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Role of corticosterone in anxiety- and depressive-like behavior and HPA regulation following prenatal alcohol exposure

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Abstract

Prenatal alcohol exposure (PAE) is known to cause dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, including hyperresponsivity to stressors. Dysregulation of the HPA axis plays a role in vulnerability to stress-related disorders, such as anxiety and depression. Thus, the effects of PAE on HPA function may result in increased vulnerability to the effects of stress and, in turn, lead to the development of stress-related disorders. Indeed, individuals prenatally exposed to alcohol have an increased risk of developing anxiety and depression. However, it is unclear whether hypersecretion of corticosterone (CORT) in response to stress *per se* is involved with mediating differential effects of stress in PAE and control animals. To investigate the role of CORT in mediating effects of stress in both adult females and males following PAE, adrenalectomy with CORT replacement (ADXR) was utilized to produce similar CORT levels among prenatal treatment groups before exposure to chronic unpredictable stress (CUS). Anxiety-like behavior was evaluated using the open field and elevated plus maze, and depressive-like behavior was examined in the forced swim test. Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA expression was assessed in the medial prefrontal cortex (mPFC), amygdala, and hippocampal formation. Under the non-CUS condition, PAE alone differentially altered anxiety-like behavior in sham but not ADXR females and males, with females showing decreased anxiety-like behavior but males exhibiting increased anxiety-like behavior compared to their control counterparts. There were no effects of PAE alone on depressive-like in females or males. PAE also decreased GR mRNA expression in the hippocampal formation in females but had no effects on MR or GR mRNA expression in any brain region in males. CUS had differential effects on

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CONTRIBUTIONS

All authors designed the experiment. VL, CR, LW, MC, GL, LE and WY collected the data. VL analyzed the data. VL and JW wrote the manuscript. All authors edited the manuscript.

DECLARATIONS OF INTEREST: none

Ethical Statement

All animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, the Canadian Council on Animal Care, and approved by the University of British Columbia Animal Care Committee.

anxiety- and depressive-like behavior in PAE and control animals, and these effects were sex dependent. Importantly, ADXR unmasked differences between PAE and control animals, demonstrating that CORT may play a differential role in modulating behavior and HPA activity/regulation in PAE and control animals, and may do so in a sex-dependent manner.

Keywords

prenatal alcohol exposure; chronic unpredictable stress; anxiety-like behavior; depressive-like behavior; MR; GR; corticosterone

1. INTRODUCTION

Prenatal alcohol exposure (PAE) can alter hypothalamic-pituitary-adrenal (HPA) function under resting conditions and following stress. Studies using animal models have found higher basal corticosterone (CORT) levels in PAE than control neonates (Angelogianni and Gianoulakis, 1989; Kakihana et al., 1980). PAE rats older than 3–5 days of age, however, typically show basal CORT levels comparable to those in control rats (Glavas et al., 2007; Kim et al., 1999b; Lam et al., 2018a; Nelson et al., 1986; Uban et al., 2013; Weinberg et al., 1996). In response to a wide range of stressors, PAE typically (with the exception of the stress hyporesponsive period) increased HPA activation and/or delayed return to basal CORT levels compared to control animals, and effects are often sex- and stressor-dependent (Angelogianni and Gianoulakis, 1989; Giberson et al., 1997; Kim et al., 1999a; Lee et al., 2000, 1990; Lee and Rivier, 1996; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982, 1981, Weinberg, 1992a, 1992b, 1988, Weinberg et al., 2008, 1996). Studies in humans have also reported that PAE increases both basal (Jacobson et al., 1999; Ramsay et al., 1996) and stress (Haley et al., 2006; Jacobson et al., 1999; Ouellet-Morin et al., 2011) levels of cortisol. However, other studies have found that PAE decreases (Ouellet-Morin et al., 2011) or does not change (Haley et al., 2006) basal cortisol levels. It is important to understand possible alterations in stress and HPA regulation following PAE, as exposed individuals are at a higher risk than unexposed individuals of encountering stressful environments during their lifetimes (O'Connor and Paley, 2006; Streissguth et al., 2004, 1991). Furthermore, dysregulation of the HPA axis has been suggested to contribute to increased risk of developing stress-related disorders, such as anxiety and depression (Jacobson, 2014; Nestler et al., 2002). It is possible that PAE-induced HPA dysregulation could predispose exposed individuals to an increased vulnerability to stress-related disorders following exposure to stressors over the life course. Indeed, studies suggest that individuals prenatally exposed to alcohol have an increased risk of developing one or more mental health problems over the life course, with anxiety and depression being among the most commonly encountered disorders (Famy et al., 1998; O'Connor et al., 2002; O'Connor and Paley, 2009; Pei et al., 2011; Streissguth et al., 2004, 1991). Preclinical studies support clinical findings that PAE can increase anxiety- and depressive-like behavior, and indicate that changes may occur in a sex-dependent manner (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Rouzer et al., 2017; Varlinskaya and Mooney, 2014; Wilcoxon et al., 2005). Moreover, it was shown that chronic unpredictable stress (CUS) differentially exacerbates anxiety- and depressive-like behavior in PAE and control animals, and that

again, effects may be sex dependent (Hellemans et al., 2010a, 2010b, Lam et al., 2018b, 2018a; Raineki et al., 2016). However, it is unclear whether HPA dysregulation is involved with mediating differential effects of CUS in PAE and control animals.

Two key receptors are involved in regulating HPA activity by mediating the effects of the glucocorticoids: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). MRs are implicated in regulating basal HPA tone, and may also play a role in setting the threshold of HPA reactivity to stress and maintaining high neuronal excitability (Joëls and de Kloet, 2017; ter Heegde et al., 2015). On the other hand, GRs appear to be involved primarily in mediating feedforward/feedback regulation of the stress response (Herman et al., 2016). Importantly, dysregulation of MR and GR has been suggested to play a role in the psychopathology underlying anxiety and depression (Holsboer and Ising, 2010, 2008; Inda et al., 2017; Joëls and de Kloet, 2017; Veenit et al., 2014). PAE has been shown to alter MR and GR mRNA expression, often in a sex-dependent manner. PAE was shown to decrease MR mRNA expression in the adult female hippocampus (Sliwowska et al., 2008; Uban et al., 2013), and to differentially decrease medial prefrontal cortex (mPFC) and amygdala GR mRNA expression in adult females and males (Lam et al., 2018a). As such, dysregulation of these key receptor systems by PAE may predispose individuals to the adverse effects of stress over the life course, which may in turn exacerbate HPA dysregulation, and increase vulnerability to stress-related disorders.

PAE also alters the role of glucocorticoids in regulating expression of these receptors. Removal of endogenous CORT feedback by adrenalectomy (ADX) revealed higher hippocampal MR and GR mRNA expression in PAE females and males, respectively, than in their control counterparts (Glavas et al., 2007). While CORT replacement at basal physiological levels normalized these PAE effects following ADX, it was insufficient in PAE compared to control males at normalizing the ADX-induced increase in hippocampal MR mRNA expression (Glavas et al., 2007). However, the role of CORT in regulating emotional behavior in the context of PAE has not been assessed.

The present study builds on and extends previous findings to investigate whether PAE-induced HPA dysregulation – in particular, the role of CORT – mediates the differential effects of CUS on anxiety- and depressive-like behavior and expression of MR and GR mRNA in PAE and control animals. We utilized ADX with CORT replacement (ADXR) at basal physiological levels to produce similar basal levels of CORT among prenatal treatment groups. Another cohort of animals underwent sham surgery and thus remained adrenal-intact. Following recovery from surgery, animals were either exposed to CUS or left undisturbed. We identified the effects of PAE and CUS on behavior and on MR and GR mRNA expression in sham (adrenal-intact) animals. In animals that underwent ADXR, we examined whether behavior, as well as MR and GR mRNA expression following CUS were normalized in PAE animals to control levels. Anxiety- and depressive-like behaviors were evaluated using the open field, elevated plus maze, and forced swim tests. Expression of MR and GR mRNA was assessed in the mPFC, amygdala, and hippocampal formation – brain areas key to both stress and emotional regulation.

2. METHODS

2.1 Animals and breeding

All animal use and care procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Canadian Council on Animal Care, and approved by the University of British Columbia Animal Care Committee. Adult virgin male (275–300g) and female (265–300g) Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, PQ, Canada). Rats were pair-housed by sex on corn cob bedding, and habituated to the Centre for Disease Modeling which was maintained on a 12:12 hour light/dark cycle (lights on at 07:00 h) with controlled temperature (21±1°C) for a 7–10-day period. During habituation, animals had *ad libitum* access to 18% protein chow (Teklad Global #2018) and water. For breeding, males were singly housed, and a female and male were paired. Presence of sperm in vaginal lavage samples taken every morning at 08:00 h indicated day 1 of gestation (GD 1).

2.2 Diets and feeding

On GD 1, females were singly housed in polycarbonate cages on ventilated racks with corn cob bedding. Dams were randomly assigned to one of three treatment groups: 1) alcohol-fed (PAE; n=13), receiving a liquid ethanol diet with 36% ethanol-derived calories, 6.7% v/v; 2) pair-fed (PF; n=11), receiving a liquid control diet with maltose-dextrin isocalorically substituted for ethanol, in an amount consumed by a PAE partner (g/kg body weight/day of gestation); and 3) *ad libitum*-fed control (C; n=10), receiving a pelleted control diet. All diets were formulated to provide optimal nutrition during pregnancy (Weinberg/Keiver High Protein Ethanol [#710324] and Control [#710109] diets; as well as Weinberg/Keiver High Protein Pelleted Control Diet [#102698] from Dyets, Inc., Bethlehem, PA, USA) (Lan et al., 2006).

The diets were presented fresh daily, one hour prior to lights off to minimize shifts in the maternal CORT circadian rhythm (Gallo and Weinberg, 1981; Krieger, 1974), and at that time the volume of liquid diet consumed since the previous night was recorded. All groups also received *ad libitum* access to water. Liquid ethanol diet was introduced gradually over the first 2 days of pregnancy with a 1:2 ratio of liquid ethanol to liquid control diet on GD 1 and 2:1 ratio on GD 2 to facilitate the transition into a full liquid ethanol diet beginning on GD 3. On GD 17, blood alcohol levels, from blood samples collected 3-hr after lights off from the tail vein, were determined using an assay from Pointe Scientific Inc. (Canton, MI, USA). Experimental diets continued through GD 21. Beginning on GD 22 and throughout lactation, ethanol-fed dams were switched to a 19% high protein laboratory chow (Teklad Global #2019) and water to minimize adverse effects of ethanol on maternal lactation. Dams from the other two diet groups were given the same 19% high protein laboratory chow (Teklad Global #2019) beginning GD22. At birth, pups were weighed, and litters randomly culled to 12 (6 males, 6 females when possible). If necessary, pups from the same prenatal group born on the same day were fostered into a litter to maintain the litter size. Dams and offspring were weighed weekly but were otherwise undisturbed until weaning on postnatal day (PN) 22, after which pups were group housed by litter and sex until they were pair-housed at PN 40±2. Animals of the same sex and from the same prenatal group, but from

different litters born ±2 days apart were paired. Weaned pups and adults were fed an 18% protein chow (Teklad Global #2018) and housed on non-ventilated racks.

