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Endocrine Active Metals, Prenatal Stress and Enhanced Neurobehavioral Disruption

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Abstract

Metals, including lead (Pb), methylmercury (MeHg) and arsenic (As), are long-known developmental neurotoxicants. More recently, environmental context has been recognized to modulate metals toxicity, including nutritional state and stress exposure. Modulation of metal toxicity by stress exposure can occur through shared targeting of endocrine systems, such as the hypothalamic-pituitary-adrenal axis (HPA). Our previous rodent research has identified that prenatal stress (PS) modulates neurotoxicity of two endocrine active metals (EAMs), Pb and MeHg, by altering HPA and CNS systems disrupting behavior. Here, we review this research and further test the hypothesis that prenatal stress modulates metals neurotoxicity by expanding to test the effect of developmental As±PS exposure. Serum corticosterone and behavior was assessed in offspring of dams exposed to As±PS. PS increased female offspring serum corticosterone at birth, while developmental As exposure decreased adult serum corticosterone in both sexes. As+PS induced reductions in locomotor activity in females and reduced response rates on a Fixed Interval schedule of reinforcement in males, with the latter suggesting unique learning deficits only in the combined exposure. As-exposed males showed increased time in the open arms of an elevated plus maze and decreased novel object recognition whereas females did not. These data further confirm the hypothesis that combined exposure to chemical (EAMs) and non-chemical (PS) stressors results in enhanced neurobehavioral toxicity. Given that humans are exposed to multiple environmental risk factors that alter endocrine function in development, such models are critical for risk assessment and public health protection, particularly for children.

Keywords

metals; lead; methylmercury; arsenic; prenatal stress; corticosterone; behavior

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Introduction

Studies examining the impact of environmental chemical exposures on endocrine systems typically focus on xenobiotics included in personal care or other consumer products, such as BPA and phthalates, as well as, brominated flame retardants, perchlorates etc. Far less consideration has been given to the fact that many metals are endocrine disruptors (Dyer, 2007; Iavicoli et al., 2009; Kortenkamp, 2010), despite the fact that exposure of children to heavy metals, including lead (Pb), arsenic (As) and mercury (Hg), remains an intractable public health problem. As seen in Flint, Michigan and other cities around the United States, children are continuously exposed to metals, particularly though drinking and food sources. Lead has been a chronic problem in the United States with many children presenting with blood leads higher than 5 μg/dl (DeWitt, 2017; Hanna-Attisha et al., 2016; Shah et al., 2017) and over a 100 million people are exposed to elevated levels of arsenic (greater than 10 μg/L) through drinking sources, including well water (Bommarito et al., 2017; Organization, 2004; Rager et al., 2017; Wasserman et al., 2004; Wasserman et al., 2016). A significant public health concern surrounding metal exposure relates to their potential to produce cognitive deficits (Canfield et al., 2004; Debes et al., 2016; Jeong et al., 2017; Lanphear et al., 2005; Wasserman et al., 2016). Pb exposure specifically is associated with reductions in IQ, learning, and attention deficits in human cohorts and these findings are paralleled in animal models, effects considered to derive from their actions on brain mesocorticolimbic circuits (i.e., prefrontal cortex, nucleus accumbens, hippocampus) (Canfield et al., 2003; Cohn et al., 1993; Jett et al., 1997; Lanphear et al., 2005; Schneider et al., 2016). As exposure has been associated with neurobehavioral disorders, including attention and cognitive function, with domains differing slightly between males and females (Rodriguez-Barranco et al., 2013; Rosado et al., 2007; Wasserman et al., 2004; Wasserman et al., 2016). MeHg exposure has been associated with neurodevelopmental delays, although co-occurring beneficial micronutrients including n-3 polyunsaturated fats (PUFAs) may modulate such effects (Cohen et al., 2005; Dzwilewski and Schantz, 2015; Myers and Davidson, 1998; Wang et al., 2014). The potential combinatorial effect of nutrients and MeHg exposure on cognition provides evidence that developmental environments may modulate metal neurotoxicity. In fact, it is becoming increasingly clear that environmental context may modulate the neurotoxic effects of a broad range of endocrine disrupting chemicals (Crews et al., 2003; Pottinger, 2003).

In the human environment, numerous risk factors exist with the potential to contribute to cognitive impairments in children, such as prenatal stress (PS), the effects of which may be more detrimental, in combination with metals exposure. The shared occurrence of risk factors are not equally distributed, as the highest blood Pb levels are often found in children of low socioeconomic status (SES) (Bellinger et al., 1988; Tsoi et al., 2016; White et al., 2016) and the same populations are repeatedly exposed to resource deprivation, both material and social, as well as dangerous neighborhood conditions, violence and racism (Keenan et al., 2007; Thayer and Kuzawa, 2014). In the context of human health, stress is a component of virtually every individual's life, which can be broadly viewed as psychosocial, environmental or physical challenges to which the body responds through activation of the hypothalamic-pituitary-adrenal (HPA) axis and production of hormonal and neurotransmitter

mediators. Via inputs to hippocampus and amygdala in brain, stressors activate the HPA axis, leading to release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus. These act on the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH), then triggering release of glucocorticoids from adrenal cortex (Fig. 1). Cortisol is the major human glucocorticoid and corticosterone plays the primary role for rodents. Via a negative feedback system, glucocorticoids act on pituitary, hypothalamus and hippocampal glucocorticoid receptors (GR) to terminate the HPA stress response. This chemical signaling produces a coordinated physiological response that restores homeostasis. HPA activation clearly conveys important developmental coordination and survival benefits to an organism. However, problems ensue in cases of allostatic overload or HPA axis dysregulation, including failure of the negative feedback, inadequate stimulation, or delayed recovery. Disruption of HPA axis function has been associated with a variety of human diseases and disorders (Denhardt, 2017; Juster et al., 2016; Korte et al., 2005; Lupien et al., 2009; McEwen, 2000; McEwen, 2017; Peters et al., 2017; Shonkoff et al., 2009; Stavrou et al., 2017). The cognitive deficits induced by exposure to endocrine active metals (EAMs) arise partially though disruptions in the HPA axis function and mesocorticolimbic (MESO) circuitry, both systems critical to mediation of rewarding properties of stimuli and to executive/cognitive functions (Figure 1). These two systems are interconnected. For example, products of HPA activation, such as corticosterone, increase dopamine signaling in the nucleus accumbens (Graff and Tsai, 2013), which in turn is critical for conditioning processes (Cory-Slechta et al., 1997a). Microinjection of the dopamine D2 receptor antagonist, sulpiride, into medial PFC of rats attenuates glucocorticoid-induced impairment of long-term memory retrieval (Pakdel and Rashidy-Pour, 2007). Cortisol administration to humans has been shown to downregulate activity of striatum in both reward and non-reward conditions (Montoya et al., 2014). Prenatal exposure to glucocorticoids during late gestation in rats changed the shape and volume of the midbrain dopamine cell bodies and size of dopaminergic neurons and astrocytes within these nuclei, and also altered their target innervation density and neurochemical transmitter functions, effects which were also profoundly sexually-dimorphic (Gillies et al., 2016). Indeed, gestational exposures to glucocorticoids have been reported to program brain dopamine circuitry (Rodrigues et al., 2011). Metals exposure disrupts behaviors mediated by brain mesocorticolimbic systems (Cory-Slechta et al., 1998; Cory-Slechta et al., 1997b; Cory-Slechta et al., 1996; Evans and Cory-Slechta, 2000); as both Pb and MeHg alter learning under a fixed interval (FI) schedule of food reward (Cory-Slechta et al., 1996; Virgolini et al., 2008a; Weston et al., 2014a). Pb, As, and MeHg have been shown to impact MESO dopamine function (Amos-Kroohs et al., 2016; Boomhower and Newland, 2017; Castoldi et al., 2006; Dreiem et al., 2009; Moreno Avila et al., 2016; Srivastava et al., 2016; Stansfield et al., 2015; Wu et al., 2017; Zuch et al., 1998).

