differences in maternal weight gain, successful pregnancies, litter size, or offspring weight at weaning (with all runts excluded) were found in the F1 and F2 breeding (see Table S2). However, in F3 offspring, a significant increase in runting was found in litters derived from Pb exposure, particularly from the combined Pb and PS exposures of the F0 generation [see Figure S1; chi-square Pearson value = 27.75, $p = 0.023$, (1/80 litters for control and 8/76 for Pb; specifically, 1 from control, 1 from Pb-only, and 7 from combined Pb- and PS-exposed F0 conditions)]. Runting did not appear to be lineage specific—with one runted litter from the control FM (F1-F2) lineage, one from the Pb-only exposed MF lineage, two from the combined Pb and PS exposed FF lineage, three from the combined Pb and PS exposed MF lineage, and two from the combined Pb and PS exposed MM lineage—but, rather, occurred more frequently in the Pb + PS lineages. These runts were excluded from any further testing.

Table 1. Summary of statistical outcomes by lineage.

Brain and behavioral outcomes ^a	F3 Females				F3 Males			
	F1 Dam		F1 Sire		F1 Dam		F1 Sire	
	FFF	FMF	MFF	MMF	FFM	FMM	MFm	MMM
Fixed interval performance								
Overall response rate	Pb × PS × T (I)	Pb × PS (I)	—	Pb × PS × T (I)	Pb (I)	—	—	—
Run rate	Pb × PS (I)	Pb × PS (I)	—	—	—	—	—	PS (I)
Mean postreinforcement pause	—	—	—	—	Pb × PS	Pb × T; PS × T	Pb × PS × T	—
Elevated plus maze								
Closed	—	—	Pb (D)	—	—	—	—	—
Open	—	—	Pb (I)	—	—	—	—	—
Locomotor time course								
Ambulatory distance	—	—	—	—	PS × T	—	—	—
Ambulatory time	—	PS; Pb × PS	—	—	PS × T	Pb × T	—	—
Ambulatory counts	—	Pb × PS	—	—	PS × T	—	—	—
Stereotypic time	—	Pb × T	—	—	—	—	—	—
Stereotypic counts	—	Pb × T	—	—	—	Pb × T	Pb × PS × T	—
Resting time	—	Pb × PS	—	—	PS × T	Pb × T	Pb × T; Pb × PS × T	—
Vertical counts	—	—	—	—	—	Pb × T	—	—
Vertical time	—	—	—	—	Pb × T	Pb × T	—	—
Serum corticosterone								
Serum corticosterone	—	—	~Pb (D)	~PS (D)	—	—	—	—
Striatal protein (nuclear and nuclear/cytosolic)								
Tyrosine hydroxylase (<i>Th</i>)	Pb (I)	—	Pb × PS	~Pb × PS (I)	—	Pb (D)	~Pb × PS	Pb (I)
Brain-derived neurotrophic factor (BDNF)	PS (I)	—	Pb × PS (I)	Pb (I)	—	—	—	—
Glucocorticoid receptor (GR)	~Pb (I)	—	PS (D)	—	PS (I)	—	—	—
Total DNA methylation								
Frontal cortex <i>Th</i>	—	Pb × PS	—	—	—	—	—	—
Frontal cortex BDNF	—	—	~Pb (I)	—	—	—	—	—
Hippocampus <i>Th</i>	Pb (I)	—	—	—	Pb (I)	~PS (D)	Pb (I)	—
Hippocampus BDNF	—	—	—	~PS (D)	—	PS (D)	—	Pb (D)

Note: ANOVA, analysis of variance; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; MFF, F1 male F2 female F3 female; MFm, F1 male F2 female F3 male; MMF, F1 male F2 male F3 female; MMM, F1 male F2 male F3 male; Pb, lead; PS, prenatal stress; T, time (minute, block, sessions).

^aDirections of effect are indicated for main effect by (I) = increase, (D) = decrease. Directions of effect are generally not indicated for interaction effects where direction differs across groups; where consistent across groups, direction is indicated. Presence of text within a cell indicates significance ($p < 0.05$); ~, significance ($p < 0.10$); —, nonstatistically significant effects as determined via repeated measures or factorial ANOVAs.

Lead Determinations

Blood Pb values of F1 pups at PND6–7 were significantly elevated compared with control pups [$F(3,20) = 488.3$, $p < 0.001$; $0.0 \mu\text{g}/\text{dL}$ vs. $12.5 \pm 0.34 \mu\text{g}/\text{dL}$, respectively]. In F3 offspring, no increases in either blood Pb or bone Pb were found above background Pb concentrations [blood: $F(3,34) = 0.94$, $p = 0.34$; bone: $F(3,19) = 0.16$, $p = 0.69$]. Specifically, F3 bone Pb means and standard errors (SEs) were $0.00 \mu\text{g}/\text{dL}$ and $5.2 \pm 5.2 \text{ ng}/\text{g}$ for control and $0.4 \pm 0.4 \mu\text{g}/\text{dL}$ and $10.3 \pm 5.1 \text{ ng}/\text{g}$ for Pb-treated mice.

Transgenerational Effects

Findings are presented in the main text in both narrative and graphical formats primarily for statistically significant outcomes, many of which were lineage specific. In the interest of clarity, readability, and transparency, all data, including nonsignificant outcomes and data by lineage or across lineage are provided in Table 1 or available in the Supplemental Material as described below.

Behavioral Changes

FI schedule-controlled behavior. Pb exposure resulted in a significant increase in overall response rates on the FI schedule of reward in F3 female offspring when considered across all lineages [main effect of Pb: $F(1,149) = 4.92$, $p = 0.028$]. Figure 2A displays these effects for the F3 female offspring across behavioral test sessions. No statistically significant effects of Pb, PS, or combined Pb and PS on F3 male offspring FI overall response rates were seen (Figure 2B). Effects on overall response rate on the FI schedule, however, differed by lineage and F3 sex, as can be seen in the FMF lineage (Figure 2C), where greater magnitude rate increases in response to Pb were seen in FMF F3 females [Pb by PS by sessions: $F(3,29) = 9.98$, $p = 0.0408$; Pb by sessions: $F(1,38) = 5.59$, $p = 0.023$], which at peak values were approximately 50% higher than those of control (0-NS) FMF females. Despite the absence of effects on overall response rates across lineages, moreover, F3 males from the FM lineage (Figure 2D) likewise showed increases in FI overall response rates [FMM, Pb by PS: $F(1,33) = 4.87$, $p = 0.034$], which averaged approximately 20% higher in Pb than in control F3 males.

Behavioral mechanisms (determinants) underlying the increases in FI overall response rates differed by sex (see Excel Tables S1 and S2), with increases in FI overall rates of F3 females of the FMF lineage resulting primarily from increases in run rates, that is, there are faster rates of responding once responding began during the fixed interval {Pb by PS [$F(1,38) = 6.67$, $p = 0.0138$]}, with run rates reaching values that were double those of 0-NS FMF females. Although some evidence of Pb-related increases in run rates were observed in FMM males, these effects were of only marginal significance [Pb by PS: $F(1,33) = 3.61$, $p = 0.0664$]. Instead, the increases in FI overall response rates in F3 males from the FMM lineage was due to the shorter period of time pausing prior to initiating responding during the fixed interval, that is, reductions in postreinforcement pause time. Although no significant treatment-related differences in postreinforcement pause time were found in F3 FMF females, these values were significantly shortened, particularly by Pb exposure in the FMM line {Pb by PS [$F(1,33) = 5.49$, $p = 0.0253$]}.

Locomotor activity. Small but statistically significant changes in various measures of locomotor activity were found in F3 females that appeared to derive primarily from F0 Pb exposure-induced increases in ambulation that were observed across lineages [Figure 3A; ambulatory distance: PS by block [$F(11,136) = 2.06$, $p = 0.0268$]; Figure 3B, ambulatory counts: Pb by PS [$F(1,146) = 3.99$, $p = 0.048$].

Changes in locomotor activity were lineage specific, with F3 females and males showing alterations in the FM lineage, as seen with FI behavior. Corresponding to the Pb-related changes in

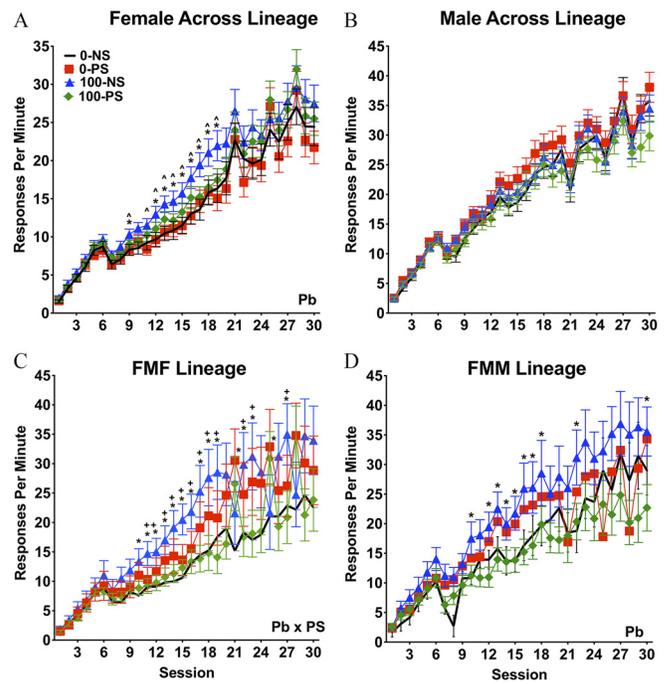


Figure 2. Fixed-interval (FI) response rates. (A,B) Group mean \pm SE overall response rates (responses per minute) on the FI schedule for F3 female (A) and male (B) offspring derived from the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS), and 100-PS (100 ppm Pb + PS) groups collapsed across lineages over the course of behavioral test sessions. Sample sizes of $n = 32$ – 40 /group. (C,D) Group mean \pm SE FI overall response rates for F3 0-NS, 0-PS, 100-NS, and 100-PS groups of the FMF (C) and FMM (D) lineages, respectively. Sample sizes of $n = 8$ – 12 /group for females; $n = 7$ – 10 /group for males. Pb = significant main effect of Pb and Pb \times PS = interaction of Pb by PS following repeated measures ANOVA. *, $p \leq 0.05$ compared with control; +, $p \leq 0.05$ compared with PS alone; ^, $p \leq 0.05$ compared with combined Pb and PS. Note: ANOVA, analysis of variance; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; Pb, lead; PS, prenatal stress; SE, standard error.

