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# Amphetamine sensitization and cross-sensitization with acute restraint stress: impact of prenatal alcohol exposure in male and female rats

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## Abstract

**Rationale—**Individuals with fetal alcohol spectrum disorder (FASD) are at increased risk for substance use disorders (SUD). In typically developing individuals, susceptibility to SUD is associated with alterations in dopamine and hypothalamic-pituitary-adrenal (HPA) systems, and their interactions. Prenatal alcohol exposure (PAE) alters dopamine and HPA systems, yet effects of PAE on dopamine-HPA *interactions* are unknown. Amphetamine-stress cross-sensitization paradigms were utilized to investigate sensitivity of dopamine and stress (HPA) systems, and their interactions following PAE.

**Methods—**Adult Sprague-Dawley offspring from PAE, pair-fed, and *ad libitum*-fed control groups were assigned to amphetamine-(1–2mg/kg) or saline-treated conditions, with injections every other day for 15 days. 14 days later, all animals received an amphetamine challenge (1mg/kg) and 5 days later, hormones were measured under basal or acute stress conditions. Amphetamine sensitization (augmented locomotion, days 1–29) and cross-sensitization with acute restraint stress (increased stress hormones, day 34) were assessed.

**Results—**PAE rats exhibited a lower threshold for amphetamine sensitization compared to controls, suggesting enhanced sensitivity of dopaminergic systems to stimulant-induced changes. Cross-sensitization between amphetamine (dopamine) and stress (HPA hormone) systems was evident in PAE, but not in control rats. PAE males exhibited increased dopamine receptor expression (mPFC) compared to controls.

**Conclusions—**PAE alters induction and expression of sensitization/cross-sensitization, as reflected in locomotor, neural, and endocrine changes, in a manner consistent with increased

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sensitivity of dopamine and stress systems. These results provide insight into possible mechanisms that could underlie increased prevalence of SUD, as well as the impact of widely prescribed stimulant medications among adolescents with FASD.

**Keywords**

prenatal alcohol; amphetamine; stress; dopamine; addiction; sex differences; sensitization; prefrontal cortex; nucleus accumbens; striatum

## INTRODUCTION

Fetal alcohol spectrum disorder (FASD) is a term encompassing the range of disorders or deficits resulting from prenatal alcohol exposure (PAE), and has an estimated prevalence of 9/1,000 births in North America (Thanh and Jonsson 2010). “Secondary” FASD-related deficits include an increased propensity for mental health problems such as substance use disorders (SUD) (Baer et al. 2003, Alati et al. 2008, O’Connor and Paley 2009). Consistent with clinical findings, rodent models of PAE demonstrate increased stress responsiveness, depressive-/anxiety-like behaviors, and preference for alcohol and other drugs (Chotro et al. 2007, Barbier et al. 2009, Hellemans et al. 2010). The present study investigated the effects of PAE on sensitivity of underlying dopamine (DA) and stress (hypothalamic-pituitary-adrenal, HPA) systems implicated in vulnerability to SUD.

The link between DA dysregulation and enhanced vulnerability to SUD is widely accepted (Sinha 2008, Le Moal 2009, Volkow et al. 2011). For example, reduced binding of DA receptors ($D_1$, $D_2$) has been associated with increased susceptibility to SUD (Hooks et al. 1994, Volkow et al. 1999, Sweitzer et al. 2012). Repeated exposure to stimulants, such as amphetamine (AMPH), can produce hypersensitivity of DA systems, resulting in an enhanced behavioral response referred to as behavioral sensitization (i.e. behavior is increasingly amplified) (Vanderschuren and Pierce 2010). Behavioral sensitization is positively correlated with stimulant self-administration (Piazza et al. 1990) and increased propensity for reinstatement following abstinence (Vanderschuren and Pierce 2010), but is not indicative of increased motivation to use (Ahmed and Cador 2006), a hallmark of addiction. The present study used repeated exposure to AMPH as a paradigm to examine sensitivity of DA systems to stimulants, which may increase DA dysregulation, and ultimately contribute to increased neurobiological vulnerability to SUD.

PAE produces marked alterations in DA systems, including reductions in neuronal activity, receptor binding sites, and metabolites (Blanchard et al. 1993, Shetty et al. 1993, Spear 1996, Shen et al. 1999, Wang et al. 2006, Shen et al. 2007), which alter tonic DA activity and change neurobiological sensitivity. For example, PAE attenuates the typical decrease in basal $D_1$ and $D_2$ expression in the nucleus accumbens (NAc) and striatum following chronic variable stress (Uban et al. 2013) and the drug-induced increases in NAc DA content (Chen et al. 1997), despite enhanced stimulant sensitization in these animals (Hannigan and Pilati 1991, Barbier et al. 2009). Thus, depending on the situation, it is possible that PAE can *reduce* tonic DA activity, but *increase* sensitivity of DA systems, to stimulants.

PAE also adversely affects the HPA axis, a major component of the stress system. Following PAE, increased HPA tone and increased stress responsiveness have been reported in infants (Ramsay et al. 1996, Jacobson et al. 1999, Haley et al. 2006, McLachlan et al. 2013) and in animal models (Taylor et al. 1988, Lee et al. 2000, Weinberg et al. 2008, Hellemans et al. 2010). Moreover, brain areas such as the medial prefrontal cortex (mPFC), NAc, and hypothalamus, which regulate HPA activity (Koob 2008), have significant bi-directional interactions with the mesocorticolimbic DA system (Pacak and Palkovits 2001, Koob and Kreek 2007, Cabib and Puglisi-Allegra 2012). For example, a range of acute stressors, including restraint stress, can induce drug relapse after abstinence (for review see (Sarnyai et al. 2001)) and acute drug exposure activates stress hormones (Koob 2008), suggesting that alterations in the cross-talk between DA and stress systems may underlie vulnerability to SUD (Lovallo 2006, Koob and Kreek 2007, Koob 2008). However, to date, very little is known about the effects of PAE on the cross-talk between DA and the stress hormone systems.

In the present study, we investigated effects of PAE on behavioral sensitization to AMPH and cross-sensitization between AMPH and acute restraint stress. $D_1$ and $D_2$ expression were investigated in brain regions (mPFC, NAc core and shell, dorsal striatum) implicated in DA-stress system interactions (Koob 2008) and altered by PAE (Uban et al. 2013). Male and female offspring were tested as sex differences are observed in SUD (Haseltine 2000, Hu and Becker 2003), HPA function (Young 1998) and PAE outcome (Weinberg et al., 2008). We hypothesized that PAE would in a sexually dimorphic manner: 1) augment behavioral sensitization to AMPH; 2) augment cross-sensitization with restraint stress (elevated stress hormone levels); 3) alter the effects of AMPH on $D_1$ and $D_2$ expression in key brain regions.