Given the critical role of HPA and MESO systems in cognitive deficits associated with metals exposure, prenatal stress (PS) may modulate a broad range of metals neurotoxicity. In fact, numerous metals, including As, Hg, Au, and Cd, have mechanistically been shown to alter HPA physiology including direct action on glucocorticoid receptor binding or activity (Brkljacic et al., 2004; Elez et al., 2001; Makino et al., 1996; Spuches and Wilcox, 2008).

EAMs alter levels of steroid hormones along the HPA axis in human studies and animal models (Appleton et al., 2017; Barros et al., 2004; Berger et al., 2002; Bodwell et al., 2006; Braun et al., 2014; Caldwell et al., 2015a; Cory-Slechta et al., 1998; Cory-Slechta et al., 1999; Davey et al., 2007; Desaulniers et al., 2013; Haider et al., 2013; Martinez-Tellez et al., 2009; Rossi-George et al., 2011; Rothenberg et al., 2016; Souza-Talarico et al., 2017; Virgolini et al., 2008a). In fact, developmental exposure to EAMs, Pb, MeHg, and As, produce protracted, lifelong HPA axis dysregulation. For example, exposure to 8 mg/kg MeHg on GD15 increased corticosterone levels 4-fold in male rat offspring measured after 90 days of age (Carratu et al., 2008). In addition, 2-fold increases in corticosterone and in ACTH were found after only 5 ppb MeHg in drinking water exposures of adult male rats over an 8 week period (Ortega et al., 1997). Prenatal As exposure at 50 ppb decreased fetal glucocorticoid receptor (GR) concentrations and created a prolonged imbalance between 11β-HSD1 and 11β-HSD2 enzymes (Caldwell et al., 2015b; Martinez-Finley et al., 2009a; Martinez et al., 2008). Additional studies showed dysregulation of the HPA axis including increased corticotrophin-releasing factor, altered corticosterone, decreased 11β-HSD1 and altered GR protein distribution in the hypothalamus (Goggin et al., 2012). The overlap of HPA targeting of EAMs and PS establishes a framework for interaction effects (Table 1). Correspondingly, we have shown that Pb and stress exposures produce unique pathology, including cognitive behaviors, levels of brain neurotransmitters, responsivity to stress challenge, post-stress corticosterone reduction times, hypercortisolism (Cory-Slechta, 1990, 1993; Cory-Slechta et al., 2009; Rossi-George et al., 2009; Rossi-George et al., 2011; Virgolini et al., 2005; Virgolini et al., 2008a; Virgolini et al., 2008b; Weston et al., 2014b). Another study extended these interactions to include the neurotoxic metal, MeHg, and found MeHg+PS induced impairment of short-term memory in a novel object recognition paradigm and alterations in brain monoamine levels (Weston et al., 2014a). EAM and stress exposures are widespread in human populations, which underscores the need to consider endocrine disruption through a multiple hits and/or mixtures framework (Cory-Slechta, 2005).

The generalizability of the hypothesis that PS can modulate EAM behavioral toxicity is an essential question. Premised on its known effects on both the HPA axis and brain dopamine function as cited above, we sought to extend these studies to another EAM with potential for developmental neurotoxicity, namely Arsenic (As). Given our prior observations and reports that interactions between stress/endocrine and mesocorticolimbic systems in development alter behavioral functions in a sex-specific manner, we will assess each endpoint in a sex-specific manner (Goel and Bale, 2009; McCarthy et al., 2009a, b; Morgan and Bale, 2017). For this purpose, comparative outcome measures were used, including the FI schedule of reinforcement and locomotor activity, as well as other behaviors mediated by MESO neurotransmitter function. Serum corticosterone levels at birth and in adulthood were measured to examine HPA axis consequences of combined As and PS exposure during development, i.e., endpoints have been previously identified to be sensitive to developmental Pb and MeHg (Table 1). Understanding the interactions between environmental exposures, including EAMs and non-chemical stressors, improves our simulation of human environmental conditions and enhances our ability to translate results to human public health

protection, which may be especially critical for developmental neurotoxicants that target the endocrine system.

Materials And Methods

Animals and Arsenic Exposure

Four-week-old female C57/Bl6 mice from Jackson Laboratories (Bar Harbor, ME) were randomly assigned to receive distilled deionized water drinking solutions of 0 or 50 ppb sodium arsenate for 2 weeks prior to breeding which continued until pup birth, chosen as an environmentally relevant dose given human exposure data and previous used in developmental rodent studies (Martinez-Finley et al., 2009b; Wasserman et al., 2016). All offspring were housed in a vivarium room maintained at 22±2°C with a 12-h light-dark cycle (lights on at 0700h). LabDiet Autoclavable Rodent Diet 5010 (St. Louis, MO) was provided ad libitum. All experiments were carried out according to NIH Guidelines and were approved by the University of Rochester Medical School University Committee on Animal Resources.

Breeding and Prenatal Stress

Female rodents were mated with males (1:1) for 4 to 5 days to cover the duration of an estrous cycle. Vaginal plugs were assessed daily from 7 to 9 am and initial presence of plug was deemed gestational day (GD) 1. Half of the dams in the As and water exposed groups were randomly selected to be subjected to resource deprivation stress beginning on GD5, to coincide with embryonic implantation, as models of resource deprivation have been shown effective for pregnant mice (Rice et al., 2008). This deprivation stressor involved access to a preferred treat, mealworms, for 5 days and then being deprived of the treat, while the resource-enriched mice received a mealworm daily. Females were individually housed for the duration of gestation and lactation, but dams were housed in adjacent cages. These cages were separated by wire mesh, within the pairs of dams only one dam received mealworms (non-stress), while the other dam (deprived) could smell, see, etc. but never access mealworms (deprivation stress). This yielded 4 treatment groups of offspring per sex: 0-NS (no arsenic, no PS), 0-PS (no arsenic, PS), 50-NS (As only) and 50-PS (As+PS) with no more than 1 pup/sex/dam to preclude litter specific effects.

Offspring Procedures

Following parturition, designated postnatal day 0 (PND0), litter size, sex ratios and whole litter body weights were recorded. Pups were weighed and weaned at PND25, separated by sex and group housed by sex and treatment group. From weaning, pups were provided with unrestricted access to the same rodent chow and to water (0 ppm As) regardless of prenatal treatment conditions.

Serum Corticosterone Determinations

Approximately 200 μl of blood was collected from into pre-chilled tubes that were processed to serum by centrifugation at 3500 rpm for 20 min. Serum corticosterone was measured in duplicate using the commercially available and well-characterized enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA).

Behavioral Battery

Offspring behavioral testing was initiated at 90 days of age, and included locomotor behavior, novel object recognition, elevated plus maze, and fixed interval (FI) schedule-controlled behavior. An additional set of offspring was retained without behavioral testing over this same time frame, as our prior studies demonstrate that behavioral experience can significantly alter the impacts of Pb and PS on brain (Cory-Slechta et al., 2013; Cory-Slechta et al., 2009). Brains were harvested from both behaviorally- and non-behaviorally tested offspring at the completion of behavioral testing. For all outcome measures, a single pup/sex/treatment group/dam was used to preclude litter specific effects.