ambulation observed across F3 female lineages, when analyzed by lineage, increases in ambulation were seen in the FMF lineage (Figure 3C,D), again deriving primarily from F0 Pb exposure-induced increases and confirmed by significant Pb by PS interactions in statistical analyses [ambulatory time: $F(1,37) = 7.82$, $p = 0.0082$; ambulatory episodes: $F(1,37) = 4.13$, $p = 0.0495$]. Changes in locomotor behavior were also evident in F3 males from the FMM lineage (Figure 3E,F). These included Pb-related reductions in vertical counts [Pb by T ($F(11,23) = 2.71$, $p = 0.021$)] and vertical time [Pb by T ($F(11,23) = 2.93$, $p = 0.0142$)], which can be seen in both the Pb-only and combined Pb and PS groups. Summary data and data from each lineage is included in Excel Tables S3 and S4.

Elevated plus maze. Across lineages, F3 Pb females showed reduced time spent in the closed arms [Figure 4A; $F(3, 150) = 5.6$, $p = 0.02$] and increases in time spent in the center and open arms of the elevated plus maze, as indicated by main effects of Pb [$F(1,150) = 5.8$, $p = 0.017$]. These effects appeared to derive from Pb effects in F1 sire lineages in particular [Figure 4B, closed: $F(1,73) = 7.8$, $p = 0.006$; open: $F(1,73) = 7.8$, $p = 0.006$], whereas there were no effects in F1 dam lineages [Figure 4C, closed: $F(1,75) = 0.20$, $p = 0.69$]. There were no significant changes in F3 males across all lineages or as related to F1 sire or F1 dam (Figure 4D–F).

Serum Corticosterone

Serum corticosterone levels were measured in nonbehaviorally tested F3 offspring, as behavioral testing alone significantly

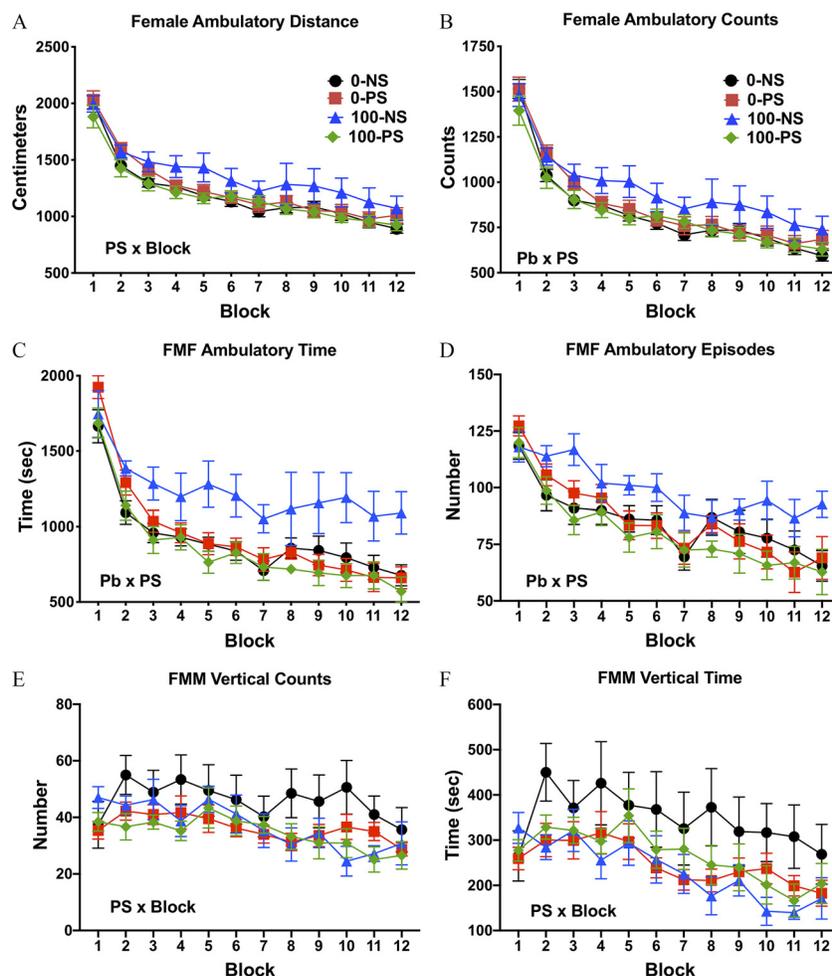


Figure 3. Locomotor activity. Group mean \pm SE levels of (A) ambulatory distance (cm) and (B) ambulatory counts of the female F3 offspring from the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS), and 100-PS (100 ppm Pb + PS) groups analyzed across F3 female lineages during locomotor activity assessment; sample sizes of $n = 35\text{--}40$ per group. Group mean \pm SE levels of (C) ambulatory time and (D) ambulatory episodes in the FMF lineage; sample sizes of $n = 10$ /group. Group mean \pm SE levels of (E) vertical counts and (F) vertical times in the FMM lineage, sample sizes of $n = 8\text{--}10$ /group, across 5-min bins of the 60-min assessment. PS by Block = significant interaction at $p \leq 0.05$ between prenatal stress (PS) exposure and block in statistical analyses; Pb \times PS = significant interaction of Pb exposure and PS at $p \leq 0.05$ of Pb, PS following repeated measures ANOVA. Note: ANOVA, analysis of variance; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; Pb, lead; SE, standard error.

elevated serum corticosterone concentrations [$F(1,262) = 11.18$, $p = 0.0009$]. Analyzed across all lineages, Pb decreased serum corticosterone in F3 females [Figure 5A; $F(3,57) = 4.78$, $p = 0.032$]. Effects in F3 females however, again differed by lineage (Figure 5B,C), with a Pb-related reduction in serum corticosterone in the F3 females originating from the F1 sire lineages, with the main effect of Pb [$F(3,28) = 4.45$, $p = 0.045$]. No F3 female changes were seen in the F1 dam lineages [$F(3,29) = 1.6$, $p = 0.21$]. No treatment-related differences in serum corticosterone were seen across lineages of F3 male offspring in nonbehaviorally tested groups or in relation to F1 sire or F1 dam (Figure 5D–F). See Excel Tables S7 and S8 for summary data.

Striatal Levels of Th, GR, and BDNF Protein

When analyzed across all lineages, there were no F3 effects for nuclear or cytosolic Th or GR. However, significant differences were found by lineage, including changes in expression levels of striatal Th (Figure 6A). Analyses of Th nuclear/cytosolic fractions revealed an effect of Pb exposure in the FFF lineage [$F(1,19) = 5.21$, $p = 0.036$]. Striatal nuclear cytosolic Th expression levels were significantly reduced in the FMM lineage Pb group [$F(1,19) = 6.25$, $p = 0.0237$], and significant Pb by PS interaction was found in

the MFF lineage [$F(1,19) = 5.07$, $p = 0.0389$], likely due to the reductions in the PS-only and Pb only groups, but post hoc comparisons did not reveal any significant between group differences. For striatal Th nuclear expression (Figure 6B), marginal trends for Pb by PS interactions and Pb effects were observed in the MFM and MMM lineages, respectively [$F(1,19) = 4.18$, $p = 0.057$ and $F(1,19) = 4.14$, $p = 0.051$]. Data for each lineage is shown in Figures S2 and S3 and Excel Tables S9 and S10.

Through the maternal lineage for F3 females and males, nuclear GR was marginally elevated in the FFF Pb group [$F(1,19) = 4.4$, $p = 0.053$] and significantly increased in the male FFM PS group [$F(1,19) = 10.95$, $p = 0.004$]. There were no significant changes in GR expression for any additional lineages (see Excel Tables S9 and S10).