## MATERIALS AND METHODS

### Breeding

Sprague-Dawley rats (Charles River Laboratories, St Constant, QU, Canada) were pair-housed by sex in clear polycarbonate cages with corn-cob bedding, and given *ad libitum* access to water and laboratory chow (18% Protein Extruded Rodent Diet, #2019, Teklad Global). Colony rooms were maintained on 12:12 hr light/dark cycle (lights on 0800 hr) at 20–23°C. Nulliparous females (225–335 grams (g); n=38) and males (275–375g; n=18) were paired and the presence of sperm in vaginal lavages indicated gestation day 1 (GD1). All animal procedures were in accordance with the National Institutes of Health guidelines, and approved by the UBC Animal Care Committee. All efforts were made to minimize suffering and the number of animals used.

### Prenatal diets and feeding

On GD1, females were single housed and randomly assigned to one of three groups: 1) Alcohol-treated (PAE) - liquid ethanol diet, *ad libitum* (n = 13); 2) Pair-fed (PF) - liquid control diet, maltose dextrin isocalorically substituted for ethanol, in the amount consumed by a PAE partner (g/kg/body wt/day of gestation) to control for the reduced food intake typical with alcohol consumption (n = 12); 3) control (C), pelleted form of liquid control diet, *ad libitum* (n = 13): all dams had *ad libitum* access to water. Alcohol containing liquid

diets were formulated to provide optimal nutrition (Dyets Inc. Bethlehem, PA, USA), with 36% of total calories derived from ethanol (Commercial Alcohol Inc., ON, Canada, catalog no. P210EAAN). Fresh diet was presented daily (1800–1900 hr) from GD1-21. This feeding schedule maintains the normal corticoid circadian rhythm in the Pair-fed dams, which are fed a restricted ration (Krieger 1974, Gallo and Weinberg 1981). On GD21, all diets were replaced with laboratory chow *ad libitum* for the remainder of the study. Dams were weighed weekly. Blood alcohol levels ranged from ~80–150 mg/dl (mean=108.6mg/dl) on GD16, 2 hours after lights off, using previous protocols (Hellemans et al. 2010, Uban et al. 2010). On postnatal day 1 (PND 1), litters were culled to 10 (5/sex) and weighed weekly. Pups were weaned on PND 22 and group-housed by litter and sex with an enrichment tube. One male and one female were selected from each litter for testing to control for litter effects.

## Amphetamine sensitization

Adult rats (70±2.5 days) were randomly assigned to either AMPH- or saline-treated conditions. There were 240 rats utilized (n= 20 per prenatal group × AMPH condition × sex [other than 2 control females removed from analysis owing to equipment malfunction]). All subjects received a total of 8 injections (intraperitoneal, i.p.), 1 injection every other day for 15 days (Figure 1), modified from a previous sensitization/cross-sensitization protocol (Piazza et al. 1990). AMPH- treated rats received escalating doses of AMPH (*d*-Amphetamine hemisulphate salt; Sigma Aldrich, England, UK), with 1 mg/kg AMPH for the first 4 injections (days 1–7) and 2 mg/kg AMPH for the last 4 injections (days 8–15). Saline-treated rats received injections of physiological (0.9%) saline, in a volume equivalent to the AMPH injection volume. Rats then remained undisturbed (other than routine feeding and husbandry) for a 14-day washout period (Deroche et al. 1992, Vanderschuren and Pierce 2010), at the end of which (day 29) ***all*** subjects (both AMPH- and saline-treated) received a single injection of the lower dose (1 mg/kg, i.p.) of AMPH. All injections and behavioral testing (0900–1330 hr) occurred in a separate procedure room with contextual cues different from those of the home cage (i.e. dimmed lighting, novel carefresh bedding, black cage lining (41 $cm^3$), single housing for 80 min). This was done as development of sensitization is strengthened by contextual cues associated with a novel environment (Browman et al. 1998, Wang et al. 2010). The doses of AMPH utilized (1–2 mg/kg) were low relative to the standard dosing in most sensitization paradigms (Robinson and Becker 1986, Browman et al. 1998) in order to maintain face validity (Doig et al. 2008) and to prevent ceiling effects in behavioral responsivity, as we anticipated enhanced sensitivity to AMPH exposure in PAE offspring.

## Behavior

Experimental design is shown in Figure 1. Locomotor behavior was recorded (SONY Handycam DCR-SR68) on days 1 (first injection), 15 (last injection), and 29 (AMPH challenge following 14 day washout) of testing. On each test day, a 20 minute baseline period was assessed prior to the AMPH/saline injection, followed by a 60 minute post-injection period to assess: 1) total distance travelled; 2) total number of rotations (atypical behavior); 3) frequency of rears; and 4) stereotypy level. Distance and rotations were quantified by ANY-maze video tracking software (version 4.75, Stoelting) in 5 min blocks.

Rearing and stereotypy were quantified manually by three independent scorers (blind to treatment) in 10 min blocks (inter-rater reliability > 90%). Level of stereotypy was scored with a Likert scale modified from (MacLennan and Maier 1983, Barr et al. 2002). Blood samples were collected via the tail vein, 10 minutes after the end of testing on day 29 (70 minutes post-AMPH injection) for analysis of plasma corticosterone (CORT) levels.

### Stress test

A 5-day washout period followed testing on day 29, to eliminate confounding effects of previous AMPH exposure on subsequent stress reactivity. The next morning (day 34, 0900–1030 hr), pairs of rats were removed from their home cages, and blood samples collected from one animal immediately (within 20 sec) to assess basal CORT and adrenocorticotropin (ACTH) levels, and from the other cage mate following a 30 min restraint stress to assess basal and activated hormone levels (n=10 per group).

### Collection of blood and vaginal lavage samples

Blood was collected on day 29 (tail knick; n=20/group) and day 34 (live decapitation; n=10/group) into tubes containing EDTA (2 cc), centrifuged for 15 min at 4°C, and stored at −80 °C until assayed. Vaginal lavage samples were collected on days 1, 15, 29 and 34, and cytology assessed as previously described (Uban et al. 2012) to determine stages of the estrous cycle.

### Radioimmunoassays (RIA) for CORT and ACTH levels

ImmuChemTM Corticosterone $I^{125}$ RIA kit (MP Biomedicals, Cat. # 07-120103), minimum detectable concentration of 7.7 ng/ml, and ACTH RIA kit (Diasorin Inc., Stillwater, MS, USA) with all reagent volumes halved, and a minimum detectable concentration of 20 pg/ml. The intra- and interassay coefficient of variations were under 8% for all assays.