Locomotor Activity—Locomotor activity was assessed in automated chambers equipped with 48-channel infrared photobeams (Med Associates, Inc, St Albans, Vermont). Photobeam breaks were recorded every 5 minutes for 60 min to assess horizontal, vertical, and ambulatory movements for a total of 12 blocks. Ambulatory counts were defined as the number of beam breaks while in ambulatory movement that broke at least 3 successive photobeams. Vertical activity was defined as movement that broke photobeams placed in the z-axis. Horizontal movement was defined as number of photobeam breaks in a 2 × 2 beam box that were non-ambulatory. Resting time was defined as time spent with no new photobeam breaks. Stereotypic time and stereotypic time measure activity within a defined space in the arena.

Fixed Interval Schedule of Control Behavior (FI)—Following the locomotor activity assessment, mice were reduced to approximately 85% of free fed weight to provide motivation for food-reinforced operant responding and maintained at those weights for the duration of the experiment by scheduled feeding. Behavioral testing was conducted in operant chambers (Med Associates, St. Albans, VT) housed in sound-attenuating cabinets equipped with white noise and fans for ventilation. Three levers were located horizontally across the back wall of the chamber, with a pellet dispenser for reinforcer delivery on the front (opposite) wall. Mice were initially trained on a variable time 60s fixed ratio 1 schedule (VT60FR1) in which a reinforcer (20 mg food pellet) was delivered on average every 60s regardless of level-presses along with a light and sound cue; a lever press during that time also triggered the light and sound cue along with delivery of a reinforcer. Following 10 correct lever press responses or 20 min on the VT60 sec component, whichever occurred first, the schedule shifted to a fixed ratio 1 schedule in which a lever press on the designated correct lever was required for each food delivery until subjects earned 50 reinforcers.

After each animal was trained to the lever-press criterion under the VT60FR1 schedule, the schedule was shifted to a 60s FI schedule (FI60) carried out in 30-minute behavioral test sessions over a total of 35 sessions (30 consecutive fixed intervals / session / day) to assess acquisition of behavior prototypical for an FI schedule (i.e., learning). On the FI schedule, the first lever press response on the designated lever after completion of a 60s interval produced food delivery and initiated the next 60 sec interval, until 30 minutes had elapsed. Responses during the interval had no scheduled consequence. Measures of FI performance presented here included overall response rate (total responses /total session time), post-

reinforcement pause (amount of time between food reward delivery and the first lever press response in the next 60 sec interval), run rate (rate of responding within an interval after post-reinforcement pause time has been subtracted out) and inter-response time (median time-lapsed between FI responses). These measures allow assessments of both the rate and pattern of responding and the acquisition of temporal control behavior by the 60-sec requirement.

Novel Object Recognition (NOR)—NOR testing consisted of two phases and was conducted in an open plexiglass arena (dimensions: 30.5 cm × 30.5 cm × 30.5 cm). In the first session, mice were placed for 10 min in the arena which contained two objects, during this time, side preference, exploration time, and patterns of exploration among treatment groups were measured. In the second session, occurring 24 h after session 1, mice were returned for 5 min to the arena, in which a novel object had now replaced one of the previous two objects. The second session of the NOR paradigm assessed short-term memory, premised on an animal's awareness of novelty and its memory of already familiar objects. Placement of the novel object was counterbalanced across treatment conditions to preclude bias. All sessions were videotaped and scored by a reviewer blinded to treatment group. Exploration was defined as a mouse oriented toward with nose touching or sniffing the object (Antunes and Biala, 2012). A recognition index was calculated based on the time spent with novel object compared to the familiar object (time spent with novel object/(time spent with novel object + time spent with familiar object). Time per approach was calculated by the average time spent per bout of investigation for either the novel or familiar object. Sessions were video recorded and videos were scored by a blinded observer using Observer XT 13.0 (Noldus).

Elevated Plus Maze (EPM) is a well characterized assay that measures time spent in closed vs. open (non-preferred) arms to evaluate fear behavior associated with spending time in an open area, as a proxy for ‘anxiety-like’ behavior in humans (Komada et al., 2008; Pellow et al., 1985). Mice were placed on an apparatus that included open (with no sides) and closed (with sides) platforms for 5 minutes with entries, into and time spent in the open vs. closed arms measured. Sessions were video recorded and videos were scored by a blinded observer using Observer XT 13.0 (Noldus).

Statistical Analyses: Given that sexes characteristically maintain different hormone profiles and our previous data indicated robust sex specific differences in behavioral responses to both Pb and MeHg with PS, it was critical that As exposure be considered with sex as a factor. As a result, all analyses were first carried out using three-factor ANOVA, with As, PS and sex as between group factors. If sex or sex*TX were trending significant ($p < 0.05$) or trending significance ($p < 0.1$), two-factor ANOVA, with As and PS as between group factors, were conducted separately by sex. Post-hoc tests were conducted as appropriate dependent upon main effect or interaction outcomes. Behavioral performances across sessions, including locomotor behavior and FI schedule were analyzed using linear models with session, As and PS included in a three-factorial design with nested session and subject terms set as a random effect. Locomotor and FI behavioral analyses were carried out separately by sex based on its clear differentiation of sex-specific effects in our prior studies

(Allen et al., 2014; Cory-Slechta et al., 2013; Sobolewski et al., 2014; Weston et al., 2014a; Weston et al., 2014b). For FI behavior, sessions 15 – 35 were analyzed as these sessions represent learning following the early acquisition phase. In all cases, post-hoc assessments were carried out based on main effects or interactions using ANOVAs or Fisher's least significant differences tests. Outliers were removed following a statistically significant Grubb's test (Graphpad Software Inc.). Statistical analyses were conducted using JMP Pro 13.0 (SAS Institute Inc., Cary, N.C.). P values < 0.05 were considered statistically significant, while near significant values (p values < 0.10) are also reported.

Results

Pregnancy Biometrics

There were no significant differences in the number of viable litters, with the 0-NS group losing 1 out of 15 litters, 0-PS losing 4 out of 15 litters, 50-NS losing 3 out of 14 litters and 50-PS losing 1 out of 14 litters. There were also no significant differences in litter size, sex ratios or litter weights (data not shown).

Serum Corticosterone Concentrations

Alterations in serum corticosterone concentrations in offspring were found at birth and in adulthood (Figure 2A). PS exposure increased serum corticosterone levels at birth ($F = 7.99$, $p < 0.01$). Given that the effect of sex was marginally significant in the overall ANOVA ($F = 3.81$, $p = 0.059$), corticosterone analyses were conducted separately by sex. At PND0, female, but not male pups showed a significant PS-induced increase in corticosterone ($F = 5.1$, $p = 0.037$, Figure 2B **left and 2B right**). At PND60 (Figure 2A **right**), As exposure was found to have significantly decreased baseline corticosterone in treated animals ($F = 4.6$, $p = 0.039$), an effect that did not indicate a significant effect of sex ($F = 0.4$, $p = 0.51$).