2.3 Adrenalectomy and corticosterone replacement (ADXR)

A pilot experiment was first done in adult (PN 55–65) control females and males (n=10 per sex) that were not part of the present study to determine the effectiveness of the ADXR procedure in simulating typical morning (trough) and evening (peak) basal CORT levels. Bilateral ADX was carried out via a dorsal approach under isoflurane anesthesia; sham surgery involved the same procedures except the adrenal glands were not removed. Immediately following ADX, CORT was provided in the drinking water (0.9% NaCl, 0.2% ethanol), at concentrations of 75 and 25 μg⁄ml for females and males, respectively. The replacement level for females was determined through previous pilot testing in our laboratory and was shown to normalize thymus weight (Glavas, 2003). The CORT concentration for males was chosen based on previous studies, which demonstrated that this level of CORT replacement results in basal plasma CORT levels, and normalizes basal ACTH levels and thymus weight compared with ADX alone (Akana et al., 1985; Jacobson et al., 1988). Animals were followed for 21 days, with blood samples collected from the tail vein at 09:00h for the AM samples and 22:00h for the PM samples on days 5, 14, and 21 for determining plasma CORT levels.

ADXR procedures in the present study were identical to those in the pilot experiment above, except blood sampling was not done on days 5, 14 and 21 to avoid additional stress. In adulthood (PN 55–65), females and males from each prenatal group (C, PF, and PAE) were randomly assigned to either the Sham or ADXR surgical condition. Only one female and one male offspring from each litter were assigned to any one condition. CORT was provided in drinking water immediately following ADX in concentrations as described above to provide similar plasma CORT at low basal levels among prenatal treatment groups. Twenty-four-hr CORT intakes pre-CUS (the day before and Day 1 of CUS) and post-CUS (Day 10, and the day after CUS ended) by ADXR animals were determined by multiplying the volume of solution consumed by the concentration of the solution (i.e. 75 μg⁄ml for females and 25 μg⁄ml for males).

Thymus weight was not measured in the present study. However, previous experiments in our laboratory (Glavas, 2003; Glavas et al., 2001) found that the 75 and 25 μg⁄ml replacement levels for females and males, respectively, normalize thymus weight among prenatal treatment groups (see TABLE S1 in Supplementary Materials).

2.4 Chronic unpredictable stress (CUS) paradigm

Following a 6-day rest from surgery, animals from each experimental group were randomly assigned to either stressed (CUS) or non-stressed (non-CUS) condition. Only one female and one male offspring from each litter were assigned to any one condition. CUS involved 10 days of twice daily unpredictable exposure to mild stressors, with a minimum of 2 hr between stressors. All CUS rats were exposed, in variable order, to the same number of each stressor over the 10-day period. Stressors used included: Platform: Animals placed on 20 × 20 cm transparent Plexiglas platforms, mounted on 90 cm high posts, for 20 min. Restraint:

Animals restrained in PVC tubes (15 cm × 6 cm for females and 19 cm × 7 cm for males) with ventilation holes for 30 min. Soiled Cage: Animals placed in a cage with soiled bedding from other same-sex animals for 1 hr. Social isolation: 12 hr of isolation in smaller cages (28 cm length X 17 cm width x 12.5 cm height) beginning at lights off with food and either water (Sham) or water with CORT (ADXR animals). Animals are returned to the home cage in the morning. Cage Tilt: Home cages tilted to a 30° angle for 2 hr. Novel Cage: Animals housed in pairs in a new cage without bedding, food, or water for 1 hr. Non-CUS animals were left undisturbed with the exception of regular cage changes.

2.5 Behavioral testing

Behavioral testing began on the third day after the end of CUS. All animals were assessed on consecutive days, with at least a one-day break between tests, in the open field (OF), elevated plus maze (EPM), and forced swim tests (FST; see FIGURE 1 for an experimental timeline). 24 hr prior to each behavioral test, animals were habituated to the testing room for 20 min. n=9–11 per prenatal treatment/CUS exposure/surgical condition/sex.

The OF apparatus was a square arena (80 × 80 cm) enclosed by transparent Plexiglas walls (40 cm). Animals were started in the periphery at the mid-point along one of the four walls, and were allowed to roam freely for 5 min under bright lighting. Total distance travelled (cm) in the field, as well as distance travelled, time spent, and entries into the center zone were analyzed. Data for the open field were recorded and automatically analyzed using the MotorMonitor™ software.

The EPM was elevated 40 cm above ground and consisted of two open arms and two arms enclosed by 40 cm opaque walls (each arm is 50 × 10 cm, and the center area is 10 × 10 cm). Under dim lighting, animals were individually placed on the center of the maze facing an open arm and were allowed to roam freely for 5 min. Time spent on the open arms as a percent of open and closed arms time, and frequency of closed arm entries were assessed. Entry into an arm is defined as when all four paws are in the zone. EPM behaviors were recorded and scored using The Observer 5.0 software (Noldus, Wageningen, The Netherlands).

The FST apparatus was a transparent Plexiglas cylinder (20 cm diameter, 60.5 cm height). The cylinders were filled with 25 ± 1°C water to a 44.5 cm depth to prevent animals' tails from touching the bottom of the tank (Detke et al., 1995). Twenty-four hour before testing, animals were placed in the cylinders for 15 min to experience the fact that escape was impossible. On the day of testing, they were tested for 5 min. Activity on the testing day was recorded by a video camera placed directly facing the cylinder for later scoring. Animals were towel-dried after each exposure and returned to preheated home cages. Duration of immobility was measured, following the criteria of Armario et al. (Armario et al., 1988); i.e., the rat performs the minimum movement necessary to stay afloat.

All testing was done during the light phase of the circadian cycle. White noise was played at 30 dB in the background during testing to dampen random noise. All behaviors were analyzed by an observer blind to the prenatal group and stress exposure.

2.6 Blood sampling and tissue collection

Basal blood samples were collected via tail nick on Day 1 of CUS prior to stress and on the day after CUS ended. Blood samples from animals in the non-CUS condition were collected at the same times. Samples were collected within 2 min of touching the cage to obtain a true basal measure. Blood was collected on ice in plastic centrifuge tubes containing 5μl/100 μl blood of EDTA (anti-coagulant; Fisher Scientific, ON, Canada) and 2.64 μg/100 μl blood of aprotinin (anti-protease; Sigma-Aldrich, ON, Canada). Blood was centrifuged within 60 min of sample collection at 2190g for 10 min at 4°C, and the plasma was stored at −20°C until assayed.

Whole brains were collected via decapitation 30 min after testing on Day 2 of the FST, snap frozen on powdered dry ice, and stored at −80°C.

2.7 Radioimmunoassays

Corticosterone: Total CORT levels (bound plus free) were measured using a modification of the ImmuChem™ Corticosterone I^{125} Radioimmunoassay Kit (MP Biomedicals, Orangeburg, NY); all reagents and samples were halved and the assay was performed according to the vendor instructions. The minimum detectable range was 7.7 ng/ml, and the inter- and intra-assay coefficients of variation were <7.2% and <10.3%, respectively, as provided by the vendor.

ACTH: The ImmuChem™ Double Antibody hACTH I^{125} RIA Kit (MP Biomedicals, Orangeburg, NY) was used. The minimum detectable range was 5.7 pg/ml, and the inter- and intra-assay coefficients of variation were <10.7% and <6.8%, respectively, as provided by the vendor.

2.8 *In situ* hybridization

20μm coronal sections were collected on a cryostat at −16 °C, mounted on slides (Superfrost slides, Fisher Scientific, ON, Canada), and stored at –80°C. For all brain areas, n=6–7 per prenatal treatment/CUS exposure/surgical condition/sex.

Probe and labeling: Ribonucleotide probes were used to detect GR mRNA in the medial prefrontal cortex (mPFC; prelimbic [PrL] and infralimbic [IL] cortices), amygdala (central, medial, lateral, and basal nuclei), and the hippocampal formation (dentate gyrus [DG], CA3, CA1, and ventral subiculum) [see FIGURES S1–3 in Supplementary Materials]. The rat GR ribonucleotide probe was prepared using a 456 bp template (complementary to the coding region and 3' untranslated region of rat GR mRNA) and was provided by Dr. James Herman (Department of Psychiatry and Behavioral Neuroscience, College of Medicine, University of Cincinnati, USA) (Herman et al., 1999). A ribonucleotide probe was also used to detect MR mRNA in the hippocampal formation, and it was prepared using a 550 bp template (complementary to the coding region and 3' untranslated region of rat MR mRNA) also from Dr. James Herman (Herman et al., 1999). The ribonucleotide probes were labeled with 35S-UTP (Amersham Biosciences, NJ, USA) using Polymerase T7 (GR) or T3 (MR) and Promega Riboprobe System (Promega Corporation, Madison, WI, USA). All probes were

purified using Micro Bio-Spin 30 Columns (Bio-Rad, CA, USA). 1M of DTT was added to prevent oxidation.

Hybridization: The slides were thawed for 20 min, fixed in formalin for 30 min, and then pre-hybridized as follows: 1X PBS twice for 10 min each, proteinase K (100 μg/L) digestion at 37°C for 9 min, 0.1M triethanolamine-hydrochloride (TEA) for 10 min, acetylation by 0.1M TEA with 0.25% acetic anhydride for 10 min, 2X sodium saline citrate (SSC) twice for 5 min each, dehydration through a graded series of ethanol, delipidation in chloroform for 5 min, and finally 100% ethanol before being air-dried. Hybridization buffer (75% formamide, 3X SSC, 1X Denhardt's solution, 200 μg/mL yeast tRNA, 50 nM sodium phosphate buffer (pH 7.4), 10% dextran sulphate, and 10mM DTT) was applied (1×10^6 cpm/slide) and covered with HybriSlips (Sigma-Aldrich, ON, Canada). The sections were incubated overnight at 55°C in humidified chambers (75% formamide). HybriSlips were then removed and slides were rinsed as follows: 2X SSC twice for 20 min, 2X SSC for 30 min, 50 μg/L RNAse A solution at 37 °C for 60 min, 2X SSC with 0.01M DTT for 10 min, 1X SSC for 10 min, 0.5X SSC with 0.01M DTT for 10 min, 0.1X SSC with 0.01M DDT at 60 °C for 60 min, and 0.1X SSC for 15 min. The sections were then dehydrated with a graded series of ethanol, and finally air dried overnight.

Kodak BioMax MR autoradiography film was exposed to hybridized slides of the medial prefrontal cortex (mPFC), amygdala, and hippocampal formation. Exposure time were as follows: 14 days for GR in the mPFC; 26 days for GR in the amygdala; and 8 days for GR and 7 days for MR in the hippocampal formation. The exposed autoradiography films were developed using Kodak GBX developer and fixer.

Densitometric analysis: The autoradiographic films were scanned and analyzed using Scion Image 4.0.3.2 (National Institutes of Health, USA) according to the Paxinos and Watson Stereotaxic Rat Brain Atlas, Fifth edition (Paxinos and Watson, 2004). Mean grey density levels were measured from Bregma 3.00 mm to 2.76 mm for the mPFC; Bregma −2.64 mm to −3.00 mm for the amygdala; and Bregma −4.80 mm to −5.28 mm for the hippocampal formation. Consistent with other studies in the literature, the Bregma range chosen for the hippocampal formation includes the ventral/temporal hippocampus which primarily relates to emotion and stress regulation (Abela et al., 2013; Bast et al., 2009; Burton et al., 2009; Consolo et al., 1994; Fanselow and Dong, 2010; Ferbinteanu and McDonald, 2000; Strange et al., 2014). As previously described (Lam et al., 2018b), the left and right sides of each brain subregion were traced freehand in two sections per animal for a total of four measurements to determine mean gray density levels. Background was measured from the forceps minor for mPFC, the internal capsule for the amygdala, and corpus callosum for the hippocampal formation. Corrected gray levels were obtained by subtracting the background level from each of the four measurements and the four measurements were then averaged together for analysis.