### Brain preparation

Brains were removed under RNAse free conditions, flash frozen over dry ice, wrapped in parafilm, covered in aluminum foil, and stored at −80°C (n=10/group). Brains were sectioned on a cryostat (MICROM HM 505 E), to obtain coronal sections (30 μm, Bregma: 4.00 – 7.32mm), with every fifth section mounted onto chilled glass slides (total of 6–8 sections per subject per brain region [mPFC, NAc, striatum]), which were stored at −80°C.

### Immunohistochemistry

Fluorescent double-staining for DA receptors ($D_1$ and $D_2$) was performed (n=7–10/group, 6–8 sections per subject per region) on subjects terminated under basal conditions, as protein expression was not expected to change after a 30 min stressor. Every $5^{th}$ section from the mPFC (3.72 – 2.52mm; prelimbic and infralimbic), NAc (core and shell) and striatum (dorsal) (2.52 – 1.20mm) was analyzed (Paxinos and Watson 2005). Sections were post-fixed for 30 min in 3.7% paraformaldehyde in phosphate buffer solution (PBS; pH = 7.4), rinsed in TBS (0.1 M tris-phosphate buffer in 0.9% saline; pH 7.4), then blocked in 4% goat serum (NDS) in TBS + 0.3% Triton-X (Vector Laboratories, Burlington, Ontario, Canada) for 2 hr. Slides were incubated in mouse monoclonal Anti-Dopamine $D_1$ Receptor (1:450,

Novas Biologicals) and rabbit polyclonal Anti-Dopamine $D_2$ Receptor (1:300, Millipore Canada) in a Nunc box lined with moistened Benchkote© with TBS at 4°C for 22 hr on a shaker. Slides were rinsed in TBS and incubated in goat anti-mouse Alexa 594 for $D_1$ with goat anti-rabbit Alexa 488 for $D_2$ for 1 hr (1:450; Invitrogen, Burlington, Ontario Canada). Sections were rinsed in TBS, $dH_20$, allowed to dry and cover-slipped with 2.5% PVA-DABCO (Sigma, Oakville, Ontario, Canada).

### Quantification of data

**Densometric Analyses—**$D_1$ and $D_2$ expression (Olympus FV1000 confocal microscope (20×)) were analyzed with ImageJ (Rasband 1997–2011). Imaging parameters were set as previously described (Uban et al. 2013) and background measurements were obtained from adjacent areas. For each subject, corrected optical density values were averaged across hemispheres and sections for each brain region.

**Statistical Analyses—**When referring to rats tested on Days 1–15 we will use the term 'treated' (groups received either AMPH or saline treatment Days 1–15) but when referring to animals tested on Day 29 we will use the term 'pre-treated' as all animals were treated with AMPH on Day 29. Repeated measures analyses of variance (ANOVAs) [Statistica (StatSoft, Inc)] were utilized. Males and females were analyzed together for pre-weaning data [prenatal group × postnatal day], and separately for all other analyses. For adult females, estrous stage was a covariate in hormonal, behavioral and neurobiological analyses, but was not significant. ANOVAs included the factors of prenatal group, drug condition, stress condition, hormone type, DA-R subtype, and test day, as appropriate, with day tested as a repeated measures factor. *Post-hoc* tests utilized Newman-Keuls comparisons. When the omnibus F test did not achieve significance, Bonferroni corrections were applied to *a priori* pair-wise comparisons based on our hypotheses.

## RESULTS

### Behavioral sensitization: typical and atypical behaviors are altered in PAE subjects

**PAE males and females showed greater locomotor behavior (distance travelled) than their C and PF counterparts following repeated AMPH exposure—**For males, *post-hoc* analyses revealed that across prenatal groups, AMPH-treated males traveled significantly greater distances than saline-treated males on both day 1 (C: 20–50 min; PF: 25 and 35–40 min; PAE: 25–50 min) and day 15 (C: 35 min; PF: 50 min; PAE: 15 and 25–50 min) (ps<0.01) (Figure 2A) [prenatal group × drug × day × time ($F_{52, 2756}$=1.41, p<0.05)]. Importantly, AMPH-treated PAE males travelled greater distances on day 15 than their AMPH-treated C (30 and 40–50 min), and PF (35 and 45 min) counterparts (ps<0.01), suggesting behavioral sensitization at an earlier time point. Intriguingly, following the washout period, saline-pretreated PAE males that received AMPH for the first time on day 29 travelled greater distances than their C (35–40 min) and PF (40 min) counterparts (ps<0.01), indicating that PAE males also demonstrate enhanced behavioral sensitization on their first AMPH exposure following the mild stress of repeated i.p. saline injections.

For females, similar to males, *post-hoc* analyses revealed that on day 15, AMPH-treated PAE females travelled significantly greater distances than both AMPH-treated C (20–50 min) and PF (25–30 min) females (ps<0.01; Figure 2B) [prenatal group × day × time ($F_{52, 2730}$=1.64, p<0.01); drug × day × time ($F_{26, 2730}$=18.43, p<0.001]. In addition, however, on day 29, when all rats received AMPH, AMPH-pretreated PAE females travelled greater distances than AMPH-pretreated C (25–50 min) and PF (35–40 min) females (p's<0.01). Thus, PAE females not only show enhanced behavioral sensitization at an earlier time point (day 15 vs. day 29), but also show enhanced behavioral sensitization to the lower dose of AMPH following the washout period (day 29) compared to C and PF females. Furthermore, as seen in males, saline-pretreated PAE females that received AMPH for the first time on day 29, travelled greater distances compared to both saline-pretreated C (20–30 and 50 min) and PF (45 min) females (ps<0.01), suggesting enhanced behavioral sensitization to the first AMPH exposure following the mild stress of repeated i.p. saline injections.

To confirm behavioral sensitization, locomotor activity was compared among test days (days 1, 15, 29) in AMPH-treated males and females immediately following injection. There was a main effect of Day (Males: F(2,954)=6.43, p<0.01; Females: F(2,900)=36.75, p<0.001). In males, significantly higher levels of locomotion were observed on day 29 compared to days 1 and 15 (ps<0.01), but no significant difference between day 15 and day 1. In females, significantly higher levels of locomotion were observed on day 29 compared to days 1 and 15 and on day 15 compared to day 1 overall (ps<0.0001).

Enhanced sensitivity to amphetamine is also reflected in body weight gain (i.e. percent change from baseline), which was significantly attenuated in PAE compared to C and PF males and females following the 15 day AMPH exposure period: For males: PAE: 35.9±1.7%, PF: 39.7±1.5%, C: 40.9±1.8%; For females: PAE: 7.3±1.98%, PF: 10.3±.56%, C: 11.0±.58% (ps<0.01) [prenatal group × day ($F_{8, 908}$=3.5, p=0.05); sex × day ($F_{4, 908}$=118.248, p<0.001)].