Locomotor Behavior

Developmental As and PS exposure altered adult locomotor behavior in males and females (Figure 3). In females (Figure 3A), ambulatory time exhibited a significant As*PS interaction ($F = 4.06$, p 0.04), as only As+PS treated animals showed decreases of approximately 8-18% relative to controls ($t = -4.79$, $p < 0.001$). Similarly, stereotypic time showed a significant As*PS interaction ($F = 4.5$, $p = 0.03$), again with As+PS decreasing stereotypic time compared to controls ($t = -3.36$, $p < 0.001$). Equivalently, only As+PS increased resting time compared to control (As*PS interaction, $F = 6.4$, $p = 0.01$, with ($t = 3.5$, $p < 0.001$). Vertical time was increased by As exposure alone ($F = 17.4$, $p < 0.001$), while As*Block interactions were found for jump time suggesting a steeper slope across the locomotor session for As-exposed females ($F = 4.5$, $p = 0.04$).

For males (Figure 3B), significant As*PS interactions were observed for jump time ($F = 21.8$, $p < 0.001$), ambulatory time ($F = 12.8$, $p < 0.001$), and resting time ($F = 21.4$, $p < 0.001$), which post-hoc testing revealed to be due to significant effects of As and/or PS, but not a significant difference between combined As+PS and controls, suggesting an antagonistic effect of As+PS on these behaviors. PS alone increased vertical time ($F = 14.5$, $p < 0.001$). There were also treatment-related differences in habituation over time, as

ambulatory time showed a significant PS*Block effect ($F = 15.0$, $p < 0.001$), stereotypic time showed a significant As*Block effect ($F = 6.1$, $p = 0.02$), and resting time showed significant As*Block ($F = 5.9$, $p = 0.01$) and PS*Block ($F = 12.4$, $p < 0.001$) effect.

Fixed Interval Schedule of Reinforcement

Both As and PS alone significantly reduced FI overall response rates in female offspring (Figure 4A, As: $F = 29.6$, $p < 0.001$ and PS: $F = 34.9$, $p < 0.001$), with decreases in overall further rate eventually further reduced by combined As+PS, while FI overall rate reductions were also seen in males (Figure 4B) but only in response to combined As and PS (As*PS interaction; $F = 22.8$, $p < 0.001$), with post hoc analyses confirming significantly lower overall response rates in the As+PS group compared to all other treatment groups (0-PS vs. 50-PS: $t = -8.9$, $p < .001$; 0-NS vs. 50-PS: $t = -7.7$, $p < .001$; 50-NS vs. 50-PS: $t = -5.1$, $p < 0.001$). Changes in run rates showed similar patterns, with females showing reductions in response to both As and PS (As: $F = 12.8$, $p < 0.001$; PS: $F = 42.4$, $p < 0.001$), and males showing a synergistic interaction between As and PS decreasing run rates (As*PS: $F = 42.2$, $p < 0.001$). As and PS both significantly increased post-reinforcement pause times of females (As: $F = 33.7$, $p < 0.001$; PS: $F = 5.4$, $p = 0.02$), while a significant As*PS interaction characterized interresponse times (As*PS: $F = 8.9$, $p = 0.003$), as the combined As+PS group had the longest inter-response times (0-PS vs. 50-PS: $t = 2.7$, $p = .006$; 0-NS vs. 50-PS: $t = 3.6$, $p < .001$; 50-NS vs. 50-PS: $t = 4.8$, $p < 0.001$). In males, As alone increased post-reinforcement pause times ($F = 102$, $p < 0.001$), and PS marginally increased post-reinforcement pause times ($F = 2.0$, $p = 0.1$).

Novel Object Recognition

There were no significant differences during session 1 (the habituation phase) of the NOR paradigm for total numbers or duration of contacts with objects or mean bout length (data not shown). For the recognition phase (session 2), a recognition index (RI) was calculated for each animal for total approaches (total approaches to the novel object/ (total approaches to the novel + total approaches to the non-novel) *100) and correspondingly for total duration of approaches. For the three-factor ANOVA, there was a trend towards a significant main effect of As ($F = 3.3$, $p = 0.07$), however there was also a trend towards a sex*As*PS interaction ($F – 3.1$, $p = 0.08$). Subsequent two-factor ANOVA confirmed a significant reduction in RI following As in males (Figure 5: $F = 2.15$, $p = 0.04$), whereas females showed no significant difference (data not shown). This decrease in RI was driven by a marginal increase in total time spent with the familiar object (Figure 5: $F = 3.8$, $p = 0.06$).

Elevated Plus Maze

For EPM testing (Figure 6), total durations of time spent in the closed arms, open arms, and center of the maze were determined. In the three-factor ANOVA, there were no significant effects for time spent in the center of the maze or in closed arms (data not shown). However, both sex and As had a significant effect on time spent in the open arms. Males (Figure 6, **right**) spent significantly more time in the open arms than females ($F = 4.3$, $p = 0.04$). As-exposed animals spend more time in the open arms, as well ($F = 5.9$, $p = 0.02$). Given that sex was a significant factor, these data were also analyzed separately by sex. These ANOVAs

revealed that females (Figure 6, **left**) did not show a significant effect of As ($F = 1.9$, $p = 0.17$) despite similar trends, while As exposed males showed a significant increase in time spent in the open arms ($F = 4.0$, $p = 0.05$). The two-factor analysis also revealed a trend of PS-exposed males to spend more time in the center of the maze ($F = 3.2$, $p = 0.08$).

Discussion

Early CNS development has been shown to be a critical window for life-long reprogramming of behavior and cognition, consistent with developmental origins of adult disease hypothesis (Bale and Vale, 2004; Barker and Osmond, 1986; Heindel et al., 2015; Wadhwa et al., 2009). Previously, developmental exposure to EAMs, such as Pb and MeHg, has been shown to alter adult HPA axis physiology, MESO neurotransmitter concentrations and behavioral function (Cory-Slechta, 1997; Cory-Slechta et al., 1998; Cory-Slechta et al., 1996; Rossi-George et al., 2009; Weston et al., 2014a; Zuch et al., 1998). Furthermore, the neurodevelopmental effects of EAMs have been shown to be modulated by environmental stressors, including prenatal maternal stress (Cory-Slechta et al., 2010; Weston et al., 2014a). Here, we further test the hypothesis PS modulates EAM neurotoxicity by demonstrating similar consequences for combined developmental exposure to As in combination with PS (Table 1). As we previously observed with Pb+PS and MeHg+PS, the effects of As could be enhanced under conditions of PS treatment, with sex-dependent consequences. These data support the hypothesis that a broad range of EAMs (Pb, MeHg, and As) may show enhanced toxicity in combination with other environmental risk factors, such as early life stress. Importantly, maternal exposure to As±PS did not result in overt toxicity with respect to pregnancy and litter-related outcomes, as there were no differences in litter viability, pup numbers, sex ratios, or litter weights.

Developmental As and PS altered circulating serum glucocorticoid concentrations at birth and into adulthood. PS increased corticosterone levels of females but not males at PND0, reaching nearly adult concentrations at this time point, while As decreased corticosterone in both sexes in adulthood. These data indicate sex-specific glucocorticoid exposure at birth, with decreases following As exposure in both sexes in adulthood. One suggested mechanism for the sex-specific effects of PS relates to placental physiology, including glucocorticoid transmission differences dependent on the sex of the fetus (Montano et al., 1993). Previous research has demonstrated differences in serum corticosterone by sex, related to greater transport of corticosterone across placenta of female as compared to male fetuses. Additionally, the placental response to metals exposure, including As, has been shown to vary with the sex of the fetus (Bommarito et al., 2017; Rager et al., 2017; Winterbottom et al., 2015; Winterbottom et al., 2017). Future research should focus on mechanisms of early sex-specific variation in placental biology, as well as physiological differences in development and metabolism in offspring.