2.9 Statistical Analyses

Due to known sexual dimorphisms of the HPA axis, all data were analyzed separately for females and males. AM and PM basal CORT data from control animals from the pilot study

were compared using a paired t test using IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software (IBM, Armonk, NY, USA). Pre-CUS CORT intake (mg per kg body weight) of ADXR animals was analyzed by one-way analysis of variance (ANOVA) for the factor of prenatal treatment (C/PF/PAE). Pre-CUS basal CORT data were analyzed by two-way ANOVA for the factors of prenatal treatment and surgical condition. Post-CUS CORT intake (mg per kg body weight) of ADXR animals was analyzed by two-way ANOVA for the factors of prenatal treatment and CUS exposure. All other data were analyzed by three-way ANOVA for the factors of prenatal treatment, surgical condition, and CUS exposure. All ANOVAs were followed by *post hoc* pairwise compairisons with Šídák correction to examine significant main effects and interactions. Differences were considered significant at $p \leq 0.05$. Further analyses on the behavioral and brain data utilized planned comparisons to test the *a priori* hypotheses that: 1) PAE will alter depressive- and anxiety-like behavior and mRNA levels of MR and GR in brain areas involved with both HPA and emotional regulation; 2) CUS will differentially alter behavior and mRNA levels of MR and GR in PAE compared to control animals; and 3) CORT will mediate the effects of PAE on behavior and MR and GR mRNA levels. *A priori* hypotheses 1 and 2 refer to animals in the sham condition, and planned comparisons were made between PAE and control animals within the same CUS condition (e.g. non-CUS PAE vs non-CUS C). *A priori* hypothesis 3 refers to animals in the ADXR condition, and the same planned comparisons used to address hypotheses 1 and 2 were performed. P values from ANOVAs and *a priori* analyses are cited in the Results section; p values from *post hoc* and *a priori* comparisons are indicated in the Figures and noted in the Figure Legends.

3. RESULTS

3.1 Pilot Study: AM and PM basal CORT levels in ADXR control animals

Morning and evening CORT levels were significantly different from each other in both female [$t(9) = -7.037$, $p < 0.001$] and male [$t(9) = -9.521$, $p < 0.001$] controls following ADXR (FIGURE 2). Importantly, replacing CORT by putting it in the drinking water of ADX animals resulted in a typical circadian rhythm and relatively low variability in CORT levels.

3.2 Blood alcohol levels, CORT intake (mg/kg body weight), and changes in body weight

Blood alcohol levels were 122.0 ± 10.1 mg/dl in PAE dams and undetectable in PF and control dams. In the offspring, there were no effects of prenatal treatment on pre-CUS CORT intake, and no effects of prenatal treatment or CUS exposure on post-CUS CORT intake in ADXR animals (TABLE S2 in the Supplementary Materials).

There were no significant effects of prenatal treatment, surgical condition, or CUS exposure on body weight in females (FIGURE 3A). By contrast, in males, CUS decreased weight gain (main effect of CUS exposure: $F_{1,111} = 38.727$, $p < 0.001$; FIGURE 3B). In addition, prenatal treatment interacted with surgical condition to alter body weight (prenatal treatment x surgical condition interaction: $F_{2,111} = 4.208$, $p = 0.017$; FIGURE 3B). In the ADXR condition, PAE gained less weight than control males, and both PAE and PF males gained less weight than their sham counterparts.

3.3 Basal CORT and ACTH levels

Pre-CUS CORT.—Pre-CUS basal CORT levels were differentially altered in females and males, with lower levels in ADXR females and higher levels in ADXR males compared to their respective sham counterparts (main effect of surgical condition – females: $F_{1,108}$ = 11.838, p = 0.001; male: $F_{1,109}$ = 4.313, p = 0.040; FIGURES 4A,C).

Pre-CUS ACTH.—In both females and males, pre-CUS basal ACTH levels were higher in the ADXR than the sham condition (main effect of surgical condition – females: $F_{1,106}$ = 27.482, p < 0.001; males: $F_{1,112}$ = 19.145, p < 0.001; FIGURES 4B,D).

Post-CUS CORT.—In females, post-CUS basal CORT levels were lower overall in the ADXR than the sham condition (main effect of surgical condition: $F_{1,106}$ = 23.108, p < 0.001; FIGURE 5A). Furthermore, as expected, CUS increased basal CORT levels in all prenatal groups under the sham condition, while there were no differences between non-CUS and CUS females under the ADXR condition (surgical condition x CUS interaction: $F_{1,106}$ = 3.990, p = 0.048; FIGURE 5A).

There were no effects of prenatal treatment, surgical condition, or CUS exposure in males (FIGURE 5C).

Post-CUS ACTH.—In females, post-CUS basal ACTH levels were higher in the ADXR than the sham condition (main effect of surgical condition: $F_{1,92}$ = 15.692, p <0.001; FIGURE 5B).

In males, both PAE and PF animals had higher post-CUS basal ACTH levels than control males (main effect of prenatal treatment: $F_{2,100}$ = 4.452, p = 0.014). In addition, a surgical condition x CUS exposure interaction ($F_{1,100}$ = 8.019, p = 0.006; FIGURE 5D) indicated that while there were no effects of CUS under the sham condition, CUS increased basal ACTH levels overall under the ADXR condition compared to both their non-CUS and sham counterparts. However, inspection of FIGURE 5D indicates that this effect is driven primarily by PAE and PF males.

3.4 Behavior in the OF, EPM, and FST

OF.—In females, the effects of prenatal treatment and CUS exposure on time in center depended on surgical condition (prenatal treatment x surgical condition x CUS exposure interaction: $F_{2,108}$ = 3.412, p = 0.037; FIGURE 6A). In the sham non-CUS condition, PAE females spent more time in the center than PF females, whereas in the ADXR condition, CUS increased time in center in control females compared to their sham counterparts and decreased time in center in PF females compared to their control counterparts; however, CUS had no effects on PAE females. Furthermore, frequency of center entries differed among prenatal treatment groups depending on surgical condition (prenatal treatment x surgical condition interaction: $F_{2,108}$ = 3.476, p =0.034; FIGURE 6B). Under the sham condition, PAE females entered the center more frequently than both C and PF females, with the greatest frequency of center entries in PAE females in the non-CUS conditions (*a priori* analysis, p = 0.011), whereas following ADXR, C but not PAE or PF females showed a

higher frequency of center entries than their sham counterparts (*post-hoc* analysis, see FIGURE 6B).

Distance travelled in the center was higher overall in PAE than PF females (main effect of prenatal treatment: $F_{2,108} = 4.321$, $p = 0.016$, FIGURE 6C).

In males, CUS differentially affected the three prenatal treatment groups (prenatal treatment x CUS exposure interaction – time: $F_{2,110} = 5.835$, $p = 0.004$, FIGURE 6D; entries: $F_{2,110} = 4.192$, $p = 0.018$, FIGURE 6E; distance: $F_{2,110} = 4.077$, $p = 0.020$, FIGURE 6F). Specifically, CUS decreased time in center, frequency of center entries, and distance travelled in the center in control males compared to their non-CUS counterparts, and increased time in center in PF animals compared to their non-CUS counterparts, while behavior of PAE males was not altered by CUS. In addition, *a priori* analyses revealed that in the sham non-CUS condition, PAE males spent less time in the center and had fewer center entries than control males (time: $p = 0.047$, FIGURE 6D; entries: $p = 0.019$, FIGURE 6E).

There were no effects of prenatal treatment, surgical condition, or CUS on total distance travelled in the OF in either females or males (data not shown).

EPM.—In females, prenatal treatment and CUS exposure interacted to alter percent time on open arms (prenatal treatment x CUS exposure interaction: $F_{2,107} = 3.912$, $p = 0.023$; FIGURE 7A). Specifically, under the non-CUS condition, PAE females spent more time on open arms than their PF counterparts. However, PAE but not PF or control females showed reduced time on open arms following CUS compared to their non-CUS counterparts. Overall, PAE females also entered the closed arms more frequently than both C and PF females (main effect of prenatal treatment: $F_{2,108} = 5.182$, $p = 0.007$; FIGURE 7B).

In males, percent time on open arms was lower overall in the ADXR than the sham condition (main effect of surgical condition: $F_{1,107} = 4.556$, $p = 0.035$; FIGURE 7C). As well, prenatal treatment and CUS exposure interacted to alter frequency of closed arm entries (prenatal treatment x CUS exposure interaction: $F_{2,111} = 4.038$, $p = 0.020$; FIGURE 7D). Under non-CUS conditions, PAE had more closed arm entries than PF males.

FST.—There were no effects of prenatal treatment, surgical condition, or CUS on time immobile in the FST in females. By contrast, in males, the effects of prenatal treatment and CUS exposure depended on surgical condition (prenatal treatment x CUS exposure x surgical condition interaction: $F_{2,110} = 3.335$, $p = 0.039$; FIGURE 8B). Under the sham condition, CUS increased time immobile in PAE males compared to their control counterparts, whereas under the ADXR condition, non-CUS PAE males showed more time immobile than their sham counterparts. In addition, *a priori* analyses revealed that non-CUS PAE males showed more time immobile than non-CUS C males ($p = 0.024$; FIGURE 8B).

3.5 GR and MR mRNA expression

mPFC – GR mRNA expression.—In females, ADXR and CUS differentially affected PrL and IL GR mRNA expression among prenatal treatment groups (prenatal treatment x

surgical condition x CUS exposure interaction – PrL: $F_{2,69} = 3.196$, p =0.047, FIGURE 9A; IL: $F_{2,69} = 5.202$, p = 0.008, FIGURE 9B). Non-CUS control females in the non-CUS ADXR condition had higher GR mRNA expression in both the PrL and IL than their sham counterparts. Moreover, under ADXR conditions, CUS decreased GR mRNA expression in the PrL of control females compared to their non-CUS counterparts, and in the IL of control females compared to both their non-CUS and sham counterparts. This resulted in higher GR mRNA expression in the PrL and IL following CUS in ADXR PAE than ADXR control females (*a priori* analyses for PrL: p = 0.023; FIGURE 9A; *post-hoc* comparisons for IL: p = 0.024, FIGURE 9B).

There were no effects of prenatal treatment, surgical condition, or CUS exposure in males (FIGURES 9C,D).

Amygdala – GR mRNA expression.—There were no effects of prenatal treatment, surgical condition, or CUS exposure on GR mRNA expression in any nuclei of the amygdala measured in either males or females (data not shown).

Hippocampal formation – GR mRNA expression.—In females, ADXR increased GR mRNA expression overall in the DG, CA3, and CA1 hippocampal subregions compared to the sham condition (main effect of surgical condition – DG: $F_{1,69} = 8.448$, p = 0.005, FIGURE 10A; CA3: $F_{1,69} = 8.370$, p = 0.005, FIGURE 10B; CA1: $F_{1,69} = 8.559$, p = 0.005, FIGURE 10C;). In addition, PF showed lower GR mRNA expression than control females in the CA3 and ventral subiculum, independent of CUS exposure or ADXR condition (main effect of prenatal treatment – CA3: $F_{2,69} = 3.656$, p = 0.031, FIGURE 10B; ventral subiculum: $F_{2,69} = 3.242$, p = 0.045, FIGURE 10D). In the ventral subiculum, *a priori* analyses further revealed that PAE females in the sham non-CUS condition had lower GR mRNA expression than their control counterparts (p = 0.049; FIGURE 10D).