**PAE males and females showed more rotation behavior than their C and PF counterparts following repeated AMPH exposure—**For males, *post-hoc* analyses revealed that more 360° rotations (direction not indicated) were observed in AMPH-treated compared to saline-treated males on days 1 (15–50 min) and 15 (20–50 min; ps<0.05), but not on day 29 (Figure 3A) [drug × day × time ($F_{26, 2704}$=6.43, p<0.001)]. Importantly, *a priori* analyses revealed that AMPH-treated PAE males rotated more than both AMPH-treated C and PF males throughout testing (i.e. day 1 (C: 10 and 30–50 min; PF: 5 min during baseline and 40–45 min); day 15 (C: 30–50 min; PF: 30–40 and 50 min), and day 29 (C: 15 min; PF: 10 and 50 min), ps<0.01) [main effect of prenatal group (p<0.01)]. In addition, and consistent with the findings on locomotor behavior, on day 29, saline-pretreated PAE males rotated more than their C and PF males counterparts upon first exposure to AMPH (C: 35–50 min; PF: 40–45 min; ps<0.01).

For females, *post-hoc* analyses revealed increased 360° rotations in AMPH- compared to saline-treated females overall on day 1 (10–50 min), day 15 (5–50 min), and day 29 (5 min) (ps<0.05; Figure 3B) [drug × day × time ($F_{26, 2730}$=21.17, p<0.001)]. Further, *a priori*

analyses revealed that AMPH-treated PAE females rotated more than their C and PF counterparts on day 15 (C: 25–40; PF: 25–40 min) and day 29 (C: 5 and 25 min; PF: 5 and 40 min; ps<0.01).

Similar to males, AMPH administration in naïve saline-pretreated PAE females (day 29) increased rotations relative to saline-pretreated C and PF females (C: 10–25 min; PF: 10 min; ps<0.01). Thus, for both males and females, unmasking of atypical behaviors in PAE compared to C and PF animals occurs following both single and repeated AMPH exposures, as well as following their first AMPH exposure after the mild stress of repeated saline injections.

Overall, the relatively low doses of AMPH used in the present study did not induce high levels of atypical behaviors indicative of severe stereotypies, eliminating the possibility of a ceiling effect in observed behaviors. While stereotypy was enhanced in AMPH- compared to saline-treated males (on all test days, ps<0.05) and females (test days 1 and 15, ps<0.05), overall levels of AMPH-induced stereotypy, rearing and CORT (day 29 only) were similar across prenatal groups (data not shown).

## Cross-sensitization between AMPH and stress was observed in PAE, but not in PF and C, rats

For males, *post-hoc* analyses revealed no significant differences in *basal* CORT levels among prenatal groups (Table 1A). Across AMPH/saline conditions, acute restraint stress resulted in greater ACTH levels in PAE compared to PF and C (ps<0.01), and in PF compared to C (p<0.05) males [prenatal group × stress × hormone interaction ($F_{2,108}$=3.23, p<0.05)]. Moreover, *a priori* analyses revealed that, following restraint stress, PAE males pretreated with AMPH showed significantly higher ACTH levels (ps<0.01) than PAE males pretreated with saline, whereas PF and C males in the AMPH and saline conditions showed similar ACTH levels.

For females, across AMPH/saline conditions and prenatal groups, both ACTH and CORT were increased over basal levels following restraint stress [stress × hormone ($F_{1,100}$=65.87, p<0.001)]. *A priori* analyses revealed a statistical trend for enhanced CORT in AMPH- compared to saline-pretreated PAE (p<0.05 but did not meet Bonferroni correction levels [critical p=0.013]), but not C or PF, females (Table 1A).

## AMPH altered DA receptor expression in PAE, but not PF and C, males in a region-specific manner

For males, *post-hoc* analyses revealed that in the PL subregion, DA-R expression was lower in PAE compared to PF males following saline- (p<0.001), but not AMPH-pretreatment, and there were no significant differences compared to C males (Table 1B) [prenatal group × drug × mPFC subregion ($F_{2, 36}$=4.61, p<0.05)]. $D_1$ was greater than $D_2$ expression overall ($F_{1, 36}$=51.24, p<0.001). $D_1$ and $D_2$ expression were greater throughout the mPFC in saline-pretreated PF compared to saline-pretreated C males (ps<0.05), but no differences among groups following AMPH. In the IL subregion, by contrast, there were *lower* densities of both

$D_1$ and $D_2$ in saline-pretreated PAE males, but *higher* densities in AMPH-pretreated PAE males compared to their C and PF counterparts (ps<0.001).

For females, greater $D_1$ and $D_2$ expression was observed in the PL compared to IL subregion (Table 1B) [main effect of mPFC subregion ($F_{1, 39}$=4.85, p<0.05)], but no other significant main or interaction effects were observed.

Within the NAc and striatum, there were no differences in $D_1$ or $D_2$ expression among prenatal groups or between drug conditions in either males or females (data not shown).

## DISCUSSION

These data demonstrate, for the first time, that PAE increases behavioral sensitization to both single *and* repeated AMPH exposures and cross-sensitization with acute restraint stress in adult males and females compared to their control counterparts. Specifically, PAE males and females exhibit: 1) more rapid development of, and a lower threshold for, behavioral sensitization; 2) greater behavioral responsivity of drug-naïve rats to AMPH exposure following the mild stress of repeated saline injections; 3) a lower threshold for AMPH-stress cross-sensitization; 4) more atypical behavior (360° rotations) following repeated AMPH exposure; and 5) altered expression of $D_1$ and $D_2$ within the IL subregion (in PAE males only). These findings suggest that, overall, PAE results in enhanced sensitivity of, and altered cross talk between, DA and stress hormone systems, which is unmasked with AMPH exposure. This neurobiological phenotype is consistent with increased dysregulation of DA and stress systems, and their interactions, in PAE rats following stimulant exposure.

### Rapid sensitization of ‘typical’ behaviors in AMPH-pretreated PAE subjects

Among all prenatal groups, there was the expected increase in locomotion in AMPH- compared to saline- exposed rats on the first day of AMPH exposure. However, by day 15 of repeated injections, PAE subjects showed behavioral sensitization (i.e. greater distances travelled compared to the first AMPH exposure), whereas Control and Pair-fed subjects showed no increase in locomotion beyond that on day 1, likely due to the relatively low dose of AMPH utilized. Locomotion is related to enhanced activity of mesocorticolimbic dopaminergic pathways (Ikemoto 2002). Thus, AMPH exposure may sensitize mesocorticolimbic DA systems more rapidly in PAE compared to Control and Pair-fed subjects. It is also possible that PAE reduced the threshold for AMPH sensitization. Our findings are consistent with those of a previous study where PAE male rats exhibited an earlier onset of behavioral sensitization following repeated exposure to cocaine (Barbier et al. 2009). Together, these findings suggest that PAE accelerates and enhances sensitization to a range of stimulants, suggesting increased sensitivity of the mesocorticolimbic dopaminergic pathways under multiple drug and exposure conditions.