Analyzed across all lineages, levels of striatal nuclear BDNF were marginally increased by F0 PS exposure in female F3 offspring with all lineages combined (see Figure S4, top left) [$F(1,78) = 4.16$, $p = 0.07$], with highest levels in the Pb + PS group. For F3 females exposed to PS through the maternal lineage (FFF), both nuclear and nuclear/cytosolic BDNF was significantly increased [Figure 6C, main effect of PS: $F(\text{nuclear/cytosolic:cytosolic}, 1, 19) = 8.18$, $p = 0.0114$; Figure 6D, $F(\text{nuclear: } 1, 19) = 7.26$, $p = 0.016$], and for BDNF nuclear/cytosolic ratios, there was also a significant

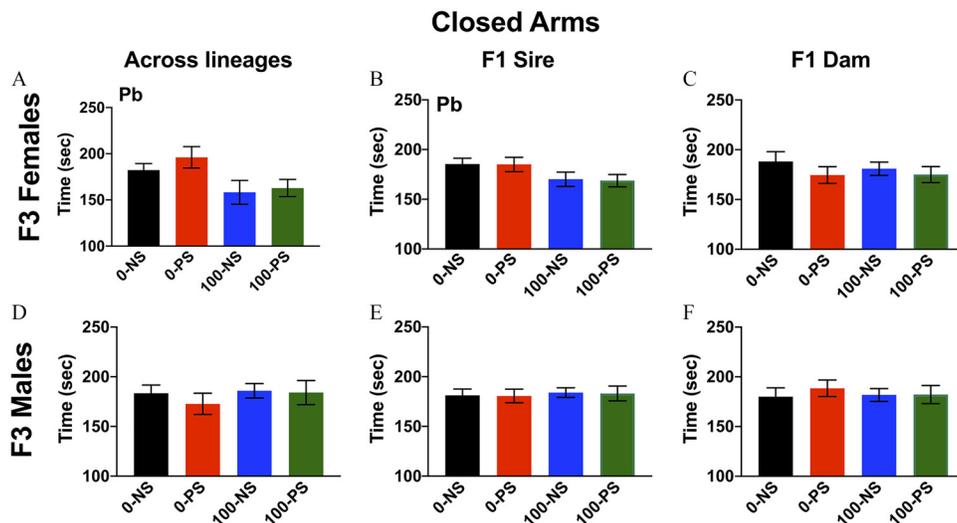


Figure 4. Elevated plus maze. Duration of time in the closed arms of the elevated plus maze (group mean \pm SE) of (A–C) F3 female and (D–F) F3 male offspring of the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS) and 100-PS (Pb + PS) groups across all F3 lineages (A,D: sample sizes of $n = 37$ – 40 /group) and separated by F1 sire lineages. (B) MMF and MFF collapsed for F3 females. (E) MFM and MMM for F3 males; sample sizes of $n = 18$ – 20 /group, and F1 dam lineages (C) FFF and FMF for F3 females collapsed; (F) FMM and FFM for F3 males; sample sizes of $n = 18$ – 20 /group. Pb indicates an overall main effect of lead at $p \leq 0.05$ following two-way ANOVA analysis. Note: ANOVA, analysis of variance; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; MFF, F1 male F2 female F3 female; MFM, F1 male F2 female F3 male; MMF, F1 male F2 male F3 female; MMM, F1 male F2 male F3 male; Pb, lead; PS, prenatal stress; SE, standard error.

interaction between Pb and PS in the MFF lineage [Pb \times PS, $F(3,19) = 4.72$, $p = 0.045$], which subsequent post hoc tests showed to be due to the significant increases in BDNF expression levels in the combined Pb and PS group. MMF mice derived from F0 dams treated with Pb exhibited significantly higher nuclear expression of BDNF [$F(1,19) = 4.72$, $p = 0.04$]. No changes were found in any striatal nuclear or cytosolic BDNF protein expression levels in F3 males. Data for each lineage is shown in Figures S4 and S5 and Excel Tables S9 and S10.

Frontal Cortex and Hippocampal DNA Methylation Changes

Significant changes in total DNA methylation when analyzed across lineages (Figure 7A,B) were limited to a Pb by PS

interaction for frontal cortex methylation of *Th* in F3 females [$F(3,74) = 4.16$, $p = 0.045$], although post hoc comparisons did not provide any significant differences among treatment groups. A significant reduction in total methylation of hippocampal *Bdnf* in F3 males derived from F0 Pb exposure was also observed [$F(3,76) = 4.81$, $p = 0.031$].

As with other outcome measures, lineage-specific changes in total DNA methylation were observed. A Pb by PS interaction was observed for FC *Th* in the FMF lineage (Figure 7C) that generally mirrored the outcome across F3 female lineages [$F(3,19) = 5.5$, $p = 0.032$]. Interestingly, several lineages showed Pb-related increases in percent total hippocampal *Th* DNA methylation in hippocampus {Figure 7D,E; FFF: [$F(3,19) = 5.41$, $p = 0.033$]; FFM: [$F(3,17) = 6.46$, $p = 0.024$]; and Figure 7F [MFM: $F(3,18) =$

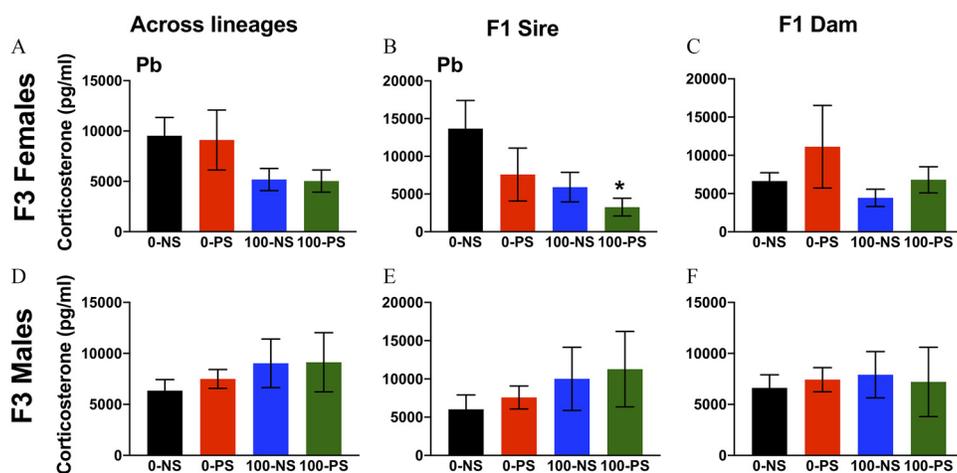


Figure 5. Serum corticosterone. Group mean \pm SE levels of corticosterone (pg/mL) analyzed across lineages of (A–C) F3 female and (D–F) F3 male offspring of the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS), and 100-PS (100 ppm Pb + PS) with sample sizes of $n = 12$ – 18 /group across all lineages (A,D), separated by F1 sire [(B) MMF and MFF collapsed for F3 females; (E) MFM and MMM for F3 males; sample sizes of $n = 6$ – 8 /group] and by F1 dam lineage (C) FFF and FMF for F3 females collapsed; (F) FMM and FFM for F3 males; sample sizes of $n = 6$ – 8 /group]. None of these animals had undergone behavioral testing. Pb = significant main effect of lead at $p \leq 0.05$ following two-way ANOVA analysis, * = significantly different from 0-NS in post hoc *t*-test analysis. Note: ANOVA, analysis of variance; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; MFF, F1 male F2 female F3 female; MFM, F1 male F2 female F3 male; MMF, F1 male F2 male F3 female; MMM, F1 male F2 male F3 male; Pb, lead; PS, prenatal stress; SE, standard error.

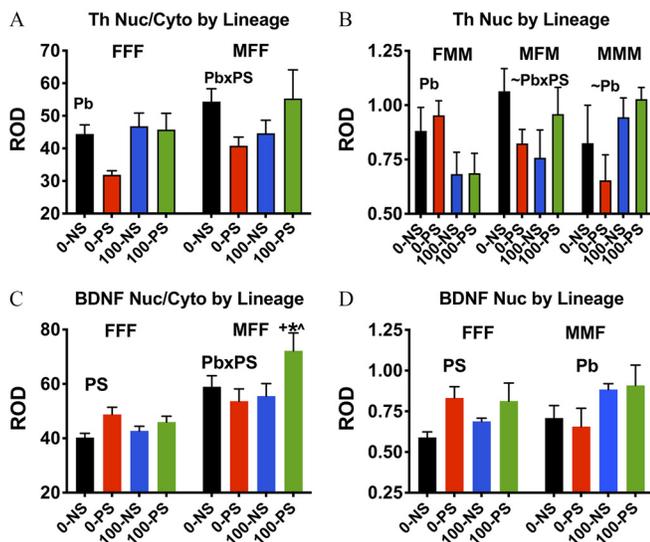


Figure 6. Striatal protein levels of tyrosine hydroxylase (Th), glucocorticoid receptor (GR), and brain-derived neurotrophic factor (BDNF). Group mean \pm SE levels [relative optical density (ROD)] of expression striatal Th nuclear/cytosolic fraction in offspring of the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS), and 100-PS (100 ppm Pb + PS) groups (sample size of $n = 4\text{--}5$ /group) from the FFF and MFF F3 female offspring (A) and Th nuclear fraction in FMM, MFM, and MMM male F3 offspring (B). Group mean \pm SE levels (ROD) of expression of striatal (C) BDNF nuclear (nuc)/cytosolic (cyto) fraction and (D) nuclear BDNF in the FFF and MFF lineages of F3 female offspring (sample sizes of $n = 5$ /group). Pb = significant main effect of Pb at $p \leq 0.05$, PS = significant main effect of PS at $p \leq 0.05$, and Pb \times PS = significant interaction of Pb and PS at $p \leq 0.05$ following two-way ANOVA analysis. \sim Pb by PS = marginal interaction of Pb by PS in statistical analysis with a $p \leq 0.10$. * = significantly different from 0-NS; ^ = significantly different from 0-PS; + = significantly different from 100-NS in post hoc t -test analysis. Note: ANOVA, analysis of variance; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; MFF, F1 female F2 female F3 female; MFM, F1 male F2 female F3 male; MMF, F1 male F2 female F3 female; MMM, F1 male F2 male F3 male; Pb, lead; PS, prenatal stress; SE, standard error.

13.13, $p = 0.0025$], with the highest mean value as determined from post hoc tests in the combined Pb and PS group in each case.

In the FMM lineage (Figure 7G,H), PS exposure significantly reduced percent total hippocampal *Bdnf* DNA methylation [$F(3,17) = 6.46$, $p = 0.024$], whereas an F0 Pb exposure related reduction in total hippocampal *Bdnf* percent DNA methylation was found in the MMM lineage [$F(3,18) = 8.06$, $p = 0.0125$] that was most pronounced in the 100-PS group as indicated by post hoc t -test analysis.