In males, CUS overall decreased GR mRNA expression in the CA3, independent of prenatal treatment or surgical condition (main effect of CUS exposure: $F_{1,72} = 4.405$, p = 0.039; FIGURE 10F).

Hippocampal formation – MR mRNA expression.—In females, there were no effects of prenatal treatment, surgical condition, or CUS exposure on MR mRNA expression. By contrast, in males, ADXR decreased MR mRNA expression in the DG (mean grey value ± SEM of 43.7 ± 1.2 for ADXR vs 48.3 ± 1.5 for sham; main effect of surgical condition: $F_{1,72} = 5.290$, p = 0.024).

4. DISCUSSION

The present study aimed to investigate whether PAE-induced HPA dysregulation mediates the differential effects of stress on behavior and expression of glucocorticoid receptors in PAE and control animals. We tested adrenal-intact (sham surgery) animals and utilized ADXR to produce similar CORT levels among prenatal treatment groups, and then examined the effects of CUS on adult females and males in behavioral tasks that measure anxiety- and depressive-like behavior. Following behavioral testing, we examined central

HPA regulation by measuring MR and GR mRNA expression in key limbic brain areas. We found that under the non-CUS condition, PAE differentially altered anxiety-like behavior and GR mRNA expression in females and males, but had no effects on depressive-like behavior. Specifically, PAE females exhibited decreased anxiety-like behavior while males showed increased anxiety-like behavior compared to their control counterparts. In the brain, PAE decreased GR mRNA expression in the hippocampal formation in females but had no effects on MR or GR mRNA expression in any brain region in males. In addition, CUS had differential effects on anxiety- and depressive-like behavior in PAE and control animals, and these effects were also sex dependent. CUS increased anxiety-like behavior in PAE females compared to their non-CUS PAE counterparts, but had no effects on depressive-like behavior. By contrast, CUS increased anxiety-like behavior in control but not PAE males, and increased depressive-like in PAE males compared to their non-CUS PAE counterparts. ADXR, at least at the current replacement concentrations of CORT, unmasked differences between PAE and control animals, suggesting that CORT may play a differential role in modulating behavior and HPA activity/regulation in PAE compared to control animals.

4.1 PAE males may have decreased sensitivity to negative feedback by CORT

Replacing CORT by adding it to the drinking water resulted in a typical circadian rhythm and relatively low variability in CORT levels. Consistent with our previous findings (Glavas et al., 2007, 2001), while ADXR maintained CORT levels within the basal physiological range in both females and males, levels were at the lower end of the physiological range for females and were insufficient to normalize ACTH levels for both females and males. Nevertheless, our previous data demonstrate that CORT replacement at these levels normalizes thymus weight among prenatal treatment groups (Glavas, 2003). Thymus weight is a sensitive indicator of normal plasma CORT levels, as slight deviations from this range were shown to result in thymic enlargement or atrophy (Akana et al., 1985; Glavas et al., 2007). Although our ADXR procedure did not normalize all CORT-mediated functions (e.g. ACTH levels), it is important to note that CORT intake and basal CORT levels were not different among prenatal treatment groups.

While there were no effects of prenatal treatment in females, we found that post-CUS basal ACTH levels were increased overall in both PAE and PF compared to control males. Inspection of Figure 5D indicates that this effect was driven primarily by CUS-induced increases in PAE and PF males in the ADXR condition. That ADX with CORT replacement unmasked higher ACTH levels in PAE compared to control males following CUS indicates that CORT replacement at the same levels normalized, at least partially, ACTH levels in control but not PAE males, providing support for the suggestion that PAE males may have decreased sensitivity to negative feedback regulation by CORT (Glavas et al., 2007). Furthermore, our previous data demonstrated that PAE males show increased HPA drive (Glavas et al., 2007, 2001; Lan et al., 2015) and increased pituitary sensitivity to CRH following dexamethasone suppression (Osborn et al., 2000) compared to control males. Together, these alterations in HPA regulation may contribute to the higher basal ACTH levels observed following CUS in PAE than control males.

4.2 Differential roles of CORT in regulating anxiety-like behavior and GR mRNA expression in PAE and control females

Previous studies on the effects of PAE on anxiety-like behavior in females have shown mixed results, with increased, decreased, or no effects of PAE on anxiety-like behavior in the EPM and/or OF reported (Fish et al., 2018; Gabriel et al., 2006; Hellemans et al., 2008; Lam et al., 2018a; Osborn et al., 1998b; Staples et al., 2013). In the current study, we found that in adrenal-intact females, PAE alone (the non-CUS condition) increased time in the center, frequency of center entries, and distance travelled in the OF, and also increased percent time on the open arms in the EPM compared to control and/or PF females, suggesting decreased anxiety-like behavior. Differences in behavior between PAE and control females cannot be attributed to differences in activity, given that locomotor activity was not different among prenatal treatment groups in the OF and EPM under sham conditions. However, data from other studies suggest that PAE animals may have deficits in response inhibition (Barron and Riley, 1990; Caul et al., 1979; Molina et al., 1984; Riley et al., 1979), and we cannot rule out the possibility that such deficits may contribute to the behavior observed in PAE animals.

Analysis of glucocorticoid receptor indicated that PAE decreased GR mRNA expression in the ventral subiculum compared to that in controls while there were no effects on GR mRNA expression in the IL, where we found decreases in GR previously (Lam et al., 2018a). These differential effects of PAE on GR mRNA expression between studies may help explain, at least partly, why anxiety-like behavior appeared to be decreased in the present study but increased in our previous study (Lam et al., 2018a). Although both the IL and the ventral subiculum play a role in feedback inhibition of the HPA axis (Herman et al., 2016), the ventral subiculum may also subserve associative learning of adaptive coping strategies in a stressful or arousing context, possibly through its involvement in context representation and connection with brain areas involved with action selection, such as the nucleus accumbens (NAc) (Floresco, 2015; Lipski et al., 2017). In fact, the ventral subiculum directly projects to the NAc, and this pathway is activated by acute stressors (Lipski et al., 2017). Furthermore, GRs may modulate BDNF expression, and BDNF is involved with neuronal growth and survival, as well as in synaptogenesis and plasticity (Chen et al., 2017). Therefore, changes in GR mRNA expression in the ventral subiculum may, at least indirectly, impact both feedback inhibition of the HPA axis and action selection following exposure to an acute stressor, such as the OF and EPM. It is possible that this may, at least partly, underlie the alterations in behavior on the OF and EPM in PAE females in the present study.

CUS had significant effects on both HPA activity and behavior, and further unmasked significant effects of prenatal treatment. Following CUS, basal CORT levels were increased in all females under the sham condition, which is consistent with the literature demonstrating that CUS typically increases basal CORT levels (Willner, 2017). Further, these data provide support that the current CUS paradigm was of adequate intensity to effectively increase basal HPA activity. Despite similar effects on basal CORT levels among prenatal treatment groups, the effects of CUS on behavior differed between PAE and control females. While there were no effects of CUS on behavior of C and PF females in either the OF or EPM, CUS decreased frequency of center entries in the OF and percent time on open arms in the EPM in PAE females to levels comparable to those of their control counterparts.

These results suggest CUS increases anxiety-like behavior in PAE females compared to behavior observed under non-CUS conditions. This is in line with the diagnostic criteria outlined in the DSM-V for major depressive disorder, in which anxious distress is a prominent feature; i.e., "symptoms…represent a change from previous functioning" ("Depressive disorders," 2013). Furthermore, focussing on the within-group effect of CUS is akin to how drugs are assessed for their anxiolytic or anxiogenic properties during screening. That is, whether a drug has anxiolytic or anxiogenic effects depends on the direction of change in behavior (e.g., increased or decreased open arm time in the EPM, respectively) within rat strains and independent of differences in baseline behavior among strains (Hogg, 1996; Ramos et al., 1997). Another possible interpretation for our findings is that CUS "normalizes" behavior of PAE females to that of their control counterparts. It may be that while the current CUS paradigm used had no effects on control females, it acted as a stress inoculation procedure for PAE females (Brockhurst et al., 2015). Regardless of interpretation, these results build on and extend previous findings, which demonstrate that the effects of acute (Gabriel et al., 2006; Osborn et al., 1998a) and chronic (Hellemans et al., 2008) stress on anxiety-like behavior may be more pronounced in PAE compared to control females, and support the suggestion that PAE females may be differentially sensitive to the effects of CUS on anxiety-like behavior compared to control females.

Our ADXR results suggest that CORT may have differential roles in the expression of anxietylike behavior and GR mRNA expression in PAE and control females. Control females in the ADXR condition in general showed decreased anxiety-like behavior compared to their sham counterparts. However, for PAE females, ADXR normalized behavior to the levels of control females, regardless of CUS exposure. These results suggest that CORT may be involved in modulating behavior in females – with lower CORT levels and/or ADX-induced inability to secrete CORT in response to stress resulting in decreased anxiety-like behavior in control but not PAE animals – supporting a differential role for CORT in behavior of PAE and control animals.

In addition to its possible role in the behavioral alterations observed, CORT may also play a role in modulating GR mRNA expression in the ventral subiculum and the mPFC in females, and again, its role may differ in PAE and control females. In the ventral subiculum, ADXR increased GR mRNA expression in PAE females to levels of control females, thus eliminating differences between groups. This finding suggests that CORT secretion in response to stress is involved with regulating GR mRNA expression in PAE females. Additionally, in the PrL and IL, which are both involved with feedback inhibition of the HPA axis in response to stress (Herman et al., 2016), GR mRNA expression was increased in non-CUS C, but not PAE, females under the ADXR compared to the sham condition. These results suggest that PAE females may have deficits in CORT-mediated GR regulation, and consequently, negative feedback regulation of the HPA axis, compared to control females, as they failed to show the typical alterations in GR mRNA expression exhibited by their control counterparts in response to ADXR.

Additionally, under the ADXR condition, CUS decreased GR mRNA expression in the IL and PrL in control but not PAE females. It is somewhat puzzling that this CUS effect in control females was observed under the ADXR condition where CORT levels were restricted

to the basal physiological range. CUS has been shown to decrease GR mRNA expression in the rat frontal cortex, but GR downregulation has been postulated to be due to the hypersecretion of CORT following CUS (Hill et al., 2012). Our pre- and post-CUS basal CORT data provide support that CORT levels were similar among prenatal treatment and CUS groups under the ADXR condition, albeit were at the lower end of the physiological range. It is not well understood what factors may regulate transcriptional and protein expression of GR, but CORT-independent mechanisms that influence GR levels and GR-CORT interactions may exist (reviewed in (Hapgood et al., 2016)). For example, basal immune activation status may impact GR levels as well as sensitivity and responsiveness of GR to CORT (Hapgood et al., 2016). PAE has been found to alter developmental cytokine levels (Bodnar et al., 2016; Drew et al., 2015). Additionally, estrogen can regulate GR expression and function (Burgess and Handa, 1993; Turner, 1990), and PAE can increase circulating estradiol (Lan et al., 2009; Polanco et al., 2010) as well as induce deficits in age-related increases in estradiol levels (Sliwowska et al., 2016). As such, CORT-independent mechanisms, such as those involving alterations in immune function or estrogen levels, may potentially contribute to the differences in GR mRNA expression in the mPFC in PAE and control females that were unmasked by the ADXR procedure. Nevertheless, our findings overall suggest that CORT may play a role in modulating GR mRNA expression in females, and that prenatal treatment may influence the effects of CORT on receptor expression.