In accordance with the hypothesis proposed for this study, differential responses to As+PS were seen by sex and behavioral function, as summarized in Table 1. Both sexes exhibited behavioral impairments, but the patterns of effects differed by sex. Of particular note are behavioral deficits of As that were only observed under conditions of AS+PS exposure. For example, in males, neither As nor PS alone had any impact on FI schedule-controlled

behavior, only the combined exposures, which resulted in FI overall response rate decreases ranging from approximately 25-45%. In females, only combined exposures to As+PS altered locomotor activity, including decreased ambulatory time and stereotypic time, while increasing resting time, consistent with a reduction in activity levels.

The FI schedule of reinforcement generates highly prototypical behavioral patterns that are seen across a wide variety of species from humans to rodents (Kelleher and Morse, 1968). As subjects learn the temporal length of the interval preceding reinforcement availability, a characteristic pause emerges at the beginning of the interval followed by a gradual increase in rate of response. This pattern of responding, that attains maximal levels at the completion of the interval when reinforcement is available, results in high rates of responding at the interval terminus and ensure reinforcement delivery is nearly immediate following the lapse of the interval. Both males and females exhibited reductions in FI overall response rates following developmental As+PS, with decrements in males ranging from 25-45% under conditions of As+PS. These decreases in overall response rate were produced by reductions in run rate and corresponding increases in inter-response times, as well as by elevations in post-reinforcement pause time. Collectively, this indicated that As+PS resulted in longer pauses before responding within the interval was initiated, and lower rates of responding once initiated. Such findings could suggest a combination of motivational deficits and altered timing behavior. To date, no other studies have examined the impacts of As on FI schedule-controlled behavior, although other studies have reported learning impairments can occur in response to As+PS (Benoit et al, 2015; Guan et al., 2017; Negron-Oyarzo et al., 2015; Pandey et al., 2017; Sun et al., 2017; Tyler and Allan, 2014; Wang et al., 2016).

In at least one context, male mice might be considered more susceptible to As+PS in relation to the number of behavioral functions altered by these exposures (Table 1). In addition to alterations in FI schedule controlled behavior and in locomotor activity shown by both sexes, As-exposed male pups showed unique deficits on EPM and NOR. As males spent more time in the open arms during EPM testing, suggesting decreased fear-mediate or historically described as ‘anxiety-like’ behavior' (Carobrez and Bertoglio, 2005). This trend was also seen in females, but was not significant in analyses separated by sex. There was an additional trend for PS-exposed males to exhibit increased total time spent in the center of the maze, suggesting increased freezing behavior at the beginning of each trial. As-exposed males also showed decreased novel object recognition, suggesting memory deficits into adulthood (Antunes and Biala, 2012). As-induced changes in NOR have been previously reported (Martinez-Finley et al., 2009a), although it is not indicated whether these effects differed by sex. In contrast to our findings with elevated plus maze in which As increased the time spent in the open arms and thus could be considered ‘anxiogenic’, others have reported anxietylike phenotypes (Aktar et al., 2017; Chang et al., 2015), whereas in another study, anxiety and anti-anxiogenic effects were observed depending upon As exposure level (Umezu et al., 2012). Taken together, our work indicates that prenatal stress can modulate the developmental neurotoxicity of multiple metals including Pb, MeHg, and now As (Cory-Slechta et al., 2010; Cory-Slechta et al., 2004; Rossi-George et al., 2009; Rossi-George et al., 2011; Weston et al., 2014a; Weston et al., 2014b). These findings suggest broad potential for cumulative neurotoxicity of multiple risk factors for cognitive development (Appleton et al., 2017; Barros et al., 2004; Berger et al., 2002; Bodwell et al., 2006; Braun et al., 2014;

Caldwell et al., 2015a; Cory-Slechta et al., 1998; Cory-Slechta et al., 1999; Davey et al., 2007; Desaulniers et al., 2013; Haider et al., 2013; Martinez-Tellez et al., 2009; Rossi-George et al., 2011; Rothenberg et al., 2016; Souza-Talarico et al., 2017; Virgolini et al., 2008a).

The ability for environmental stressors to modulate neurotoxic EAMs has several implications. First, it may be that at higher doses As itself would alter these behavioral baselines, but this is complicated by the fact that many endocrine active chemicals have non-monotonic dose response curves. Our prior studies indicate that in males developmental Pb exposure effects circulating glucocorticoids in a non-monotonic dose-response, with low doses decreasing serum concentrations and high doses increasing corticosterone levels (Table 1). Increasingly, research identifies non-monotonic consequences of EAM and other endocrine active compound exposures (Vandenberg et al., 2012). Notable examples include, the non-monotonic influence of As exposure on glucocorticoid receptor function (Bodwell et al., 2006; Bodwell et al., 2004) and Pb effects on long-term potentiation of the excitatory postsynaptic potential and population spikes in the hippocampus and Ca+ dependent glutamate release both show biphasic dose-response relationships with Pb (White et al., 2007). Neurotransmitter functions (catecholamine concentrations within MESO brain regions) display non-monotonic responses to Pb and MeHg exposure (Weston et al., 2014a). Considered in the context of environmental modulation of metals toxicity, these dose-response relationships may vary in different contexts: stress exposure, vitamin deficiency, iron-deficiency, etc. In the absence of a relevant context, we may not be able to estimate true neurotoxicity with respect to complex human environmental conditions.

These data indicate that to appropriately translate our research to human populations, studies should consider frequent co-occurring risk factors that share biological targets. As the human environment is comprised of mixtures, understanding how these insults combine to inhibit an organism's ability to maintain homeostasis is critical to estimate risk of exposure to endocrine active chemicals (McEwen, 2017). However, the plethora of possible endocrine modulating xenobiotic and non-xenobiotic exposures encountered by developing organisms and the vast number of endocrine active compounds necessitates narrowing of targets for combinatory research. This narrowing can begin by using defined criteria for inclusion, some of which are suggested as follows. First, risk factors should be broadly co- or sequentially occurring in vulnerable populations, such as children. In the case of EAMs, early Pb exposure and poverty often occur simultaneously, requiring research focused on developmental Pb and PS exposures. Future research should consider unique sources and routes of exposure of metals exposure (and metal mixtures exposure) such as air pollution also associated with developmental behavior toxicity (Allen et al., 2015; Klocke et al., 2017). A second and critical criterion is that risk factors should also share common downstream biological targets. One suggested alternative example would be EAMs in the context of iron-deficiency in pregnant women. Pregnant women are prone to iron-deficiency and heavy metals such as Pb and As mimic essential metals, such as manganese and iron, and can be transported directly into the cell and across biological barriers through metal transporters such as the divalent cation transporter-1 (DCT1) (Ballatori, 2002). Metals species such as lead, mercury, and arsenic have all been showed to alter neurodevelopment by co-opting endogenous metal mechanisms, including Ca+ signaling, Zn+ binding sites,

and Fe+ mediated cellular maturation. For example, metal toxicity can occur through interference with protein metal-binding sites, such as zinc finger protein mediated transcription, although these vary based on protein-specific metal ion sensitivity (Asmuss et al., 2000a; Asmuss et al., 2000b; Basha et al., 2003; Razmiafshari et al., 2001; Razmiafshari and Zawia, 2000; Zawia et al., 2000; Zhou et al., 2015). One additional potential mechanism for convergence is epigenetic reprogramming, as epigenetic profiles, like HPA axis modulation, is generally responsive to environmental fluctuations. In fact, research on developmental Pb and PS indicate that combined exposures can uniquely alter epigenetic profiles across the lifespan (Anderson et al., 2015; JS and Cory-Slechta, 2016; Schneider et al., 2016; Schneider and Cory-Slechta, 2016; Varma et al., 2017). Future research on PS and EAMs should identify potential cross-talk between these molecular modes of action and endocrine interactions with CNS functions to identify critical pathways for behavioral development (Weiss, 2012).