Discussion

Knowledge of the neurotoxicity of Pb exposure dates back centuries (Hernberg 2000), with current understanding indicating that no safe level of Pb with respect to impaired cognitive function in children can be identified (CDC 2019a). More recent studies have documented consequences of developmental Pb exposure for brain and behavior enduring beyond childhood (Bellinger 2008; Cecil et al. 2008; Reuben et al. 2017). The consequences and nature of Pb are greater in lower socioeconomic status communities (Lefmann and Combs-Orme 2014), where a higher proportion of U.S. children younger than 6 years of age were found to have high or very high blood levels compared with those living in communities with a lower percentage of poverty (McClure et al. 2016). Because developmental Pb and PS share the HPA axis and brain MESO circuits as targets (Barros et al. 2004; Berger et al. 2002; Martínez-Telléz et al. 2009), our laboratory has studied these risk

factors in combination. In these rodent studies, combined exposures led to enhanced neurotoxicity for some outcome measures as compared with Pb or PS alone (Virgolini et al. 2006, 2008a; Weston et al. 2014), findings confirmed in studies since carried out in human cohorts (Surkan et al. 2008; Tamayo y Ortiz et al. 2017). Recent studies have suggested that effects of PS may occur multigenerationally, and even transgenerationally (Gutteling et al. 2006; Schwabe et al. 2012; Weiss et al. 2011); whether similar passage occurs with Pb exposure is unknown. Using a mouse model, the present study sought to determine whether Pb, PS, or combined Pb and PS could produce transgenerational consequences for brain and behavior and to determine whether such effects differed by offspring sex and lineage.

In this study, we found that Pb, PS, and combined Pb and PS exposures produced transgenerational effects on brain and behavior. Analyzed across lineages, consistent effects that were largely associated with Pb exposure were observed in F3 females, with increases in rates of responding on the FI schedule, increases in ambulatory locomotor activity, decreases in time spent in the closed arms of the elevated plus maze, and reductions in serum corticosterone levels. For these F3 females, PS-only exposure also significantly increased striatal expression levels of BDNF. Fewer changes were seen across the F3 males, with transgenerational changes observed in the Pb group consisting of reductions in postreinforcement pause time on the FI schedule, altered locomotor activity, altered striatal Th concentrations, and decreased total percent DNA methylation of the *Bdnf* gene in the hippocampus.

Given the breeding strategy employed in this study, individual lineages could be tracked and specific lineages frequently showed greater magnitude effects than those observed in analyses collapsed across all lineages (summarized in Table 1). Lineage-specific analyses revealed that Pb-related increases in FI response rates of F3 females largely derived from lineages with F1 dams (grandmothers), including male parentage (FMM lineage) and mother parentage (FFF). Further, Pb-related locomotor changes were also seen in F3 female offspring from F1 dams, with male parentage (FMM lineage). In nonbehaviorally tested animals, Pb-related corticosterone reductions were seen in F3 female offspring through F1 sire lineage, with greater reductions in the MFF lineage. F3 female offspring through the F1 sire showed decreased time in the closed arms of the EPM. Increases in striatal BDNF in response to Pb alone or to combined Pb and PS were seen in F3 females from F1 sires, seen in the MFF and MMF lineages, whereas PS-based increases were found in the FFF lineage. Pb-related changes in total DNA methylation were seen in F3 females of the FFF lineage.

For F3 male offspring, lineage-specific changes were observed in FI response rates, locomotor activity, striatal Th expression, and hippocampal BDNF methylation profiles. Lineage-specific analyses revealed Pb, PS, or combined Pb and PS-related reductions in postreinforcement pause time, particularly in F3 male offspring from the MF, FM, and FF lineages. Similarly, although locomotor activity effects were relatively minor when analyzed across lineages, analyses of individual lineages revealed reductions in response to Pb, PS, or combined Pb and PS following exposures to the F1 grandmothers, particularly the FF and FM lineages. Pb-based reductions in F3 male offspring total hippocampal DNA methylation of *Bdnf* were primarily due to greater magnitude changes through the paternal MMM lineage. Collectively, these findings suggest that transgenerational passage is influenced by the sex composition of the lineage and that different lineages exhibit different profiles of transgenerational effects. Unfortunately, few studies of transgenerational effects include outside breeding or follow lineages other than FF and MM, yet all such lineages are present in human populations.

Notably, effects seen in response to Pb, PS, or combined Pb and PS in the F3 lineages are consistent with changes in behaviors and

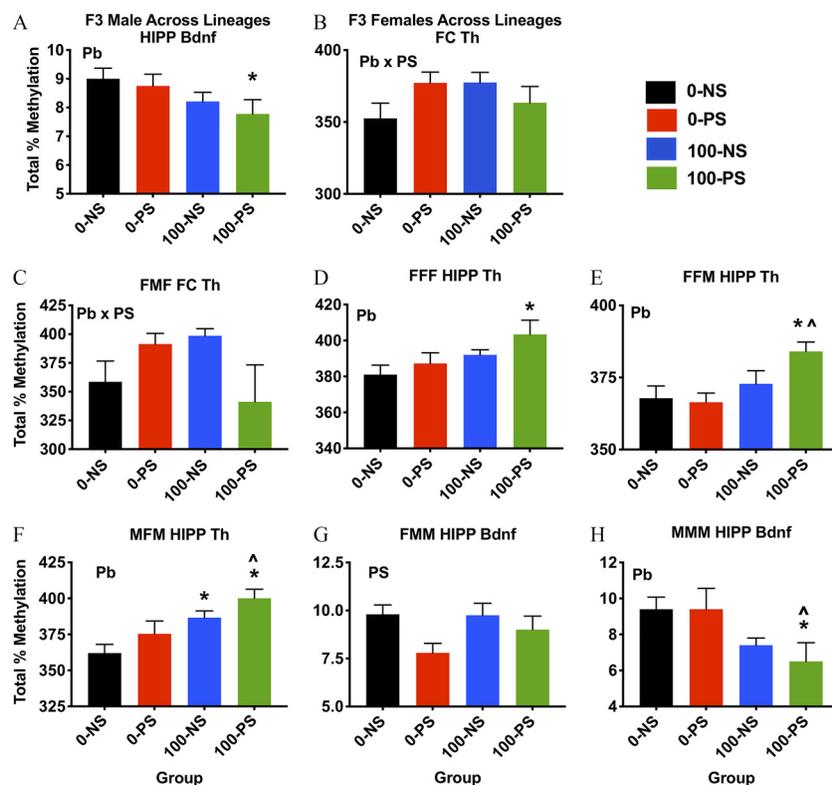


Figure 7. DNA methylation changes in frontal cortex and hippocampus. (A,B) Group mean \pm SE levels of total percentage methylation of hippocampal Bdnf (brain-derived neurotrophic factor) across lineages of F3 male offspring [(A) sample sizes of $n = 18\text{--}20/\text{group}$] and frontal cortex tyrosine hydroxylase (Th) in female F3 offspring [(B) sample sizes of $n = 17\text{--}20/\text{group}$] of the F0 0-NS [control; 0 ppm Pb + no stress (NS)], 0-PS (0 ppm Pb + PS), 100-NS (100 ppm Pb + NS) and 100-PS (100 ppm Pb + PS) groups. (C–E) Group mean \pm SE levels of total percentage methylation of Th in the frontal cortex of FMF F3 females (C), group mean \pm SE levels of total percentage DNA methylation in FFF (D) and FFM (E) F3 offspring (sample size of $n = 4\text{--}5/\text{group}$). (F–H) MFM Group mean \pm SE levels of total percentage DNA methylation of hippocampal Th in MFM (F), hippocampal Bdnf in the FMM (G), and hippocampal Bdnf in the MMM lineages of (H) F3 male offspring (sample size of $n = 4\text{--}5/\text{group}$). Pb = significant main effect of Pb at $p \leq 0.05$ in statistical analysis, PS = significant main effect of PS at $p \leq 0.05$ in statistical analysis; Pb \times PS = significant interaction of Pb and PS at $p \leq 0.05$ following two-way ANOVA analysis. * = significantly different from 0-NS; ^ = significantly different from 0-PS; + = significantly different from 100-NS in post hoc *t*-test analysis. Note: ANOVA, analysis of variance; FFF, F1 female F2 female F3 female; FFM, F1 female F2 female F3 male; FMF, F1 female F2 male F3 female; FMM, F1 female F2 male F3 male; HIPP, hippocampal; MFF, F1 male F2 female F3 female; MFM, F1 male F2 female F3 male; MMM, F1 male F2 male F3 male; Pb, lead; PS, prenatal stress; SE, standard error.

systems previously reported in developmental studies of F1 offspring. For example, Pb- as well as PS- and combined Pb and PS-induced changes in FI behavioral performance have been repeatedly noted in F1 offspring in our prior studies, with the nature of the consequences dependent upon multiple factors, including developmental period and level of Pb exposure as well as sex of offspring (Cory-Slechta and Thompson 1979; Cory-Slechta et al. 1985, 1997, 1998, 2002; Cory-Slechta 1997; Rossi-George et al. 2011; Virgolini et al. 2005, 2008a). The present study suggests that such effects could be transmitted across generations, with Pb-induced increases in FI response rates in females of the F3 generation arising from increased run rates. In addition, F3 male offspring of the FMM, FFM, and MMM lineages displayed significantly shorter postreinforcement pause times, contributing to the trend toward Pb-related increases in FI rates. Increases in FI response rates signify inefficient behavioral function given that increased response rates cannot accelerate reinforcement availability. In addition, increased response rates on FI schedules have been directly related to reductions in self-control in both infants and children (Darcheville et al. 1992, 1993). Shorter postreinforcement pause times also signify impairments in learning the timing of the interval, normally seen as a characteristic scalloped pattern of responding with little responding early in the interval and subsequent increases as time elapses. These FI changes are further notable given that they could be reflective of impulsivity and or inability to inhibit responding. All of these are features of neurodevelopmental disorders such as autism spectrum disorder and attention

deficit hyperactivity disorder (Coghill et al. 2014; Hove et al. 2017; Lambrechts et al. 2018; López-Martín et al. 2015; Rubia et al. 2009; van Hulst et al. 2017).