4.3 Differential roles of CORT in regulating depressive- but not anxiety-like behavior in PAE and control males

By contrast to females, previous studies showed that in males, PAE typically increases anxiety-like behavior (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Rouzer et al., 2017). Consistent with these previous findings, we found that under the sham condition, PAE alone decreased time spent in the center and the number of center entries in the OF compared to that in control males. PAE alone had no effects on depressive-like behavior, or on MR or GR mRNA expression in any brain regions examined. Interestingly, although CUS exposure had no effects on post-CUS basal CORT levels, it increased anxiety-like behavior (decreased time in center, frequency of center entries, and distance travelled in the center of the OF) in control males, regardless of surgical condition. In comparison, PAE males under the CUS condition exhibited similar levels of anxiety-like behavior as both non-CUS PAE and CUS control males, suggesting that PAE males may be constitutively susceptible to the effects of the acute stress of testing in the OF, instead of to the effects of the CUS procedure. These effects of PAE and CUS were in the context of similar locomotor activity among groups, as measured by total distance travelled in the field.

In the FST, CUS had no effects on control males, but increased depressive-like/passive-coping behavior in PAE males compared to their non-CUS counterparts, as measured by time spent immobile in the FST. These results contrast with our previous findings that CUS either had no differential effects or decreased immobility in the FST in PAE males (Hellemans et al., 2010b; Lam et al., 2018b). Differences among the studies may be due to the different CUS paradigms used. For example, in our previous studies, social isolation stress occurred with no access to food or water, and/or in hanging cages with wire mesh front and floor, and it was followed by an acute stressor in the home cage the next morning.

In the present study, however, animals had access to food and water during social isolation due to the needs of animals that underwent ADX, and we did not use an additional acute stressor. Additionally, different stressors (e.g. wet bedding, wet cage) were previously used (Lam et al., 2018a). Nevertheless, the behavioral findings in the OF and FST provide support that the current CUS paradigm used was effective in inducing changes in behavior even though it had no effects on basal CORT levels. This is consistent with the literature, showing that while CUS typically induces an increase in basal CORT levels, behavioral effects of CUS may be present without changes in CORT levels (reviewed in (Willner, 2017)). More importantly, following certain experimental manipulations and testing conditions, PAE males may also show increased depressive-like/passive-coping behavior.

The effects of PAE or CUS exposure on anxiety-like behavior in males were not normalized by ADXR. However, percent time on open arms in the EPM was decreased under the ADXR condition compared to that in the sham condition, regardless of prenatal treatment or CUS exposure. Furthermore, non-CUS PAE males exhibited increased depressive-like/passive-coping behavior under the ADXR condition compared to both their control counterparts within the same surgical group and their non-CUS PAE counterparts under the sham condition. These behavioral findings suggest that CORT is involved in modulating behavior, and may be involved in mediating the effects of PAE on FST (but not OF) behavior in males, at least at the replacement concentrations used. That is, the FST findings may indicate that the replacement concentration used in males was adequate to maintain the behavior of control but not PAE animals, and that the ability to secrete CORT in response to stress – which is lost due to ADX – may be needed for PAE animals under non-CUS conditions to behave like their control counterparts. Overall, similar to females, CORT levels may be involved in modulating behavior in males, and the role of CORT in behavior may potentially differ between PAE and control animals.

4.4 Pair-feeding is a treatment in itself: PAE and PF effects may differ

While certain results were similar between PAE and PF animals, including post-CUS body weight changes and basal ACTH levels in males, we found that PAE differed from PF animals in their behavior in the OF and EPM. A PF group is traditionally included in studies of this type to serve as a control for the reduction in food intake typically observed in alcohol-consuming animals. PF animals receive a reduced ration of a nutritionally optimal diet matched in amount to that consumed by a PAE partner (Weinberg, 1985, 1984). However, pair-feeding cannot control for other nutritional effects of alcohol, such as effects on nutrient utilization and absorption. There is also a component of mild prenatal stress involved with receiving a reduced food ration. These animals are given less food than they would otherwise consume if they had *ad libitum* access; therefore, the entire ration is typically consumed within a few hours of diet presentation and the PF animals are food-deprived and hungry until feeding the next day. This may result in a mild level of prenatal stress for the fetus. As such, the PF condition is an imperfect control condition. Regardless, our findings indicate that while GR and MR mRNA expression may be similar between PAE and PF animals, PAE induces different effects on anxiety- and depressive-like behavior. Thus, effects of PAE are not simply due to a reduction in food consumption, but rather, unique to the effects of alcohol exposure during the prenatal period.

4.5 Limitations

Adrenalectomy with CORT replacement in the drinking water was used in the present study to examine whether CORT may mediate the effects of PAE, alone or with CUS, on anxiety- and depressive-like behavior, as well as on receptor expression in key limbic areas that also regulate the endocrine stress response. While it is a valid procedure for our question of interest, adrenalectomy is an invasive procedure, and removal of the adrenals may have other physiological effects. The adrenal glands produce other steroid hormones as well as neurotransmitters, and it is possible that loss or reduction in levels of these hormones and neurotransmitters may impact the behavioral and receptor expression results obtained. Additionally, we cannot know whether the possible physiological impact of the loss/reduction of these hormones and neurotransmitters is equivalent among the prenatal treatment groups or between females and males. It would be a useful extension to the current findings to isolate the contribution of the hormones and neurotransmitters lost through adrenalectomy on anxiety-/depressive-like behavior and stress-related receptor expression. Additionally, the present study utilized only one replacement level among same-sex prenatal treatment groups, aiming for typical basal levels in both females and males. It is unclear whether PAE animals may require a different level of CORT replacement than their control counterparts. A follow-up dose-response study may help enrich the present findings.

While we were able to localize differential MR and GR mRNA expression in specific nuclei and reveal potential neurobiological underpinnings for behavioral changes following PAE and CUS, we did not measure changes in protein levels and function. Although findings from a recent study provide support for the implicit assumption that protein levels would change correspondingly with alterations in mRNA expression (Koussounadis et al., 2015), we cannot rule out the possibility that PAE may exert influences on processes downstream of transcription, including protein translation and post-translational modifications, and further experimentation is warranted. Nevertheless, our findings can serve as a guide for future experiments that directly examine the relationship between changes in expression of specific genes and behavioral outcome.

Another possible limitation in the present experimental design is that multiple behavioral tests were conducted in the same animal without counterbalancing for order of testing. Behavioral testing involves a component of stress and should be considered as a manipulation in itself; in the present study, it can be considered an extension of CUS. Furthermore, there may be carry-over effects from one test to the next. The order of testing can also impact behavioral responses, and the placement of a more stressful behavioral test, such as FST, in the line-up of tests needs careful consideration (Blokland et al., 2012; McIlwain et al., 2001). Our typical practice is to order the tests from least to most stressful; e.g., FST is typically performed last. The possibility that PAE animals may respond differently from controls to the cumulative stress of multiple testing done in a particular testing order remains to be determined and could provide additional insight into the outcomes we observed. As it stands, the present study does provide important information on brain and behavioral responses to the same cumulative stress experience (CUS + behavioral testing), which has implications for the response to stress in general in individuals prenatally exposed to alcohol, and may in fact reflect the real world situation of

cumulative stress in individuals with FASD. It would be an interesting extension to the current findings to probe for possible prenatal treatment x order of testing x CUS exposure interactions to further evaluate the impact of PAE on anxiety-like behavior, which could enhance interpretations of the present results.

Lastly, that testing occurred during the light phase in the present study may impact the interpretation of the findings. Circadian phase may differentially affect behavior of male and female rats, with males showing greater anxiety-like behavior during the light than the dark phase, but females showing greater anxiety- and depressive-like behavior during the dark than the light phase (Verma et al., 2010). Therefore, testing exclusively during the light or dark phase may either mask or reveal differences between experimental treatment groups (Verma et al., 2010). Follow-up studies comparing behavior, hormone, and brain measures assessed in the dark vs. the light phase among prenatal treatment groups could help provide additional insight into the current findings.

5. CONCLUSION

PAE differentially altered anxiety-like behavior and GR mRNA expression in females and males, with females showing decreased GR mRNA expression in the hippocampal formation and males showing no changes in MR or GR mRNA expression in any brain region. In turn, CUS had differential effects on anxiety- and depressive-like behavior in PAE and control animals, and these effects were sex dependent. That is, in females, CUS increased anxiety-like behavior in PAE animals, but had no effects on depressive-like behavior. By contrast in males, CUS increased anxiety-like behavior in control but not PAE animals, possibly because behavior of PAE males under non-CUS conditions was similar to behavior of both C and PAE males under CUS conditions. Furthermore, CUS increased depressive-like behavior in PAE males compared to their non-CUS counterparts. Importantly, our ADXR findings suggest that CORT levels may be involved in mediating the differential effects of PAE on anxiety- and depressive-like behavior and GR mRNA expression in females and males, and that the role of CORT on anxiety- and depressive-like behavior, and on HPA regulation differs in a sex-dependent manner between PAE and control animals. Overall, our findings have important implications for understanding the role of stress and HPA activity in the adverse effects of PAE on the development of mental health disorders, and for the possible development of sex-specific interventions and treatments for individuals prenatally exposed to alcohol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1. PAE males may have decreased sensitivity to negative feedback by corticosterone.
2. Differential effects of PAE and CUS are sex dependent.
3. Differential role of corticosterone in modulating PAE effects.

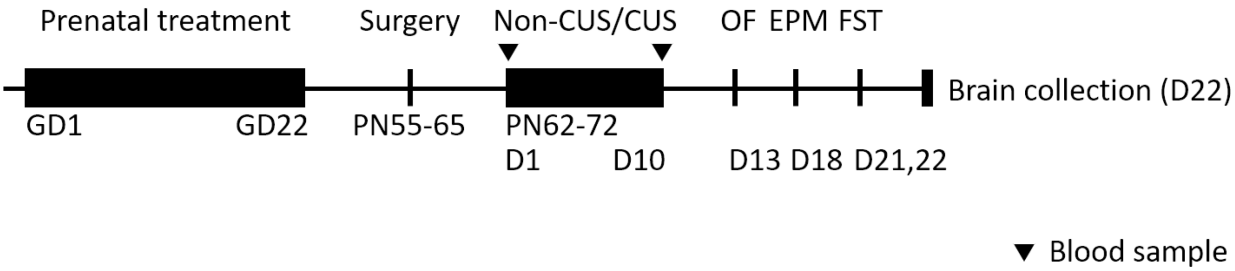

Figure 1. Experimental design.

Prenatal treatment occurred during Gestational Day (GD) 1 to 22. In adulthood (Postnatal day [PN] 55–65), animals underwent either adrenalectomy with corticosterone replacement or sham surgery. Following recovery from surgery, animals (PN 62–72) were subjected either to chronic unpredictable stress (CUS) or left undisturbed (Non-CUS), and then their behavior was assessed in a battery of behavioral tests: OF = open field test; EPM = elevated plus maze; FST = forced swim test. Blood sampling for corticosterone and ACTH measurements occurred on D1 of CUS prior to stress and the day after CUS ended, and samples were collected from Non-CUS animals at the same time. Brain collection occurred 30-min after the start of FST testing on D2.