Taken together, this research highlights the need to study EAMs exposure within a relevant environmental context to best translate our findings to human relevant conditions. Our findings support the hypothesis that early environments can modulate the neurobehavioral toxicity of metals exposures. Additionally, these outcomes are highly sex-specific supporting the importance of the NIH request to conduct toxicity testing with consideration of sex-differences.

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Highlights

1. Prenatal stress (PS) can modulate tde neurotoxicity of endocrine active metals.
2. PS enhanced developmental toxicity of arsenic (As) on behavior in adultdood.
3. Developmental PS and EAMs exposures alter serum corticosterone.
4. Tde developmental effects of endocrine active metals are often sex-specific.
5. Early environments may enhance neurotoxicity endocrine active compounds.

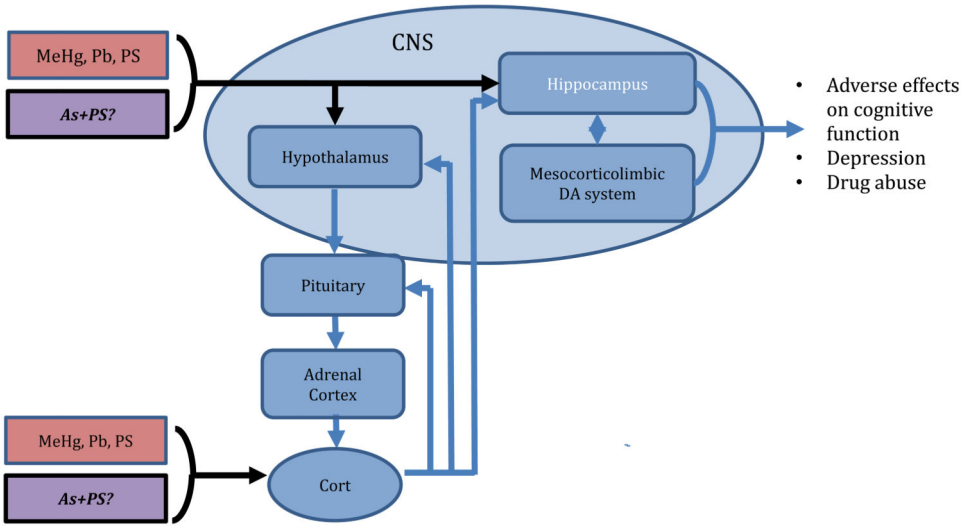


Figure 1.

Both Pb and MeHg and Stress activate the HPA axis and act on brain hippocampal and mesocorticolimbic dopamine/glutamate systems. Thus, they share multiple common biological substrates, and effects of Pb are enhanced by stress. ***Does As also interact with PS to alter the HPA axis, act on hippocampal and mesocorticolimbic systems with similar effects on behavior***?

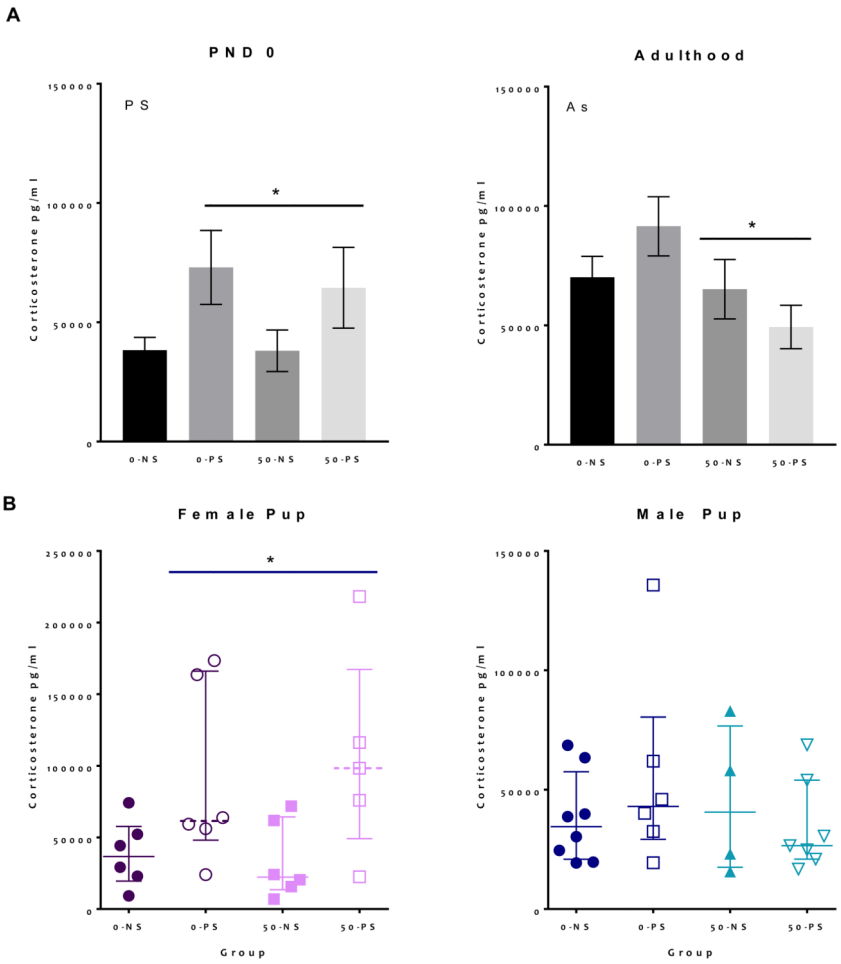
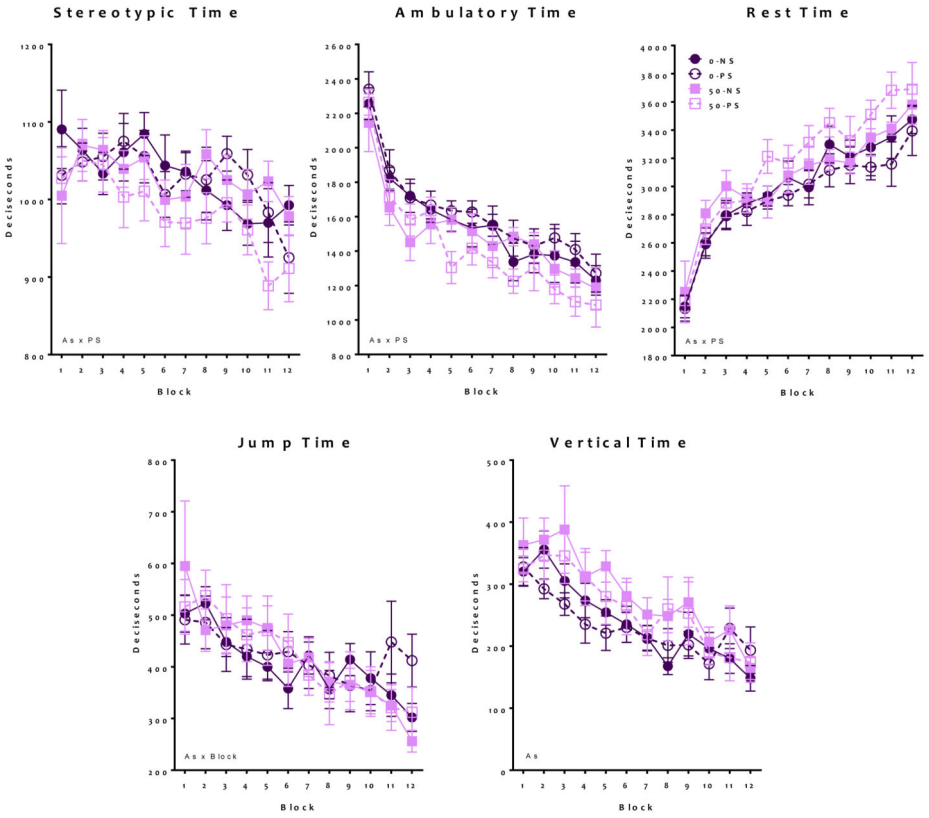