Our prior experimental studies in rodents also show Pb, PS, and combined Pb and PS-induced changes in MESO neurotransmitter systems in developmentally exposed F1 offspring (Cory-Slechta et al. 2008, 2013a, 2013b; Weston et al. 2014) consistent with effects of these factors on brain dopamine systems. We have previously demonstrated in rats the importance of MESO dopamine to mediation of performance on the FI schedule of reward, a behavior altered by Pb and PS in rats (Cory-Slechta et al. 1998, 2002; Evans and Cory-Slechta 2000). The nature of the changes in dopamine systems, as with FI performance, is dependent upon developmental periods and concentration of Pb exposure, as well as sex of F1 offspring. These findings correspond to the observed changes in female F3 lineages in expression of striatal Th, the rate-limiting enzyme in dopamine synthesis, and with alterations in BDNF, which is critical to brain dopamine function (Bustos et al. 2004). BDNF is a protein that plays a key role in MESO system synaptic plasticity and learning/memory (Kowiański et al. 2018; von Bohlen und Halbach and von Bohlen and Halbach 2018) via mechanisms that include its control of dopamine D3 receptor function (Guillin et al. 2004). Rodent studies focused on developmental exposures to PS (stress to the F0 dam, prenatal for exposure the F1 generation) indicate that *Bdnf* mRNA levels are reduced in the hippocampus, with increases in DNA methylation particularly in

females (Berry et al. 2015; Boersma et al. 2014; Dong et al. 2015; Seo et al. 2016). Our prior studies, moreover, have demonstrated that Pb exposure reduced BDNF protein levels in the frontal cortex, whereas PS increased BDNF in the nucleus accumbens and the hippocampus of male rats, and in females, PS exposure reduced nucleus accumbens BDNF (Weston et al. 2014). Correspondingly, in the F3 generation assessed in this study, PS-related increases in BDNF expression in the striatum were observed when analyzed across all F3 female lineages, particularly in the FFF lineage, whereas significant Pb-related increases occurred in the F1 male sire lineages (MMF and MFF lineages), particularly under conditions of combined Pb and PS. Alterations in BDNF by PS, including shifts in methylation and expression profiles, have likewise been related to neurodevelopmental disorders with sex-biased prevalence rates, including female-biased disorders such as anxiety and depression (Baker-Andresen et al. 2013; Zheng et al. 2016).

As mentioned above, our prior developmental (F1) studies have shown modification of the HPA axis function following Pb, PS, or both (Rossi-George et al. 2011; Virgolini et al. 2005, 2006, 2008a, 2008b). In the present study, female F3 offspring showed significant Pb-related reductions in corticosterone levels across lineages, particularly F3 females from F1 sires. In addition, the same group of females had significant reductions in time spent in the closed arms of the elevated plus maze, suggesting decreased fear or “anxiety-like” behavior. The strong influence in females may be due to female sensitivity, or due to the nature of gamete production. Future studies should also examine F2 males because these would be the first unexposed generation and may elicit more changes than F3 males in HPA axis function and behavior. Sex-dependent Pb-induced changes in stress responsivity need additional research on *a*) the functionality of the stress response because either increased or reduced serum corticosterone potentially represent disruptions to stress homeostasis (Korte et al. 2005; McEwen 2010; Peters et al. 2017), and *b*) additional generations of both F2 males and F4 females to determine if transgenerational effects are eliminated in subsequent unexposed generations. Furthermore, PS may have had a larger role using different types of stressors given that the nature and duration of a stressor have been shown to alter long-term, sex-specific neurophysiological responses (McEwen 2010; Peters et al. 2017).

Collectively, these data indicate that lineage and sex-specific transgenerational passage of brain and behavioral phenotypes can be induced by developmental exposures to risk factors such as Pb and PS. Importantly, these effects were seen even with full outbreeding using unexposed C57BL/6J mice, compared with transgenerational studies where treated animals are bred to each other. Although differences by sex in F3 consequences are certainly recognized (Dew-Budd et al. 2016; Zizzari et al. 2016), the contribution of lineage transfer in relation to sex, and potential dynamic changes in response to ongoing environmental conditions, have received far less attention (Burggren 2015).

What remains unknown from these studies is the mechanism by which these transgenerational effects are produced. Although transgenerational effects are primarily studied in the context of epigenetic inheritance, there are actually two major routes by which transgenerational effects and programming can occur that may include either direct molecular epigenetic inheritance or environmental passage, or both (Danchin et al. 2011), via parental, ecological, and cultural means. The fact that many of the behavioral and biochemical changes observed here were lineage dependent and that transgenerational effects were observed in both the FF and MM lineages could suggest that both types of mechanisms were operative. Further, although our prior studies of Pb, PS, and combined Pb and PS in F1 offspring revealed numerous instances of enhanced toxicity of combined Pb and PS, few such examples were found transgenerationally, with the majority of effects reflecting either Pb or PS exposures alone.

Interestingly, most examples of enhanced F3 effects with combined Pb and PS exposure were seen in levels of the percent total DNA methylation of the *Th* and/or *Bdnf* gene in the frontal cortex and the hippocampus. Further, it is worth investigating whether changes in methylation patterns exist broadly and whether these changes may play a role in dysregulation of imprinted genes known to tightly regulate growth considering the increased number of litters with runt pups in F3 Pb + PS litters. Future studies investigating transgenerational mechanisms of cumulative risk should focus on multiple mechanisms of environmental transmission, including small RNAs, chromatin reprogramming, and genomic imprinting and methylation, in addition to focusing on changes in maternal behavior and health and by understanding that transgenerational heredity can be directly inherited, occurring in response to inherited environments, or arise stochastically (Bale 2015; Bošković and Rando 2018). Understanding the differences in the transgenerational effects of Pb, PS, and combined Pb and PS, integrated across varied inheritance mechanisms, may help assist in defining differential contributions of lineage and offspring sex.

Understanding these complexities is critical, because in the human environment, Pb exposures are likely to be more complex. First, following birth, Pb and PS exposure will inevitably continue to occur. This dynamic sequence of events would likely induce epigenetic changes that could further influence a behavioral phenotype. Further, bone Pb stores are reduced during pregnancy and breastfeeding (Gulson et al. 2016), suggesting that Pb exposure is reduced across successive pregnancies. Hence, it might be expected that the magnitude of transgenerational passage would be altered based on birth order. These points are especially relevant to studies in human populations as well as to proposed reversals via epigenetic means of such transgenerational impacts.

Acknowledgments

The work reported here was supported by National Institutes of Health/National Institute of Environmental Health Sciences grant P30 ES001247 to B. Paige Lawrence.

References

- Ambeskovic M, Soltanpour N, Falkenberg EA, Zucchi FCR, Kolb B, Metz GAS. 2017. Ancestral exposure to stress generates new behavioral traits and a functional hemispheric dominance shift. *Cereb Cortex* 27(3):2126–2138, PMID: 26965901, <https://doi.org/10.1093/cercor/bhw063>.
- Bahari Z, Meftahi GH, Meftahi M. 2018. Dopamine effects on stress-induced working memory deficits. *Behav Pharmacol* 29(7):584–591, PMID: 30215620, <https://doi.org/10.1093/FBP.0000000000000429>.
- Baker-Andresen D, Flavell CR, Li X, Bredy TW. 2013. Activation of BDNF signaling prevents the return of fear in female mice. *Learn Mem* 20(5):237–240, PMID: 23589089, <https://doi.org/10.1101/lm.029520.112>.
- Bale TL. 2015. Epigenetic and transgenerational reprogramming of brain development. *Nat Rev Neurosci* 16(6):332–344, PMID: 25921815, <https://doi.org/10.1038/nrn3818>.
- Barros VG, Berger MA, Martijena ID, Sarchi MI, Pérez AA, Molina VA, et al. 2004. Early adoption modifies the effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. *J Neurosci Res* 76(4):488–496, PMID: 15114621, <https://doi.org/10.1002/jnr.20119>.
- Bellinger DC. 2008. Neurological and behavioral consequences of childhood lead exposure. *PLoS Med* 5(5):e115, PMID: 18507501, <https://doi.org/10.1371/journal.pmed.0050115>.
- Berger MA, Barros VG, Sarchi MI, Tarazi FI, Antonelli MC. 2002. Long-term effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. *Neurochem Res* 27(11):1525–1533, PMID: 12512957, <https://doi.org/10.1023/a:1021656607278>.
- Berry A, Panetta P, Luoni A, Bellisario V, Capoccia S, Riva MA, et al. 2015. Decreased *Bdnf* expression and reduced social behavior in periadolescent rats following prenatal stress. *Dev Psychobiol* 57(3):365–373, PMID: 25783782, <https://doi.org/10.1002/dev.21297>.
- Bock J, Wainstock T, Braun K, Segal M. 2015. Stress in utero: prenatal programming of brain plasticity and cognition. *Biol Psychiatry* 78(5):315–326, PMID: 25863359, <https://doi.org/10.1016/j.biopsych.2015.02.036>.