Interestingly, saline-pretreated PAE rats also exhibited significantly enhanced locomotion compared to controls on their first exposure to AMPH (day 29). Among AMPH-pretreated rats, there were no pre-existing differences among prenatal groups in behavioral responsivity following the first AMPH exposure (day 1). Together, these results suggest that DA systems may also be sensitized by repeated mild stress (repeated saline injections combined with

exposure to novel contextual cues) (Antelman et al. 1980) in PAE rats, thereby facilitating the enhanced behavioral sensitization to the first AMPH exposure observed in these drug-naïve subjects. These findings support a PAE-induced cross-sensitization between mild repeated (injection) stress and AMPH exposure, consistent with Barr et al (2002). Moreover, the data support and extend our previous findings demonstrating altered DA-stress (chronic variable stress) interactions in PAE animals terminated under basal conditions (Uban et al. 2013), as well as increased HPA tone and enhanced sensitivity to a range of acute and chronic stressors in PAE compared to control animals (reviewed in (Weinberg et al. 2008).

### Rapid sensitization of 'atypical' behaviors in AMPH-treated PAE subjects

Repeated AMPH exposure enhanced rotation behavior in PAE compared to Control and Pair-fed males and females. Rotation is an atypical behavior produced by DA agonists and is positively correlated with enhanced voluntary alcohol consumption and with the inability to cope with restraint and cold stressors (Carlson et al. 1993, Nielsen et al. 1999). These findings suggest that PAE increases propensity for AMPH-induced alterations in nigrostriatal dopaminergic function and activity within NAc-mPFC neural loops. Moreover, we found that saline-pretreated PAE rats also exhibited enhanced rotation behavior compared to Control and Pair-fed rats after their first AMPH exposure (day 29). This further suggests cross-sensitization between stress and AMPH in PAE subjects as a consequence of the mild stress of repeated saline injection. Although turn directionality was not investigated, only completed rotations of 360° or greater were included in analyses, and this increase in rotational behavior is known to be associated with both locomotor activity and an increased propensity for drug self-administration in male rats (Carlson and Glick 1989). Future studies are needed to further examine directionality to elucidate potential asymmetry in nigrostriatal DA function, which could reflect vulnerability to a range of mental health problems, including SUDs.

### Cross-sensitization between low dose AMPH exposure and acute stress was seen in PAE but not control animals under context-dependent test conditions

In the present study, we showed cross-sensitization between low dose AMPH and acute restraint stress in PAE, but not control, animals. These findings contrast with previous work demonstrating cross-sensitization between AMPH and acute restraint stress in control male rats (Barr et al. 2002). Two notable differences between these studies may account for the differences in results: In Barr et al, the AMPH doses were twice those in the present study (2–4 mg compared to 1–2 mg), and AMPH was administered in the home cage, rather than in a novel context-specific environment. The relatively low AMPH doses in the present study may explain why AMPH sensitization and cross-sensitization with stress hormones were not observed in controls. In addition, as context is a powerful factor in drug responsivity (Badiani and Robinson 2004), AMPH/stress exposure in a novel context may have facilitated the development of cross-sensitization in PAE animals, who are more sensitive to stimulants and restraint stress in general (Taylor et al. 1988, Blanchard et al. 1993, Lee et al. 2000), and show altered responses to environmental contexts (Berman and Hannigan 2000, Kajimoto et al. 2013). Furthermore, the hippocampus is highly susceptible to damage following PAE (Berman and Hannigan 2000, Livy et al. 2003, Sliwowska et al. 2010, Uban et al. 2010). Given the role of the hippocampus in encoding contextual cues and

regulating stress, it is possible that hippocampal deficits reduced the ability of PAE animals to form associations between contextual cues and AMPH exposure.

## PAE and AMPH exposure interact to alter DA receptor expression within the infralimbic (IL) subregion of the mPFC in males but not females

AMPH-exposure *increased* $D_1$ and $D_2$ expression in the IL mPFC of PAE compared to Control and Pair-fed males, suggesting interactive effects of PAE and AMPH exposure on DA-R expression. These alterations indicate enhanced sensitivity of specific dopaminergic loops to repeated AMPH exposure in PAE males, but not females. Repeated exposure to stimulants produces a remarkable degree of adaption in underlying DA systems (Castner and Williams 2007), and overall, the present results suggest a small but significant effect on stimulant-induced malleability of DA systems in PAE males. Paralleling previous findings (Uban et al. 2013), there were no pre-existing differences in $D_1$ and $D_2$ density in the NAc or striatum in adult PAE rats. Rather, enhanced behavioral sensitization may have resulted from alterations in location of expression sites of DA-Rs (e.g. intra- versus extra-cellular), or other closely interacting neurotransmitter systems such as the glutamatergic system. In human neuroimaging studies, stimulant-induced changes in DA binding capacity and DA levels in the striatum and nucleus accumbens were positively correlated with reinforcing properties of stimulants (reviewed in (Volkow et al. 2007). Thus the increase in DA-R expression seen in the present study may represent greater long-term plasticity of the DA system in response to stimulant exposure in PAE, but not Pair-Fed and Control, male rats. Additionally, the increased density of both $D_1$ and $D_2$ in PAE males may contribute, in part, to increased behavioral sensitization, as co-activation of both receptor subtypes is necessary for the neural and behavioral expression of stimulant sensitization in rats (Capper-Loup et al., 2002). Additionally, dopaminergic changes in other brain regions, such as the ventral tegmental area, may also contribute to increased sensitivity of dopaminergic systems to AMPH in PAE rats (Xu and Shen 2001).

## Sex differences in AMPH-induced behavioral sensitization and DA-R densities

Across prenatal groups, females exhibited significantly more locomotor activation and stereotypy following AMPH exposure, as well as higher densities of $D_1$ and $D_2$ expression in the mPFC, NAc, and dorsal striatum compared to males. In contrast, PAE males but not females exhibited AMPH-induced changes in $D_1$ and $D_2$ expression in the mPFC. Previous studies have shown that females typically exhibit greater behavioral responses and greater sensitization to psychomotor stimulants than males. The mechanisms that underlie this sex difference are likely both hormonal, as higher estradiol levels in adult females enhance these sex differences in sensitization, and neural as neural systems that underlie behavioral sensitization are sexually dimorphic (Becker et al. 2001, Hu and Becker 2003). Further research is needed to understand the sexually dimorphic effects observed in the present study, and possible implications of these findings for sex differences in vulnerability to SUD.