Figure 2. Group mean ± S.E. Serum Corticosterone Concentrations

A. Serum corticosterone concentrations were measured at birth (left) and in adulthood (right) (N = 5 - 8). **B**. Serum corticosterone at birth separated by sex, with females only showing significant increases (N = 5 - 8). Main effects stated on graphs and asterisk indicates $p < 0.05$.

A



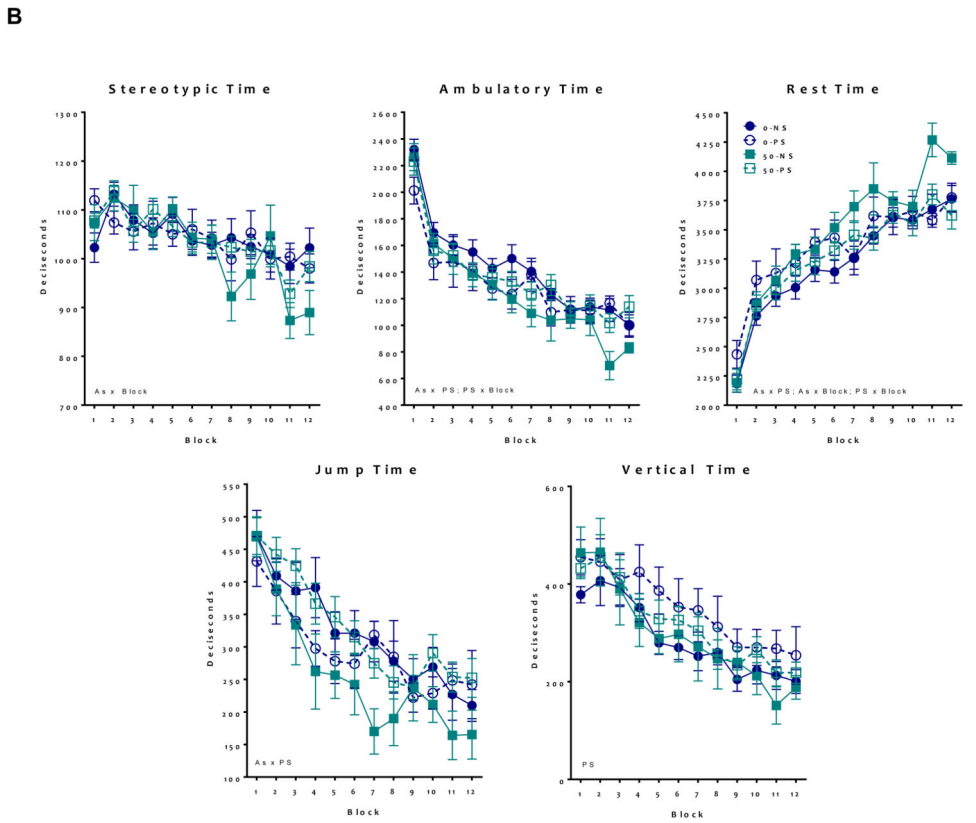


Figure 3. Group mean ± S.E Locomotor behavior in adult mice following developmental exposure to As and PS

Analysis of locomotor behavior including stereotypic time, ambulatory time, resting time, jump time, and vertical time. **(A)** Females showed significant alterations following the As +PS group, suggesting a worsening of locomotor deficits for ambulatory, stereotypic and resting time. For jump time, As exposed animals showed altered habituation patterns of block with As also increasing vertical time (N = 8-10) **(B)** Analysis of male locomotor behavior indicates that ambulatory time, resting time, and jump time showed significant alterations following As only exposure. As exposed male showed different stereotypic behavior over time, while PS exposed males elevated vertical time. Significant (* indicates $p \leq 0.05$) main effects stated on graphs (N = 7-12).