- Boersma GJ, Lee RS, Cordner ZA, Ewald ER, Purcell RH, Moghadam AA, et al. 2014. Prenatal stress decreases *Bdnf* expression and increases methylation of *Bdnf* exon IV in rats. *Epigenetics* 9(3):437–447, PMID: 24365909, <https://doi.org/10.4161/epi.27558>.
- Bošković A, Rando OJ. 2018. Transgenerational epigenetic inheritance. *Annu Rev Genet* 52:21–41, PMID: 30160987, <https://doi.org/10.1146/annurev-genet-120417-031404>.
- Burggren WW. 2015. Dynamics of epigenetic phenomena: intergenerational and intragenerational phenotype 'washout'. *J Exp Biol* 218(Pt 1):80–87, PMID: 25568454, <https://doi.org/10.1242/jeb.107318>.
- Bustos G, Abarca J, Campusano J, Bustos V, Noriega V, Aliaga E. 2004. Functional interactions between somatodendritic dopamine release, glutamate receptors and brain-derived neurotrophic factor expression in mesencephalic structures of the brain. *Brain Res Brain Res Rev* 47(1–3):126–144, PMID: 15572168, <https://doi.org/10.1016/j.brainresrev.2004.05.002>.
- Canfield RL, Gendle MH, Cory-Slechta DA. 2004. Impaired neuropsychological functioning in lead-exposed children. *Dev Neuropsychol* 26(1):513–540, PMID: 15276907, https://doi.org/10.1207/s15326942dn2601_8.
- Canfield RL, Henderson CR Jr, Cory-Slechta DA, Cox C, Jusko TA, Lanphear BP. 2003. Intellectual impairment in children with blood lead concentrations below 10 µg per deciliter. *N Engl J Med* 348(16):1517–1526, PMID: 12700371, <https://doi.org/10.1056/NEJMoa022848>.
- CDC (Centers for Disease Control and Prevention). 2019a. Childhood Lead Poisoning. <https://ephrtracking.cdc.gov/showChildhoodLeadPoisoning.action> [accessed 17 January 2020].
- CDC. 2019b. Childhood Lead Poisoning Prevention. <https://www.cdc.gov/nceh/lead/prevention/blood-lead-levels.htm> [accessed 17 January 2020].
- Cecil KM, Brubaker CJ, Adler CM, Dietrich KN, Altaye M, Egelhoff JC, et al. 2008. Decreased brain volume in adults with childhood lead exposure. *PLoS Med* 5(5):e112, PMID: 18507499, <https://doi.org/10.1371/journal.pmed.0050112>.
- Cintra A, Solfrini V, Bunemann B, Okret S, Bortolotti F, Gustafsson JA, et al. 1993. Prenatal development of glucocorticoid receptor gene expression and immunoreactivity in the rat brain and pituitary gland: a combined in situ hybridization and immunocytochemical analysis. *Neuroendocrinology* 57(6):1133–1147, PMID: 8232769, <https://doi.org/10.1159/000126480>.
- Coghill DR, Seth S, Matthews K. 2014. A comprehensive assessment of memory, delay aversion, timing, inhibition, decision making and variability in attention deficit hyperactivity disorder: advancing beyond the three-pathway models. *Psychol Med* 44(9):1989–2001, PMID: 24176104, <https://doi.org/10.1017/S0033291713002547>.
- Cory-Slechta DA. 1997. Relationships between Pb-induced changes in neurotransmitter system function and behavioral toxicity. *Neurotoxicology* 18(3):673–688, PMID: 9339816.
- Cory-Slechta DA, Brockel BJ, O'Mara DJ. 2002. Lead exposure and dorsomedial striatum mediation of fixed interval schedule-controlled behavior. *Neurotoxicology* 23(3):313–327, PMID: 12387360, [https://doi.org/10.1016/s0161-813x\(02\)00059-1](https://doi.org/10.1016/s0161-813x(02)00059-1).
- Cory-Slechta DA, Merchant-Borna K, Allen JL, Liu S, Weston D, Conrad K. 2013a. Variations in the nature of behavioral experience can differentially alter the consequences of developmental exposures to lead, prenatal stress, and the combination. *Toxicol Sci* 131(1):194–205, PMID: 22930682, <https://doi.org/10.1093/toxsci/kfs260>.
- Cory-Slechta DA, O'Mara DJ, Brockel BJ. 1998. Nucleus accumbens dopaminergic mediation of fixed interval schedule-controlled behavior and its modulation by low-level lead exposure. *J Pharmacol Exp Ther* 286(2):794–805, PMID: 9694936.
- Cory-Slechta DA, O'Mara DJ, Brockel BJ. 1999. Learning versus performance impairments following regional administration of MK-801 into nucleus accumbens and dorsomedial striatum. *Behav Brain Res* 102(1–2):181–194, PMID: 10403026, [https://doi.org/10.1016/s0166-4328\(99\)00015-7](https://doi.org/10.1016/s0166-4328(99)00015-7).
- Cory-Slechta DA, Pazmino R, Bare C. 1997. The critical role of the nucleus accumbens dopamine systems in the mediation of fixed interval schedule-controlled operant behavior. *Brain Res* 764(1–2):253–256, PMID: 9295219, [https://doi.org/10.1016/s0006-8993\(97\)00591-x](https://doi.org/10.1016/s0006-8993(97)00591-x).
- Cory-Slechta DA, Sobolewski M, Varma G, Schneider JS. 2017. Developmental lead and/or prenatal stress exposures followed by different types of behavioral experience result in the divergence of brain epigenetic profiles in a sex, brain region, and time-dependent manner: implications for neurotoxicology. *Curr Opin Toxicol* 6:60–70, PMID: 29430559, <https://doi.org/10.1016/j.cotox.2017.09.004>.
- Cory-Slechta DA, Stern S, Weston D, Allen JL, Liu S. 2010. Enhanced learning deficits in female rats following lifetime Pb exposure combined with prenatal stress. *Toxicol Sci* 117(2):427–438, PMID: 20639260, <https://doi.org/10.1093/toxsci/kfq221>.
- Cory-Slechta DA, Thompson T. 1979. Behavioral toxicity of chronic postweaning lead exposure in the rat. *Toxicol Appl Pharmacol* 47(1):151–159, PMID: 425114, [https://doi.org/10.1016/0041-008x\(79\)90082-6](https://doi.org/10.1016/0041-008x(79)90082-6).
- Cory-Slechta DA, Virgolini MB, Rossi-George A, Thiruchelvam M, Lisek R, Weston D. 2008. Lifetime consequences of combined maternal lead and stress. *Basic Clin Pharmacol Toxicol* 102(2):218–227, PMID: 18226077, <https://doi.org/10.1111/j.1742-7843.2007.00189.x>.
- Cory-Slechta DA, Weiss B. 1985. Alterations in schedule-controlled behaviour of rodents correlated with prolonged lead exposure. In: *Behavioral Pharmacology: The Current Status*. Seiden LS, Balster RL, eds. New York, NY: Alan R. Liss, 487–501.
- Cory-Slechta DA, Weiss B, Cox C. 1985. Performance and exposure indices of rats exposed to low concentrations of lead. *Toxicol Appl Pharmacol* 78(2):291–299, PMID: 4035681, [https://doi.org/10.1016/0041-008x\(85\)90292-3](https://doi.org/10.1016/0041-008x(85)90292-3).
- Cory-Slechta DA, Weston D, Liu S, Allen JL. 2013b. Brain hemispheric differences in the neurochemical effects of lead, prenatal stress, and the combination and their amelioration by behavioral experience. *Toxicol Sci* 132(2):419–430, PMID: 23358193, <https://doi.org/10.1093/toxsci/kft015>.
- Danchin E, Charmanter A, Champagne FA, Mesoudi A, Pujol B, Blanchet S. 2011. Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. *Nat Rev Genet* 12(7):475–486, PMID: 21681209, <https://doi.org/10.1038/nrg3028>.
- Darcheville JC, Rivière V, Wearden JH. 1992. Fixed-interval performance and self-control in children. *J Exp Anal Behav* 57(2):187–199, PMID: 1573372, <https://doi.org/10.1901/jeab.1992.57-187>.
- Darcheville JC, Rivière V, Wearden JH. 1993. Fixed-interval performance and self-control in infants. *J Exp Anal Behav* 60(2):239–254, PMID: 8409821, <https://doi.org/10.1901/jeab.1993.60-239>.
- Dew-Budd K, Jarnigan J, Reed LK. 2016. Genetic and sex-specific transgenerational effects of a high fat diet in *Drosophila melanogaster*. *PLoS One* 11(8):e0160857, PMID: 27518304, <https://doi.org/10.1371/journal.pone.0160857>.
- Dias BG, Ressler KJ. 2014. Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat Neurosci* 17(1):89–96, PMID: 24292322, <https://doi.org/10.1038/nn.3594>.
- Dong E, Dzitoyeva SG, Matrisciano F, Tueting P, Grayson DR, Guidotti A. 2015. Brain-derived neurotrophic factor epigenetic modifications associated with schizophrenia-like phenotype induced by prenatal stress in mice. *Biol Psychiatry* 77(6):589–596, PMID: 25444166, <https://doi.org/10.1016/j.biopsych.2014.08.012>.
- Evans SB, Cory-Slechta DA. 2000. Prefrontal cortical manipulations alter the effects of intra-ventral striatal dopamine antagonists on fixed-interval performance in the rat. *Behav Brain Res* 107(1–2):45–58, PMID: 10628729, [https://doi.org/10.1016/s0166-4328\(99\)00108-4](https://doi.org/10.1016/s0166-4328(99)00108-4).
- Farah MJ. 2017. The neuroscience of socioeconomic status: correlates, causes, and consequences. *Neuron* 96(1):56–71, PMID: 28957676, <https://doi.org/10.1016/j.neuron.2017.08.034>.
- Franklin TB, Linder N, Russig H, Thöny B, Mansuy IM. 2011. Influence of early stress on social abilities and serotonergic functions across generations in mice. *PLoS One* 6(7):e21842, PMID: 21799751, <https://doi.org/10.1371/journal.pone.0021842>.
- Franklin TB, Russig H, Weiss IC, Gräff J, Linder N, Michalon A, et al. 2010. Epigenetic transmission of the impact of early stress across generations. *Biol Psychiatry* 68(5):408–415, PMID: 20673872, <https://doi.org/10.1016/j.biopsych.2010.05.036>.
- Gapp K, von Ziegler L, Tweedie-Cullen RY, Mansuy IM. 2014. Early life epigenetic programming and transmission of stress-induced traits in mammals: how and when can environmental factors influence traits and their transgenerational inheritance? *Bioessays* 36(5):491–502, PMID: 24585414, <https://doi.org/10.1002/bies.201300116>.
- Geier DA, Kern JK, Geier MR. 2018. A cross-sectional study of the relationship between blood lead levels and reported attention deficit disorder: an assessment of the economic impact on the United States. *Metab Brain Dis* 33(1):201–208, PMID: 29134344, <https://doi.org/10.1007/s11011-017-0146-6>.
- Guillin O, Griffon N, Diaz J, Le Foll B, Bezard E, Gross C, et al. 2004. Brain-derived neurotrophic factor and the plasticity of the mesolimbic dopamine pathway. *Int Rev Neurobiol* 59:425–444, PMID: 15006497, [https://doi.org/10.1016/S0074-7742\(04\)59016-5](https://doi.org/10.1016/S0074-7742(04)59016-5).
- Gulson B, Taylor A, Eisman J. 2016. Bone remodeling during pregnancy and postpartum assessed by metal lead levels and isotopic concentrations. *Bone* 89:40–51, PMID: 27233973, <https://doi.org/10.1016/j.bone.2016.05.005>.
- Gutteling BM, de Weerth C, Zandbelt N, Mulder EJH, Visser GHA, Buitelaar JK. 2006. Does maternal prenatal stress adversely affect the child's learning and memory at age six? *J Abnorm Child Psychol* 34(6):787–796, PMID: 17063407, <https://doi.org/10.1007/s10802-006-9054-7>.
- Hernaus D, Quaedflieg CWEM, Offermann JS, Casales Santa MM, van Amelsvoort T. 2018. Neuroendocrine stress responses predict catecholamine-dependent working memory-related dorsolateral prefrontal cortex activity. *Soc Cogn Affect Neurosci* 13(1):114–123, PMID: 29087511, <https://doi.org/10.1093/scan/nsx122>.
- Hernberg S. 2000. Lead poisoning in a historical perspective. *Am J Ind Med* 38(3):244–254, PMID: 10940962, [https://doi.org/10.1002/1097-0274\(200009\)38:3<244::aid-ajim3>3.0.co;2-f](https://doi.org/10.1002/1097-0274(200009)38:3<244::aid-ajim3>3.0.co;2-f).
- Hogg S. 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol Biochem Behav* 54(1):21–30, PMID: 8728535, [https://doi.org/10.1016/0091-3057\(95\)02126-4](https://doi.org/10.1016/0091-3057(95)02126-4).