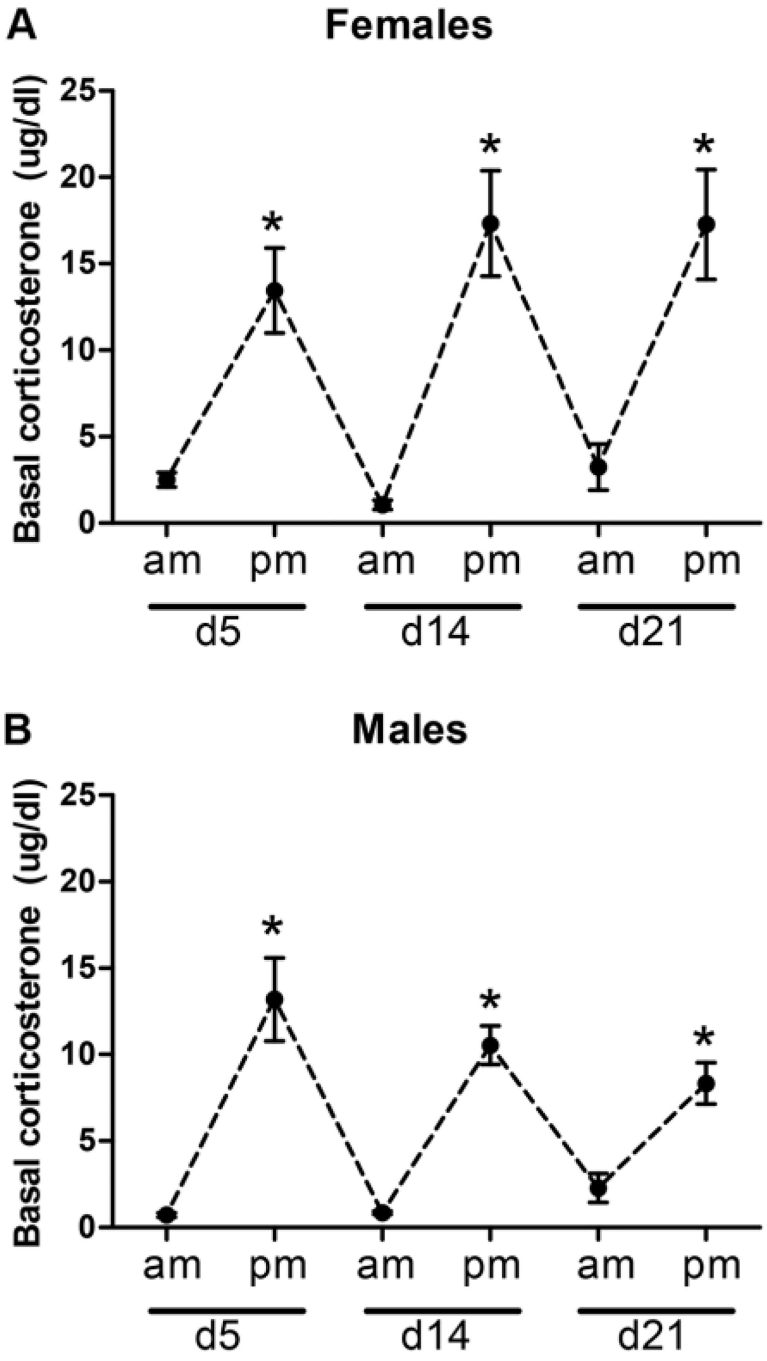

Figure 2. AM and PM basal corticosterone levels in control animals following adrenalectomy with corticosterone replacement.

PM basal corticosterone levels (mean ± SEM) were significantly higher than AM levels in both females and males (* $p<0.05$). n=10 per sex.

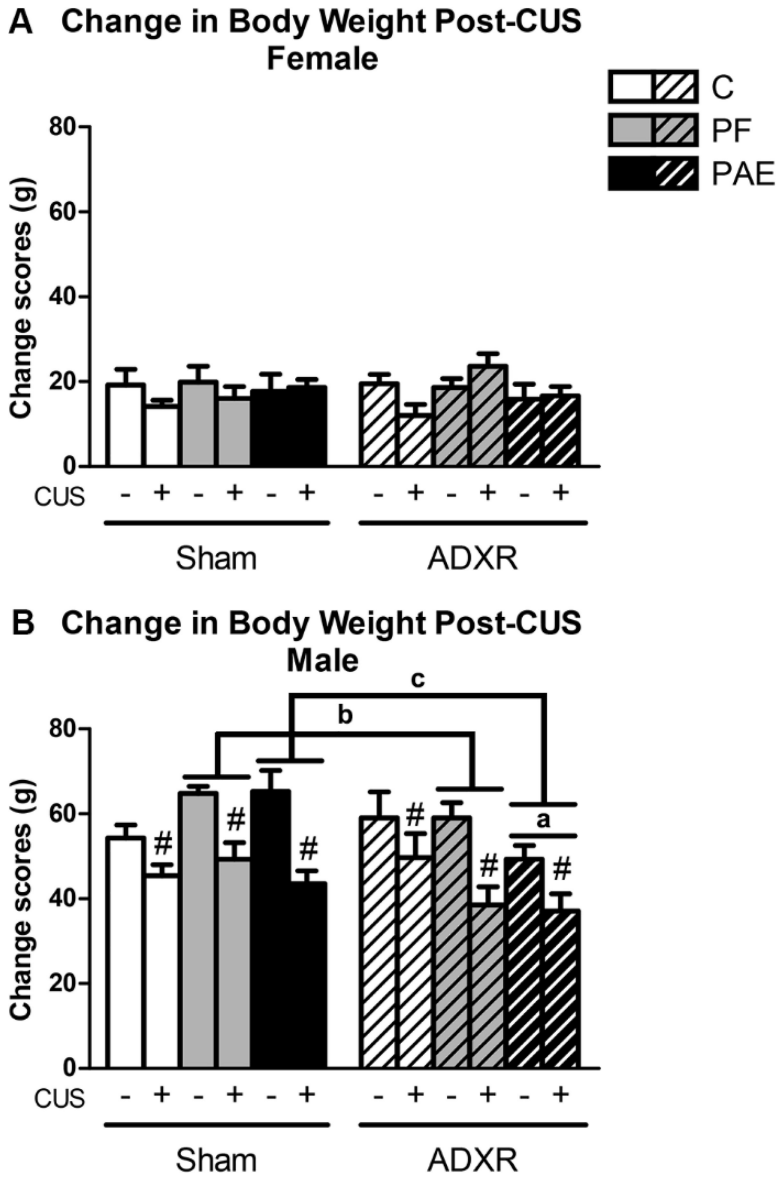

Figure 3. Change in body weight following adrenalectomy with corticosterone replacement (ADXR) and CUS exposure.

Bars represent change scores (difference between pre- and post-CUS body weight; mean ± SEM) following the 10-day period of CUS in control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) females (A) and males (B). For B, [#] CUS males overall gained less weight than non-CUS males ($p < 0.001$), regardless of prenatal treatment or surgical condition; within the ADXR condition, [a] PAE males gained less weight than their C counterparts ($p = 0.020$); and between surgical conditions, [b] PF ($p = 0.045$) and [c] PAE ($p = 0.007$) males under the ADXR condition gained less weight than their respective sham counterparts. On the x-axis, [−] indicates animals that were left undisturbed (non-CUS) and [+] indicates animals that were subjected to CUS. n = 10–11/prenatal treatment/surgical condition/CUS exposure/sex.

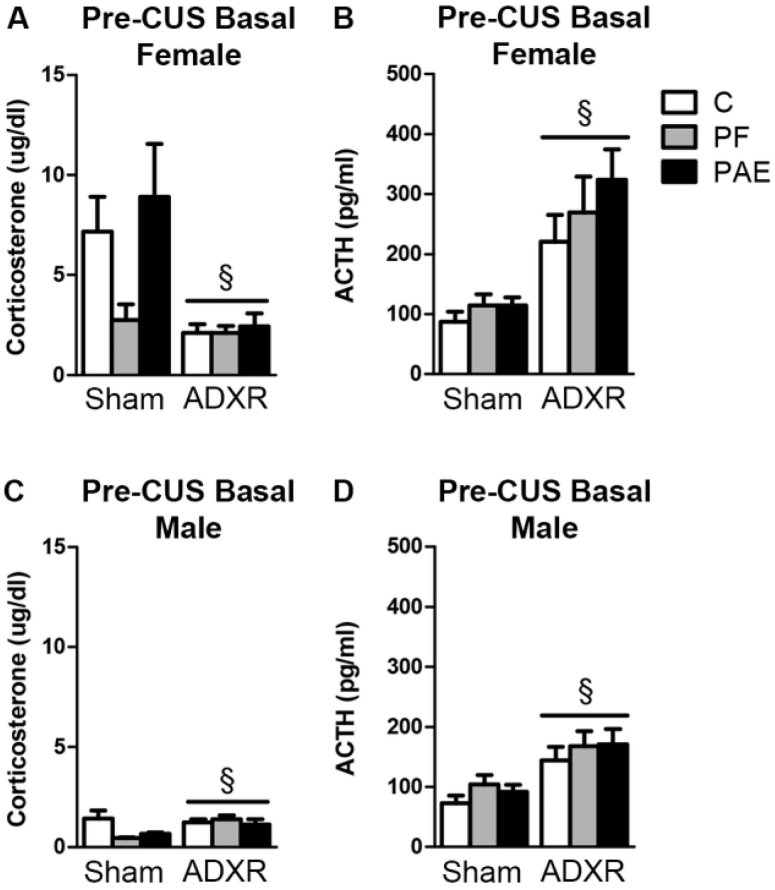

Figure 4. Effects of prenatal alcohol exposure (PAE) and adrenalectomy with corticosterone replacement (ADXR) on pre-CUS basal corticosterone (μg/dL) and ACTH (pg/ml) levels in females (A,B) and males (C,D).

Bars represent mean ± SEM. § animals under the ADXR condition were different from those under the sham condition (A: $p = 0.001$; B: $p = 0.04$; C,D: $p < 0.001$). n=16–21/prenatal treatment/surgical condition/sex.