### PAE-induced alterations are consistent with a neurobiological phenotype of enhanced vulnerability to increased substance use: limitations and future directions

Stimulant sensitization paradigms are useful for modeling particular aspects of SUD (Vanderschuren and Pierce 2010), such as neural adaptations involved in the initial development of self-administration and propensity to relapse (Castner and Williams 2007), but not for increased motivation to use (Ahmed and Cador 2006). PAE accelerated the development of sensitization, and reduced the threshold at which sensitization occurred, indicating increased sensitivity of underlying DA systems within brain regions implicated in SUD (Shen et al. 2007, Barbier et al. 2009, Uban et al. 2013). However, future studies are needed to directly link enhanced sensitization and/or cross-sensitization to increased motivation for, and consumption of, drugs in this model of prenatal alcohol exposure. Additionally, as *pre-existing* differences in DA-sensitive behaviors and DA-R expression were not observed among prenatal groups, it appears that drug or stress challenge (see also (Uban et al. 2013)) may be required to unmask significant dysregulation of underlying neural systems in adult PAE subjects.

### Conclusions

In summary, we demonstrate that PAE enhances sensitivity of both DA and stress hormone systems to acute and repeated AMPH exposure, resulting in a reduced threshold for both sensitization to AMPH and cross-sensitization between AMPH and restraint or injection stress, in a sexually dimorphic manner. These findings support the hypothesis that PAE alters sensitivity of mesocorticolimbic dopaminergic systems in a manner consistent with enhanced neurobiological sensitivity to stimulant exposure. Our data provide insight into possible mechanisms underlying a range of mental health problems related to dysregulation of DA and stress systems, including SUD, which occurs at an increased prevalence among individuals with FASD (O'Connor and Paley 2009).

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## Research Highlights

1. Amphetamine sensitization occurred earlier and at a lower threshold in males and females prenatally exposed to alcohol.
2. Cross-sensitization between a low dose of amphetamine and restraint stress was observed in prenatally alcohol exposed, but not control, males and females.
3. In males, prenatal alcohol exposure and repeated amphetamine treatment interactively altered dopamine receptor expression in the mPFC.
4. Overall, females exhibited enhanced behavioral sensitization compared to males regardless of their prenatal treatment.

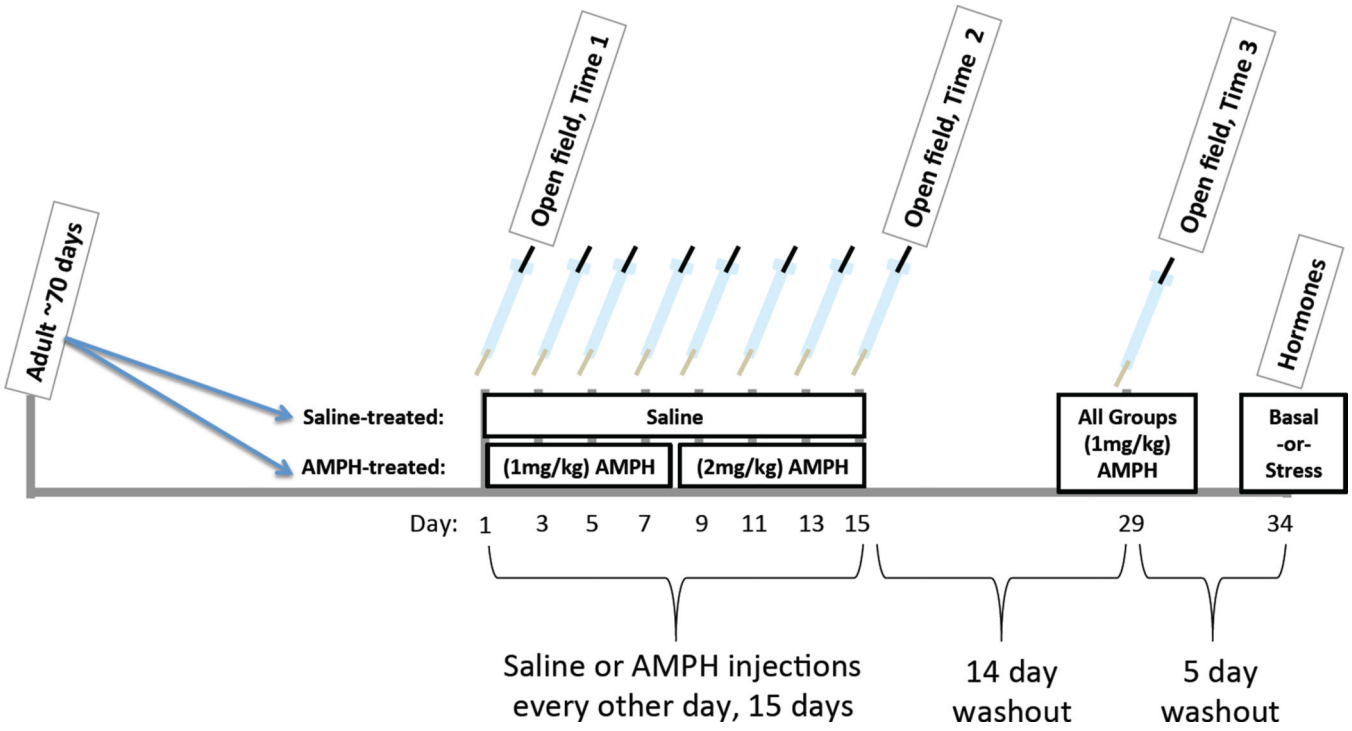


**Figure 1. Experimental Timeline**

All experimental procedures are charted above beginning with the pre-injection period. During the first 15 days of the experiment, all subjects received a total of 8 injections (intraperitoneal, i.p.), 1 injection every other day for 15 days. During these 15 days, AMPH-treated rats received 1 mg/kg AMPH for the first 4 injections and 2 mg/kg AMPH for the last 4 injections, while the saline-treated rats received only saline injections. Rats then remained undisturbed for a 14 day washout period, and on Day 29 *all* subjects (both AMPH- and saline-pretreated) received a single injection of AMPH (1 mg/kg, i.p.). On Day 34, blood was collected under either basal or stress conditions to assess HPA sensitization.