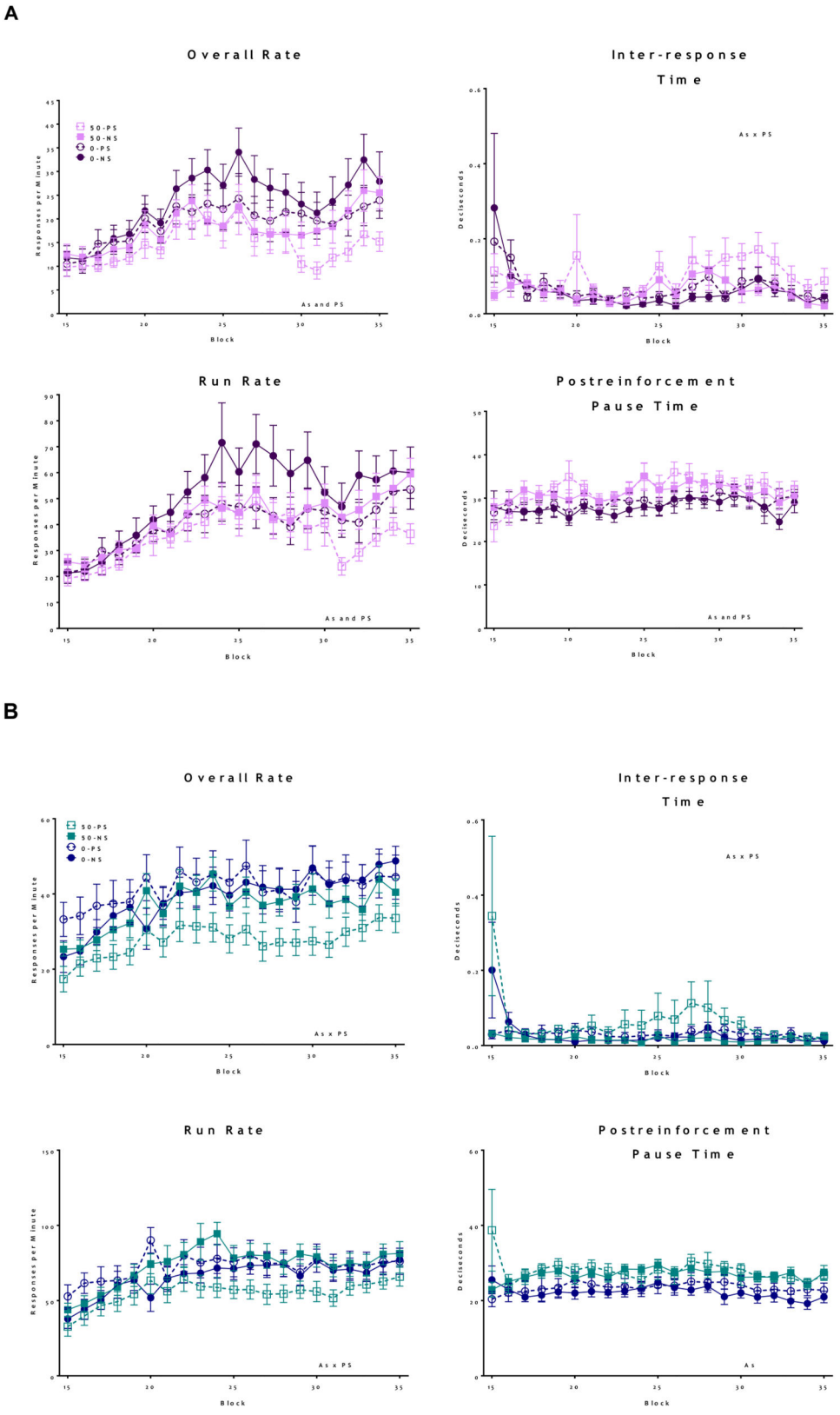


Figure 4. Group mean + S.E Fixed Interval (FI) Schedule of Reward in adult mice following developmental exposure to As and PS

Analysis of FI behavior including Overall rate, pause time, run rate and Inter-response time. Text stated on graphs indicates significance ($p \leq 0.05$) main effects. **(A)** Females showed significant alterations following the As and PS on overall rate, suggesting decreased motivation, with run rate indicating similar deficits. As+PS exposure increased post-reinforcement pause times, while both As and PS increased inter-response times, suggesting additive effects of each exposure (N = 7-10). **(B)** Analysis of male FI behavior showed significantly enhanced decreases in overall rate and run rate in Pb+PS exposed males, compared to all other treatment groups. As+PS males had increased post-reinforcement pause times and As exposure, and PS marginally, increased inter-response times (N = 8 -12).

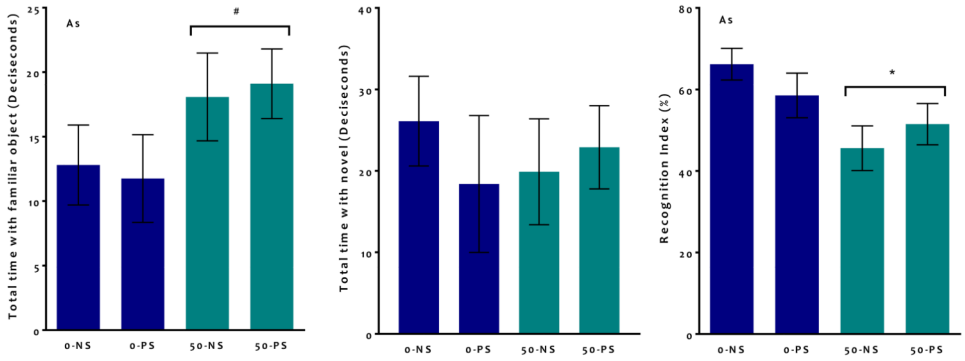


Figure 5. Group mean + S.E Novel Object Recognition Index for males

Significant (* indicates $p < 0.05$, # indicated $P < 0.1$) main effects stated on graphs (N = 7 -11). As exposure altered adult novel object recognition for total duration calculated recognition index, driven by a marginal increase in total time spent with the familiar object.

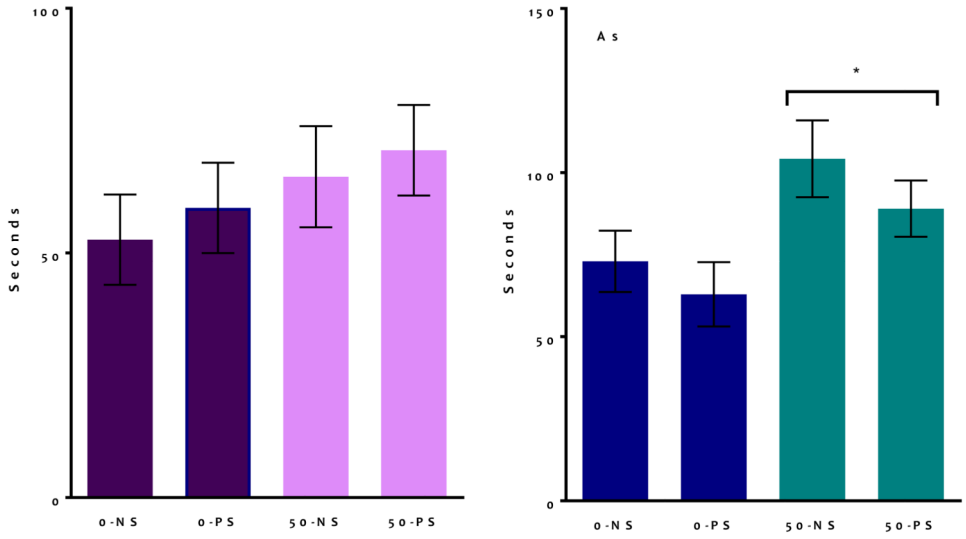


Figure 6. Group mean + S.E Elevated Plus Maze in adult mice following developmental exposure to As and PS

Analysis of male EPM behavior showed an increased in time spent in the open arms following As exposure. There was a significant decrease in time spent in the open arms for As exposed males, with similar trends observed in females. Significant (* indicates $p \leq 0.05$) main effects stated on graphs (N = 7 -11).

Table 1

Changes in Corticosterone and FI Behavior Outcomes.

Lead (Pb)	***FEMALE***					***MALE***				
	0-PS	50-NS	50-PS	150-NS	150-PS	0-PS	50-NS	50-PS	150-NS	150-PS
Cort (Adult)		↑	↑	↑	↑/↓		↓	↓		↑
FI response rates	↑	↑	↑	↑	↑	↑			↓	
FI post-reinforcement pause	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑
FI inter-response time										
FI run rate		↑	↑	↑	↑					
Metdyl-mercury (MeHg)	***FEMALE***					***MALE***				
	0-	0.5-	0.5-	2.5-	2.5-	0-	0.5-	0.5-	2.5-	2.5-
PS	NS	PS	NS	PS	PS	NS	PS	NS	PS	
Cort (Adult)		↑							↓	↓
FI response rates	↓	↓	↓	↓	↓					
FI post-reinforcement pause	↑	↑	↑	↑	↑					
FI inter-response time					↑					
FI run rate	↓	↓	↓	↓	↓					
Arsenic (As)	***FEMALE***					***MALE***				
	0-PS	50-NS	50-PS			0-PS	50-NS	50-PS		
Cort (PND0)	↑		↑							
Cort (Adult)		↓	↓				↓	↓		
FI response rates	↓	↓	↓					↓		
FI post-reinforcement pause	↑	↑	↑				↑	↑		
FI inter-response time			↑					↑		
FI run rate	↓	↓	↓					↓		

↑ indicates increase ↓ indicates decrease. Previous and current data on behavioral toxicity of EDMs and PS gatdered from studies on mice and rats. Tdis review was based on our studies witd PS only and focused on basal serum corticosterone alterations and FI behavioral assay, as endpoints were conducted for each EDM±PS. Review base on following studies (Cory-Slechta et al., 2010; Cory-Slechta et al., 2004; Rossi-George et al., 2011; Virgolini et al., 2008a; Weston et al., 2014b; White et al., 2007).