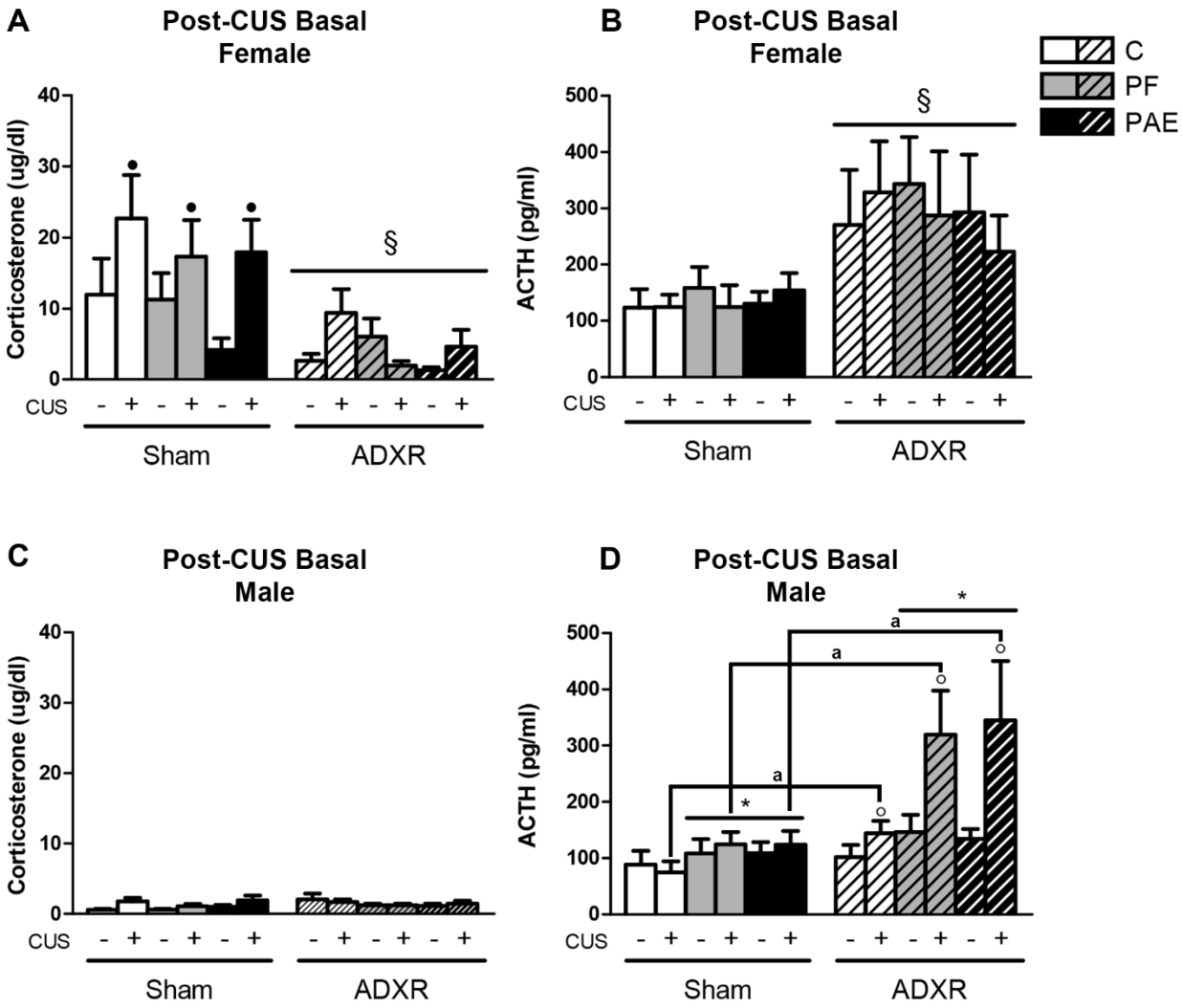

Figure 5. Effects of prenatal alcohol exposure (PAE), chronic unpredictable stress (CUS), and adrenalectomy with corticosterone replacement (ADXR) on post-CUS basal corticosterone (μg/dL) and ACTH (pg/ml) levels in females (A,B) and males (C,D).

Bars represent mean ± SEM. For A, • under the sham but not ADXR condition, corticosterone levels were higher in CUS than non-CUS females, regardless of prenatal groups ($p = 0.001$); for A,B, § animals under the ADXR were different from those under the sham condition ($ps < 0.001$); for D, * ACTH levels were higher overall in PF and PAE than C males ($ps \leq 0.015$), and under the ADXR condition, ACTH levels were higher in CUS than their ○ non-CUS and [a] sham counterparts ($ps < 0.001$). On the x-axis, − indicates animals that were left undisturbed (non-CUS) and + indicates animals that were subjected to CUS. For A,C, n=9–11/prenatal treatment/surgical condition/CUS condition/sex; for B,D, n=6–11/prenatal treatment/surgical condition/CUS condition/sex.

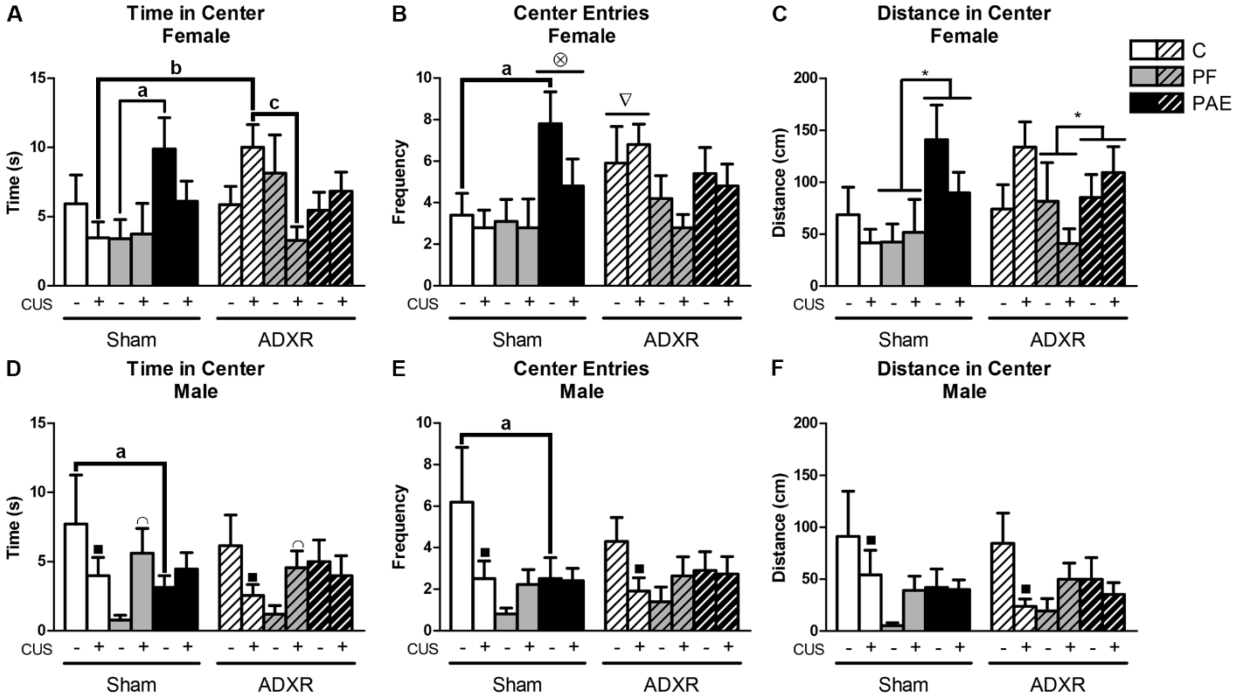

Figure 6. Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) and on behaviors of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats in the open field.

Bars represent the mean ± SEM of time in the center (A,D), frequency of center entries (B,E), and distance travelled in the center (C,F). For A, [a] within the sham condition, non-CUS PAE females spent more time in the center than non-CUS PF females ($p = 0.029$); following CUS exposure, time in center was [b] higher in C females in the ADXR than sham condition ($p = 0.009$), and [c] lower in PF than C females within the ADXR condition ($p = 0.022$); for B,D,E, [a] under the sham condition, non-CUS PAE females were different from non-CUS C females (B: $p = 0.011$; D: $p = 0.047$; E: $p = 0.019$); for B, ⊗ under the sham condition, PAE females entered the center more frequently than both C and PF females ($ps \leq 0.027$), while under the ADXR condition, ∇ C females showed a higher frequency of center entries than their sham counterparts ($p = 0.008$); for C, * distance travelled in the center was higher overall in PAE than PF females ($p = 0.004$); for D-F, ■ CUS decreased time in center in C males compared to their non-CUS counterparts (D: $p = 0.024$; E: $p = 0.006$; F: $p = 0.016$); for D, ∩ CUS increased time in the center in PF males compared to their non-CUS counterparts ($p = 0.013$). On the x-axis, $^{-}$ indicates animals that were left undisturbed (non-CUS) and $^{+}$ indicates animals that were subjected to CUS. n=9–11/prenatal treatment/surgical condition/CUS condition/sex.

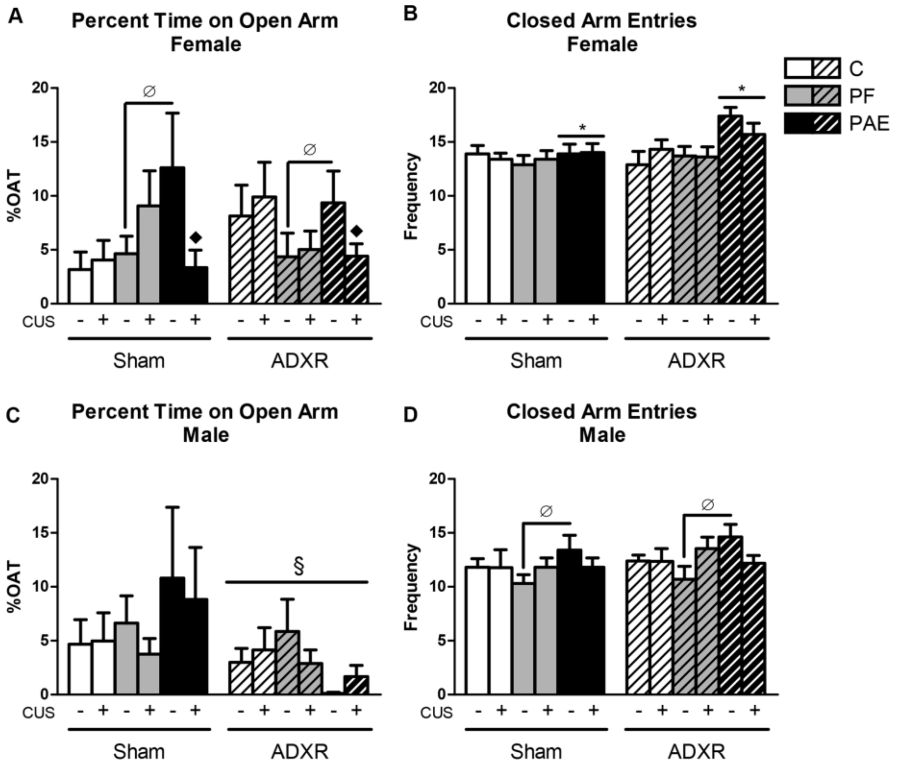

Figure 7. Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) and on behaviors of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats in the elevated plus maze.

Bars represent the mean ± SEM of percent time on open arms (A,C) and frequency of closed arm entries (B,D). For A, ∅ under the non-CUS condition, PAE females spent more percent time on open arms than their PF counterparts ($p = 0.048$), ♦ and in PAE females, percent time on open arms were lower under the CUS than non-CUS condition ($p = 0.009$); for B, * PAE females entered the closed arms more frequently than both C and PF females (ps ≤ 0.011); for C, § males under the ADXR condition spent less percent time on open arms than those under the sham condition ($p = 0.035$); for D, ∅ under the non-CUS condition, PAE males had more closed arm entries than PF males ($p = 0.004$). On the x-axis, − indicates animals that were left undisturbed (non-CUS) and + indicates animals that were subjected to CUS. n=9–11/prenatal treatment/surgical condition/CUS condition/sex.