- Hove MJ, Gravel N, Spencer RMC, Valera EM. 2017. Finger tapping and pre-attentive sensorimotor timing in adults with ADHD. *Exp Brain Res* 235(12):3663–3672, PMID: 28913612, <https://doi.org/10.1007/s00221-017-5089-y>.
- Jablónka E, Raz G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Q Rev Biol* 84(2):131–176, PMID: 19606595, <https://doi.org/10.1086/598822>.
- Ji Y, Hong X, Wang G, Chatterjee N, Riley AW, Lee L-C, et al. 2018. A prospective birth cohort study on early childhood lead levels and attention deficit hyperactivity disorder: new insight on sex differences. *J Pediatr* 199:124–131.e8, PMID: 29752174, <https://doi.org/10.1016/j.jpeds.2018.03.076>.
- Jung Y-H, Jang JH, Lee D, Choi Y, Choi S-H, Kang D-H. 2019. Relationships between catecholamine levels and stress or intelligence. *Neurochem Res* 44(5):1192–1200, PMID: 30887217, <https://doi.org/10.1007/s11064-019-02762-z>.
- Jusko TA, Henderson CR, Lanphear BP, Cory-Slechta DA, Parsons PJ, Canfield RL. 2008. Blood lead concentrations < 10 µg/dL and child intelligence at 6 years of age. *Environ Health Perspect* 116(2):243–248, PMID: 18288325, <https://doi.org/10.1289/ehp.10424>.
- Kiss D, Ambeskovic M, Montina T, Metz GA. 2016. Stress transgenerationally programs metabolic pathways linked to altered mental health. *Cell Mol Life Sci* 33(23):4547–4557, PMID: 27188285, <https://doi.org/10.1007/s00118-016-2272-4>.
- Korte SM, Koolhaas JM, Wingfield JC, McEwen BS. 2005. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci Biobehav Rev* 29(1):3–38, PMID: 15652252, <https://doi.org/10.1016/j.neubiorev.2004.08.009>.
- Kowiański P, Lietzau G, Czuba E, Waśkow M, Steliga A, Moryś J. 2018. BDNF: a key factor with multipotent impact on brain signaling and synaptic plasticity. *Cell Mol Neurobiol* 38(3):579–593, PMID: 28623429, <https://doi.org/10.1007/s10571-017-0510-4>.
- Kundakovic M, Gudsnek K, Herbstman JB, Tang D, Perera FP, Champagne FA. 2015. DNA methylation of BDNF as a biomarker of early-life adversity. *Proc Natl Acad Sci USA* 112(22):6807–6813, PMID: 25385582, <https://doi.org/10.1073/pnas.1408355111>.
- Lamb YN, Thompson JMD, Murphy R, Wall C, Kirk IJ, Morgan AR, et al. 2014. Perceived stress during pregnancy and the catechol-O-methyltransferase (*COMT*) rs165599 polymorphism impacts on childhood IQ. *Cognition* 132(3):461–470, PMID: 24955500, <https://doi.org/10.1016/j.cognition.2014.05.009>.
- Lambrechts A, Falter-Wagner CM, van Wassenhove V. 2018. Diminished neural resources allocation to time processing in autism spectrum disorders. *Neuroimage Clin* 17:124–136, PMID: 29085774, <https://doi.org/10.1016/j.nicl.2017.09.023>.
- Lanphear BP, Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, et al. 2005. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. *Environ Health Perspect* 113(7):894–899, PMID: 16002379, <https://doi.org/10.1289/ehp.7688>.
- Lefmann T, Combs-Orme T. 2014. Prenatal stress, poverty, and child outcomes. *Child Adolesc Soc Work J* 31(6):577–590, <https://doi.org/10.1007/s10560-014-0340-x>.
- Lieberman SA, Mashoodh R, Thompson RC, Dolinoy DC, Champagne FA. 2012. Concordance in hippocampal and fecal *Nr3c1* methylation is moderated by maternal behavior in the mouse. *Ecol Evol* 2(12):3123–3131, PMID: 23301177, <https://doi.org/10.1002/ece3.416>.
- López-Martín S, Albert J, Fernández-Jaén A, Carreté L. 2015. Emotional response inhibition in children with attention-deficit/hyperactivity disorder: neural and behavioural data. *Psychol Med* 45(10):2057–2071, PMID: 25708692, <https://doi.org/10.1017/S0033291714003195>.
- Martínez-Telléz RI, Hernández-Torres E, Gamboa C, Flores G. 2009. Prenatal stress alters spine density and dendritic length of nucleus accumbens and hippocampus neurons in rat offspring. *Synapse* 63(9):794–804, PMID: 19489049, <https://doi.org/10.1002/syn.20664>.
- McClure LF, Niles JK, Kaufman HW. 2016. Blood lead levels in young children: US, 2009–2015. *J Pediatr* 175:173–181, PMID: 27292707, <https://doi.org/10.1016/j.jpeds.2016.05.005>.
- McCreary JK, Truica LS, Friesen B, Yao Y, Olson DM, Kovalchuk I, et al. 2016. Altered brain morphology and functional connectivity reflect a vulnerable affective state after cumulative multigenerational stress in rats. *Neuroscience* 330:79–89, PMID: 27241944, <https://doi.org/10.1016/j.neuroscience.2016.05.046>.
- McEwen BS. 2010. Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Ann N Y Acad Sci* 1204(suppl):E38–E59, PMID: 20840167, <https://doi.org/10.1111/j.1749-6632.2010.05568.x>.
- Needleman HL, Gunnoe C, Leviton A, Reed R, Peresie H, Maher C, et al. 1979. Deficits in psychologic and classroom performance of children with elevated dentine lead levels. *N Engl J Med* 300(13):689–695, PMID: 763299, <https://doi.org/10.1056/NEJM197903293001301>.
- Needleman HL, Schell A, Bellinger D, Leviton A, Alired EN. 1990. The long-term effects of exposure to low doses of lead in childhood: an 11-year follow-up report. *N Engl J Med* 322(2):83–88, PMID: 2294437, <https://doi.org/10.1056/NEJM199001132202003>.
- Nigg JT, Knottnerus GM, Martel MM, Nikolas M, Cavanagh K, Karmaus W, et al. 2008. Low blood lead levels associated with clinically diagnosed attention-deficit/hyperactivity disorder and mediated by weak cognitive control. *Biol Psychiatry* 63(3):325–331, PMID: 17868654, <https://doi.org/10.1016/j.biopsych.2007.07.013>.
- Niwa M, Jaaro-Peled H, Tankou S, Seshadri S, Hikida T, Matsumoto Y, et al. 2013. Adolescent stress-induced epigenetic control of dopaminergic neurons via glucocorticoids. *Science* 339(6117):335–339, PMID: 23329051, <https://doi.org/10.1126/science.1226931>.
- Noorlander CW, De Graan PN, Middeldorp J, Van Beers JJ, Visser GH. 2006. Ontogeny of hippocampal corticosteroid receptors: effects of antenatal glucocorticoids in human and mouse. *J Comp Neurol* 499(6):924–932, PMID: 17072842, <https://doi.org/10.1002/cne.21162>.
- Pallarés ME, Antonelli MC. 2017. Prenatal stress and neurodevelopmental plasticity: relevance to psychopathology. *Adv Exp Med Biol* 1015:117–129, PMID: 29080024, https://doi.org/10.1007/978-3-319-62817-2_7.
- Peters A, McEwen BS, Friston K. 2017. Uncertainty and stress: why it causes diseases and how it is mastered by the brain. *Prog Neurobiol* 156:164–188, PMID: 28576664, <https://doi.org/10.1016/j.pneurobio.2017.05.004>.
- Pirkle JL, Brody DJ, Gunter EW, Kramer RA, Paschal DC, Flegal KM, et al. 1994. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *JAMA* 272(4):284–291, PMID: 8028141, <https://doi.org/10.1001/jama.1994.03520040046039>.
- Reuben A, Caspi A, Belsky DW, Broadbent J, Harrington H, Sugden K, et al. 2017. Association of childhood blood lead levels with cognitive function and socioeconomic status at age 38 years and with IQ change and socioeconomic mobility between childhood and adulthood. *JAMA* 317(12):1244–1251, PMID: 28350927, <https://doi.org/10.1001/jama.2017.1712>.
- Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. 2008. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 17(1):1–14, PMID: 17901045, <https://doi.org/10.1093/hmg/ddm280>.
- Rossi-George A, Virgolini MB, Weston D, Thiruchelvam M, Cory-Slechta DA. 2011. Interactions of lifetime lead exposure and stress: behavioral, neurochemical and HPA axis effects. *Neurotoxicology* 32(1):83–99, PMID: 20875452, <https://doi.org/10.1016/j.neuro.2010.09.004>.
- Rubia K, Halari R, Christakou A, Taylor E. 2009. Impulsiveness as a timing disturbance: neurocognitive abnormalities in attention-deficit hyperactivity disorder during temporal processes and normalization with methylphenidate. *Philos Trans R Soc Lond B Biol Sci* 364(1525):1919–1931, PMID: 19487194, <https://doi.org/10.1098/rstb.2009.0014>.
- Schneider JS, Anderson DW, Kidd SK, Sobolewski M, Cory-Slechta DA. 2016. Sex-dependent effects of lead and prenatal stress on post-translational histone modifications in frontal cortex and hippocampus in the early postnatal brain. *Neurotoxicology* 54:65–71, PMID: 27018513, <https://doi.org/10.1016/j.neuro.2016.03.016>.
- Schwabe L, Bohbot VD, Wolf OT. 2012. Prenatal stress changes learning strategies in adulthood. *Hippocampus* 22(11):2136–2143, PMID: 22605683, <https://doi.org/10.1002/hipo.22034>.
- Sen A, Heredia N, Senut MC, Land S, Hollocher K, Lu X, et al. 2015. Multigenerational epigenetic inheritance in humans: DNA methylation changes associated with maternal exposure to lead can be transmitted to the grandchildren. *Sci Rep* 5:14466, PMID: 26417717, <https://doi.org/10.1038/srep14466>.
- Seo MK, Ly NN, Lee CH, Cho HY, Choi CM, Lee JG, et al. 2016. Early life stress increases stress vulnerability through BDNF gene epigenetic changes in the rat hippocampus. *Neuropharmacology* 105:388–397, PMID: 26877199, <https://doi.org/10.1016/j.neuropharm.2016.02.009>.
- Skinner MK. 2014. Environmental stress and epigenetic transgenerational inheritance. *BMC Med* 12:153, PMID: 25259699, <https://doi.org/10.1186/s12916-014-0153-y>.
- Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9(7):671–675, PMID: 22930834, <https://doi.org/10.1038/nmeth.2089>.
- Sobolewski M, Conrad K, Marvin E, Allen JL, Cory-Slechta DA. 2018a. Endocrine active metals, prenatal stress and enhanced neurobehavioral disruption. *Horm Behav* 101:36–49, PMID: 29355495, <https://doi.org/10.1016/j.yhbeh.2018.01.004>.
- Sobolewski M, Varma G, Adams B, Anderson DW, Schneider JS, Cory-Slechta DA. 2018b. Developmental lead exposure and prenatal stress result in sex-specific reprogramming of adult stress physiology and epigenetic profiles in brain. *Toxicol Sci* 163(2):478–489, PMID: 29481626, <https://doi.org/10.1093/toxsci/kfy046>.
- Surkan PJ, Schnaas L, Wright RJ, Téllez-Rojo MM, Lamadrid-Figueroa H, Hu H, et al. 2008. Maternal self-esteem, exposure to lead, and child neurodevelopment. *Neurotoxicology* 29(2):278–285, PMID: 18261800, <https://doi.org/10.1016/j.neuro.2007.11.006>.
- Susiarjo M, Sasson I, Mesaros C, Bartolomei MS. 2013. Bisphenol A exposure disrupts genomic imprinting in the mouse. *PLoS Genet* 9(4):e1003401, PMID: 23593014, <https://doi.org/10.1371/journal.pgen.1003401>.