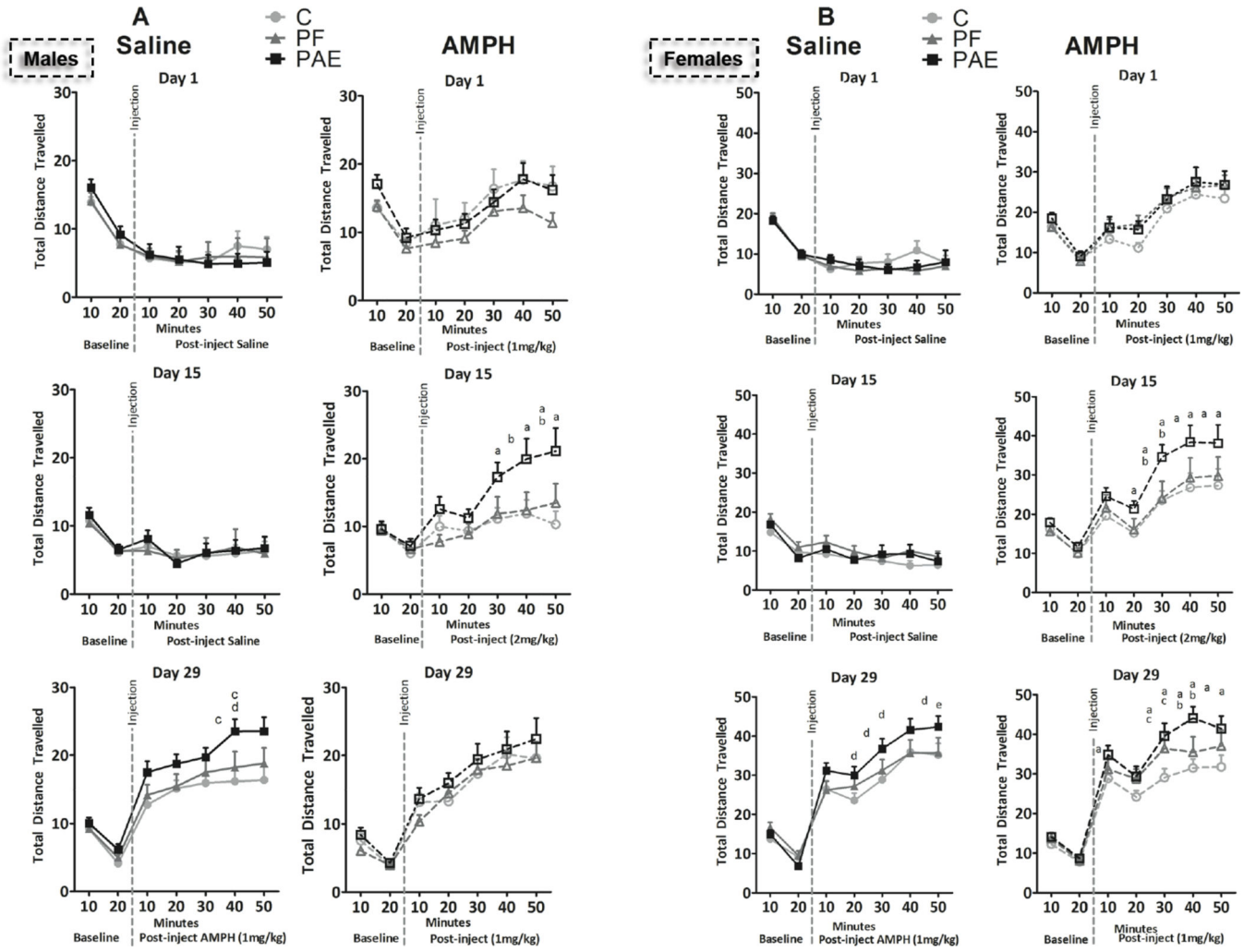


**Figure 2. Sensitization of 'typical' behaviors**

Mean±SEM ; n=18–20/group. Sensitization of 'typical' behaviors assessed by distance travelled during 80 min sessions in an open field on test days 1, 15 and 29 [behavior analyzed in 5 min bins, but presented in 10 min bins]. AMPH-treated rats received 1 mg/kg AMPH for the first 4 injections and 2 mg/kg AMPH for the last 4 injections, while saline-treated rats only received saline-injections during this time (Days 1–15 every other day). On Day 29, after a 14 day washout period, *all* subjects (both AMPH- and saline-pretreated) received a single injection of AMPH (1 mg/kg, i.p.) to examine sensitization. A) Males: Saline-treated (left): [c]PAE>C; [d]PAE>PF. AMPH-treated (right): [a]PAE>C; [b]PAE>PF. B) Females: Saline-treated (left): [d]PAE>C; [e]PAE>PF. AMPH-treated (right): [a]PAE>C; [b]PAE>PF; [c]PF>C. Time of injection indicated by dashed line. Both saline- and AMPH-pretreated groups received 1mg/kg AMPH on Day 29.