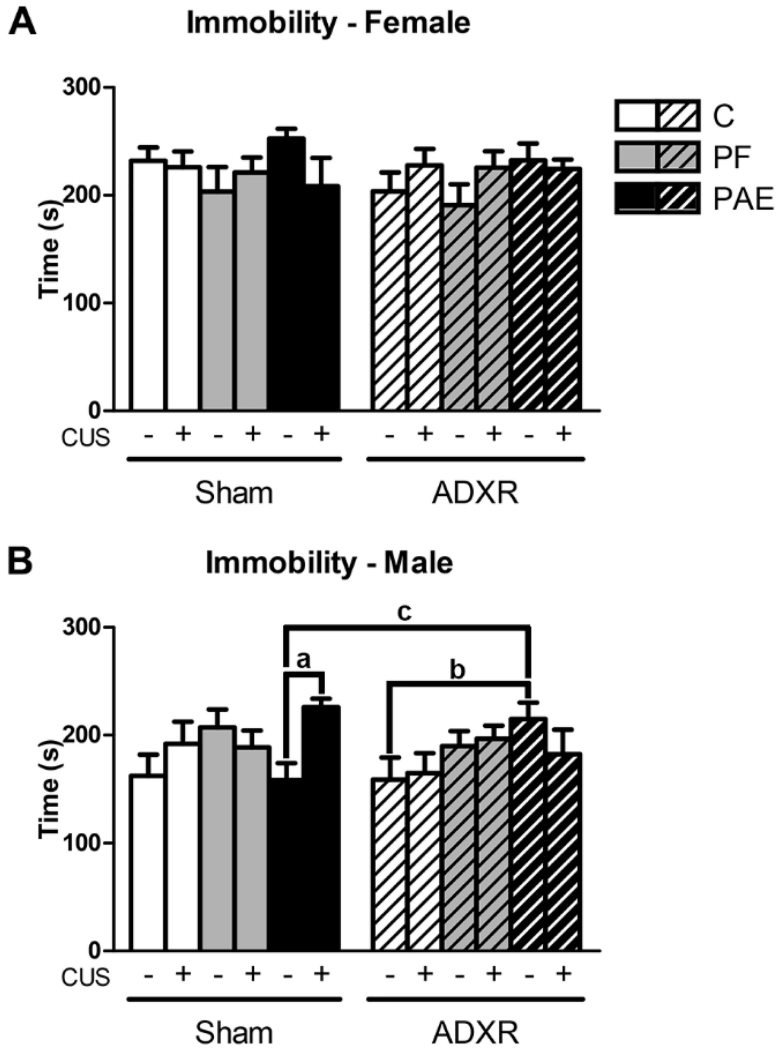

Figure 8. Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) on behaviors of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats in the forced swim test (FST).

Bars represent the mean ± SEM of time immobile in the FST in females (A) and males (B). For B, [a] under the sham condition, CUS differentially increased time immobile in PAE males compared to their non-CUS PAE counterpart ($p = 0.009$); under the ADXR condition, non-CUS PAE males showed greater immobility than both [b] non-CUS C counterparts within the same surgical condition ($p = 0.024$) and their [c] non-CUS PAE counterparts in the sham condition ($p = 0.023$). On the x-axis, $^{-}$ indicates animals that were left undisturbed (non-CUS) and $^{+}$ indicates animals that were subjected to CUS. n=9–11/prenatal treatment/surgical condition/CUS condition/sex.

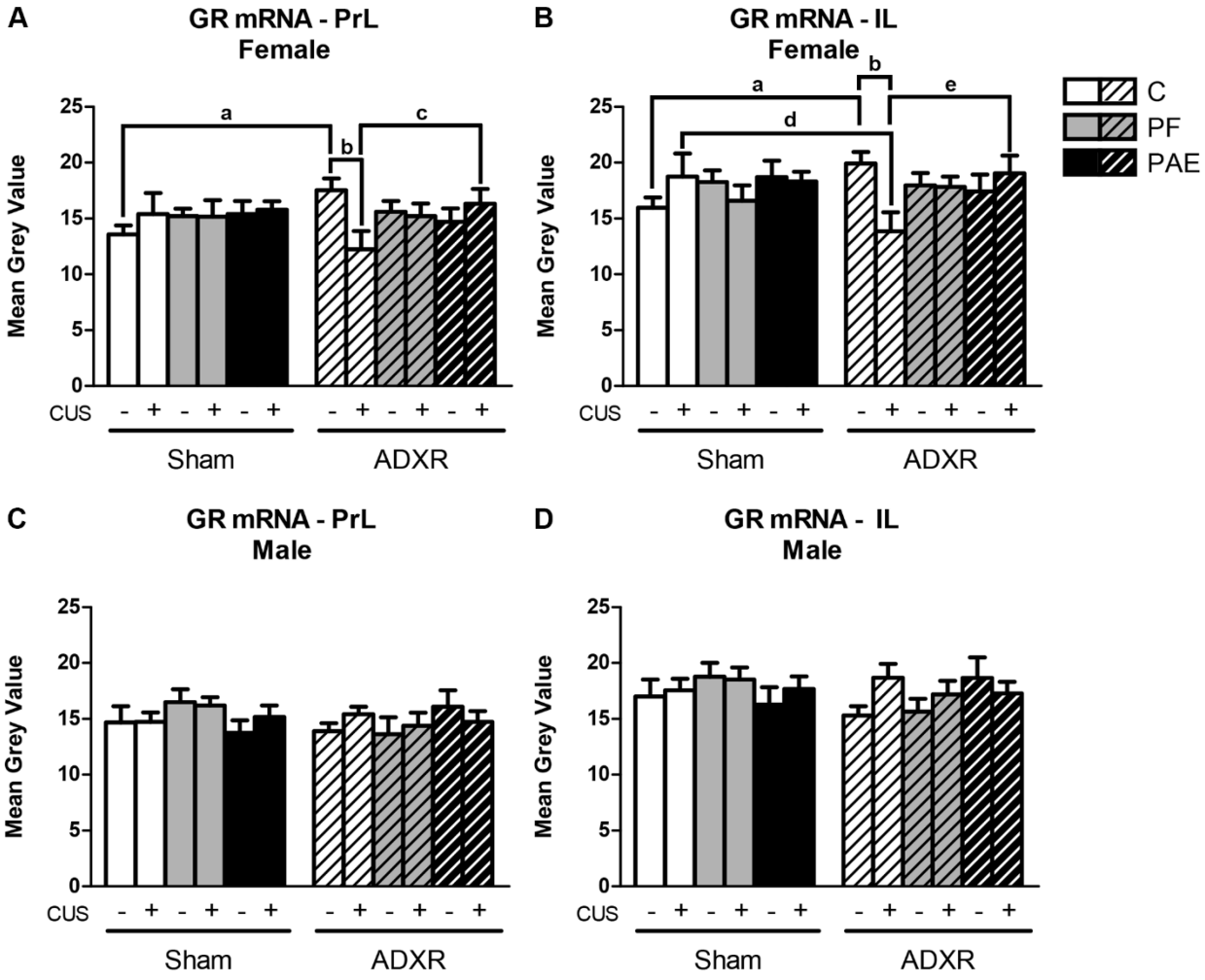

Figure 9. Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) on GR mRNA expression in the medial prefrontal cortex (mPFC) of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed

(PAE) rats. Bars represent mean grey values (mean ± SEM) of GR mRNA expression in the PrL (A,C) and IL (B,D) cortices of the mPFC. For A,B, [a] non-CUS C females in the ADXR condition had higher GR mRNA expression than their sham counterparts (A: $p = 0.027$; B: $p = 0.041$), and [b] C females in the ADXR condition had lower GR mRNA expression following CUS than their non-CUS counterparts (A: $p = 0.005$; B: $p = 0.003$); for A, [c] in the ADXR condition, GR mRNA expression following CUS was higher in PAE than C females ($p = 0.023$); for B, [d] in C females, GR mRNA expression following CUS was lower in the ADXR than sham condition ($p = 0.015$), and [e] in the ADXR condition, GR mRNA expression following CUS was higher in PAE than C females ($p = 0.024$). On the x-axis, $^{-}$ indicates animals that were left undisturbed (non-CUS) and $^{+}$ indicates animals that were subjected to CUS. n=6–7/prenatal treatment/surgical condition/CUS condition/sex.

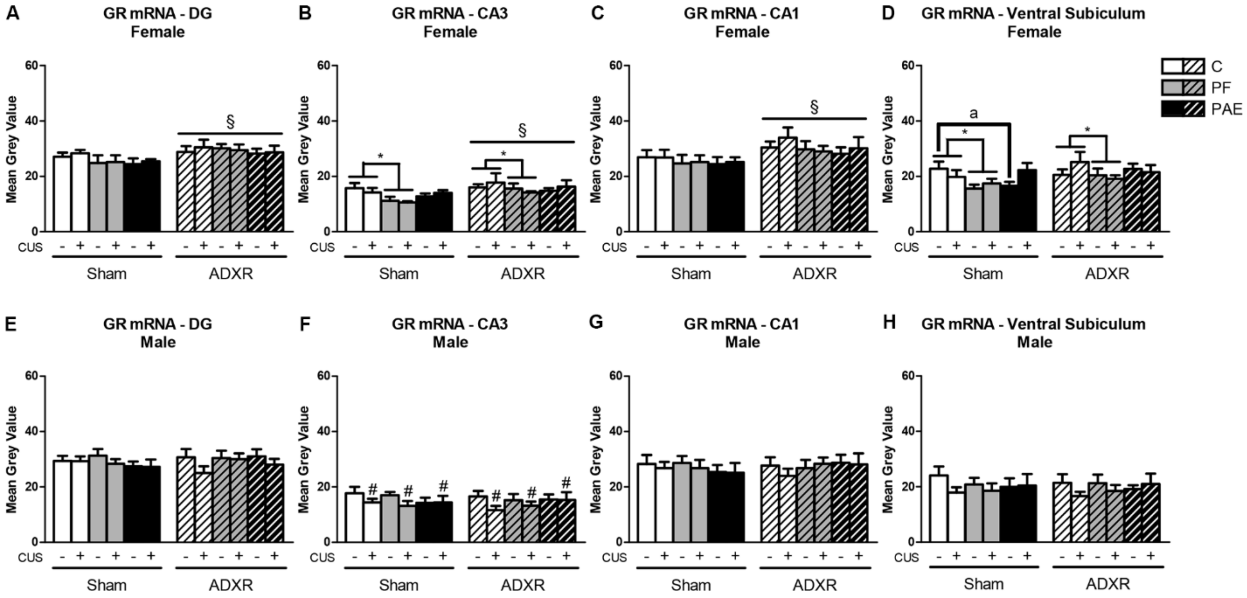

Figure 10. Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) on GR mRNA expression in the hippocampal formation of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.

Bars represent mean grey values of GR mRNA expression in the dentate gyrus (DG; A,E), CA3 (B,F), CA1 (C,G) and ventral subiculum (D,H) subregions of the hippocampal formation. For A-C, § ADXR increased GR mRNA expression compared to the sham condition (ps = 0.005); for B,D, * PF females showed lower GR mRNA expression than C females (A: p = 0.009; B: p = 0.015)); for D, [a] in the sham condition, non-CUS PAE females showed lower GR mRNA expression than their non-CUS C counterparts (p = 0.049); for F, # males under the CUS condition showed lower GR mRNA expression than their non-CUS counterpart, regardless of prenatal treatment or surgical condition (p = 0.039). On the x-axis, $^{-}$ indicates animals that were left undisturbed (non-CUS) and $^{+}$ indicates animals that were subjected to CUS. n=6–7/prenatal treatment/surgical condition/CUS condition/sex.