- Tamayo y Ortiz M, Téllez-Rojo MM, Trejo-Valdivia B, Schnaas L, Osorio-Valencia E, Coull B, et al. 2017. Maternal stress modifies the effect of exposure to lead during pregnancy and 24-month old children's neurodevelopment. *Environ Int* 98:191–197, PMID: 27865525, <https://doi.org/10.1016/j.envint.2016.11.005>.
- van Hulst BM, de Zeeuw P, Rijks Y, Neggers SFW, Durston S. 2017. What to expect and when to expect it: an fMRI study of expectancy in children with ADHD symptoms. *Eur Child Adolesc Psychiatry* 26(5):583–590, PMID: 27904952, <https://doi.org/10.1007/s00787-016-0921-7>.
- Varma G, Sobolewski M, Cory-Slechta DA, Schneider JS. 2017. Sex- and brain region-specific effects of prenatal stress and lead exposure on permissive and repressive post-translational histone modifications from embryonic development through adulthood. *Neurotoxicology* 62:207–217, PMID: 28712943, <https://doi.org/10.1016/j.neuro.2017.07.002>.
- Virgolini MB, Bauter MR, Weston DD, Cory-Slechta DA. 2006. Permanent alterations in stress responsivity in female offspring subjected to combined maternal lead exposure and/or stress. *Neurotoxicology* 27(1):11–21, PMID: 16140384, <https://doi.org/10.1016/j.neuro.2005.05.012>.
- Virgolini MB, Chen K, Weston DD, Bauter MR, Cory-Slechta DA. 2005. Interactions of chronic lead exposure and intermittent stress: consequences for brain catecholamine systems and associated behaviors and HPA axis function. *Toxicol Sci* 87(2):469–482, PMID: 16049266, <https://doi.org/10.1093/toxsci/kfi269>.
- Virgolini MB, Rossi-George A, Lisek R, Weston DD, Thiruchelvam M, Cory-Slechta DA. 2008a. CNS effects of developmental Pb exposure are enhanced by combined maternal and offspring stress. *Neurotoxicology* 29(5):812–827, PMID: 18440644, <https://doi.org/10.1016/j.neuro.2008.03.003>.
- Virgolini MB, Rossi-George A, Weston D, Cory-Slechta DA. 2008b. Influence of low level maternal Pb exposure and prenatal stress on offspring stress challenge responsivity. *Neurotoxicology* 29(6):928–939, PMID: 18951918, <https://doi.org/10.1016/j.neuro.2008.09.010>.
- von Bohlen und Halbach O, von Bohlen und Halbach V. 2018. BDNF effects on dendritic spine morphology and hippocampal function. *Cell Tissue Res* 373(3):729–741, PMID: 29450725, <https://doi.org/10.1007/s00441-017-2782-x>.
- Weiss IC, Franklin TB, Vizi S, Mansuy IM. 2011. Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Front Behav Neurosci* 5:3, PMID: 21331159, <https://doi.org/10.3389/fnbeh.2011.00003>.
- Weston HI, Weston DD, Allen JL, Cory-Slechta DA. 2014. Sex-dependent impacts of low-level lead exposure and prenatal stress on impulsive choice behavior and associated biochemical and neurochemical manifestations. *Neurotoxicology* 44:169–183, PMID: 25010656, <https://doi.org/10.1016/j.neuro.2014.06.013>.
- Zheng Y, Fan W, Zhang X, Dong E. 2016. Gestational stress induces depressive-like and anxiety-like phenotypes through epigenetic regulation of BDNF expression in offspring hippocampus. *Epigenetics* 11(2):150–162, PMID: 26890656, <https://doi.org/10.1080/15592294.2016.1146850>.
- Zhu P, Hao J-H, Tao R-X, Huang K, Jiang X-M, Zhu Y-D, et al. 2015. Sex-specific and time-dependent effects of prenatal stress on the early behavioral symptoms of ADHD: a longitudinal study in China. *Eur Child Adolesc Psychiatry* 24(9):1139–1147, PMID: 25791080, <https://doi.org/10.1007/s00787-015-0701-9>.
- Zizzari ZV, van Straalen NM, Eilers J. 2016. Transgenerational effects of nutrition are different for sons and daughters. *J Evol Biol* 29(7):1317–1327, PMID: 27018780, <https://doi.org/10.1111/jeb.12872>.