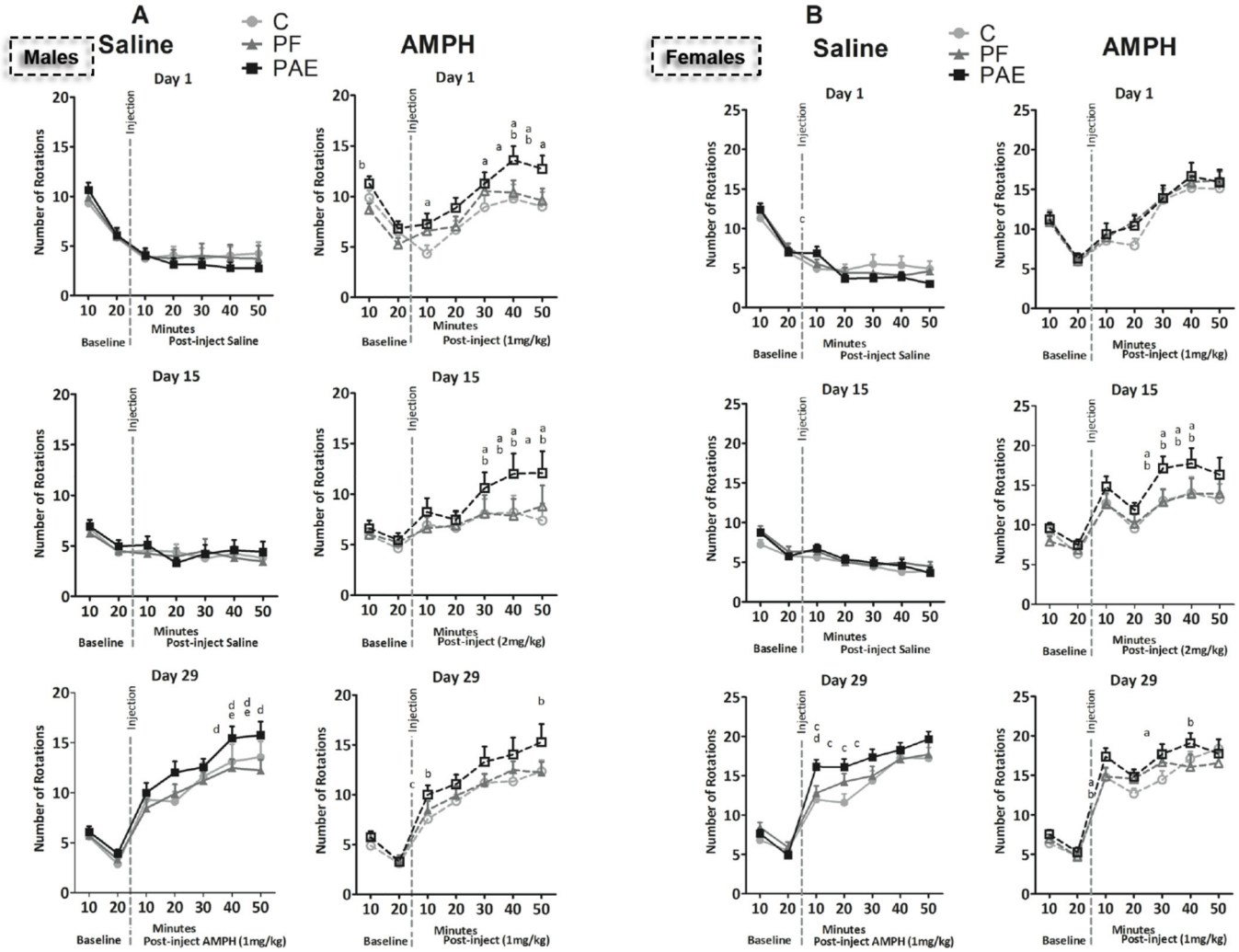


**Figure 3. Sensitization of 'atypical' behaviors**

Mean±SEM; n=18–20/group. Sensitization of 'atypical' behaviors assessed by number of 360° rotations during 80 min sessions inside an open field on test days: 1, 15 and 29 [behavior analyzed in 5 min bins, but presented in 10 min bins]. AMPH-treated rats received 1 mg/kg AMPH for the first 4 injections and 2 mg/kg AMPH for the last 4 injections, while saline-treated rats only received saline-injections during this time. On Day 29, after a 14 day washout period, *all* subjects (both AMPH- and saline-pretreated) received a single injection of AMPH (1 mg/kg, i.p.) to examine sensitization. A) Males: Saline-treated (left): $^{d}$PAE>C; $^{e}$PAE>PF. AMPH-treated (right): $^{a}$PAE>C; $^{b}$PAE>PF; $^{c}$PF<C. B) Females: Saline-treated (left): $^{c}$PAE>C; $^{d}$PAE>PF. AMPH-treated (right): $^{a}$PAE>C; $^{b}$PAE>PF. Time of injection indicated by dashed line. Both saline- and AMPH-pretreated groups received 1mg/kg AMPH on Day 29.

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**Table 1**

Stress Hormone Levels and DA-R Optical Densities

| **A) Hormone Levels** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sex** | **Group** | **Basal** | | **Stress** | | **Basal** | | **Stress** | |
| | | **Saline** | **AMPH** | **Saline** | **AMPH** | **Saline** | **AMPH** | **Saline** | **AMPH** |
| | | **Adrenocorticotropin (ACTH) (pg/ml)** | | | | **Corticosterone (CORT) (ng/ml)** | | | |
| Male | C | 46.8±6 | 40.9±3 | 201.2±37 | 202.5±36 | 1.4±0.7 | 0.7±0.1 | 30.4±4 | 38.5±7 |
| | PF | 50.6±12 | 35.7±4 | 256.1±69 | 248.5±32 | 0.7±0.1 | 2.9±1.5 | 34.9±6 | 35.0±4 |
| | PAE | 39.0±2 | 42.8±6 | 278.0±37[a] | 376.4±70[a,b] | 1.0±0.4 | 1.1±0.4 | 40.3±3 | 41.3±5 |
| Female[f] | C | 54.0±6 | 53.6±6 | 525.2±86 | 580.0±173 | 13.9±6 | 6.9±1 | 79.2±6 | 91.9±2 |
| | PF | 85.3±19 | 64.4±7 | 644.8±172 | 500.0±76 | 15.9±4 | 14.9±6 | 79.7±7 | 96.6±2 |
| | PAE | 59.3±9 | 53.6±6 | 432.1±38 | 570.3±108 | 10.5±4 | 9.4±4 | 72.6±3 | 88.1±4[c] |

| **B) DA-R expression (optical density)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sex** | **Group** | **Saline** | **AMPH** | **Saline** | **AMPH** | **Saline** | **AMPH** | **Saline** | **AMPH** |
| | | **$D_1$** | | **$D_2$** | | **$D_1$** | | **$D_2$** | |
| | | **Prelimbic** | | | | **Infralimbic** | | | |
| Male | C | 986.7±175 | 866.8±103 | 595.6±95 | 594.4±87 | 1028±140 | 876.4±112 | 597.5±110 | 560.9±87 |
| | PF | 1044.3±155[d] | 899.1±66 | 794.1±92[d] | 652.6±88 | 1034.9±159[d] | 877.6±87 | 716.6±95[d] | 627.9±87 |
| | PAE | 956.4±117 | 887.5±135 | 535.6±82 | 564.0±107 | 816.0±94[e] | 1036.0±204[b] | 475.0±61[e] | 644.5±141[b] |
| Female[f] | C | 1278.3±223 | 1341.5±214 | 1256.6±234 | 1270.7±173 | 1322.2±277 | 1382.3±124 | 1141.8±172 | 1238.9±166 |
| | PF | 1498.2±216 | 1437.5±154 | 1524.9±230 | 1308.3±115 | 1456.3±211 | 1426.4±160 | 1514.4±224 | 1271.1±113 |
| | PAE | 1305.8±132 | 1458.7±142 | 1252.3±129 | 1372.7±132 | 1285.9±122 | 1365.6±219 | 1272.5±118 | 1308.9±113 |

**A)** Stress hormone levels (ACTH (pg/ml) and CORT (ng/ml)) were assessed under basal and acute stress conditions.

**B)** DA-R expression in the mPFC (prelimbic and infralimbic subregions) as optical density assessed by ImageJ.

Mean±SEM (n=7–10/group).

[a] PAE>PF>C (p's<0.05);

[b] AMPH>Saline (p's<0.001); [b] PAE>PF=C (p's<0.01);

[c] statistical trend for AMPH>saline (p<0.05);

[d] PF>PAE=C (p's<0.05);

[e] PAE<PF=C (p's<0.01);

[f] Females>Males (p's<0.01).