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Prenatal Alcohol Exposure Increases Vulnerability to Stress and Anxiety-Like Disorders in Adulthood

Kim G.C. Helleman^a, Pamela Verma, Esther Yoon^b, Wayne Yu^b, and Joanne Weinberg^b

^aDepartment of Psychology, Carleton University, Ottawa, Ontario, Canada

^bDepartment of Cellular and Physiological Sciences, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

Children and adults with fetal alcohol spectrum disorder (FASD) have elevated rates of depression and anxiety disorders compared to control populations. The effects of prenatal alcohol exposure (PAE) on anxiety, locomotor activity, and hormonal reactivity in male and female rats tested on the elevated plus maze (EPM), a task commonly used to assess anxiety-like behaviors in rodents, were examined. Pregnant dams were assigned to PAE, pair-fed (PF), or *ad libitum*-fed control (C) groups. At adulthood, half of all male ($N = 60$) and female ($N = 60$) PAE, PF, and C offspring were exposed to 10 days of chronic mild stress (CMS); the other half remained undisturbed. Animals were then tested on the EPM, and blood collected 30 min posttest for analysis of corticosterone (CORT), testosterone, estradiol, and progesterone. Overall, CMS exposure produced a significant anxiogenic profile. Moreover, CMS increased anxiety-like behavior in PAE males and females compared to controls and eliminated the locomotor hyperactivity observed in nonstressed PAE females. CMS also increased post-EPM CORT, testosterone, and progesterone levels in all groups, with CORT and progesterone levels significantly higher in PAE than in C females. By contrast, CMS selectively lowered estradiol levels in PAE and PF, but not C, females. CMS exposure reveals sexually dimorphic behavioral and endocrine alterations in PAE compared to C animals. Together, these data suggest the possibility that fetal reprogramming of hypothalamic–pituitary–adrenal (HPA) and –gonadal (HPG) systems by alcohol may underlie, at least partly, an enhanced susceptibility of fetal alcohol-exposed offspring to depression/anxiety-like disorders in adulthood.

Keywords

chronic mild stress; prenatal alcohol exposure; elevated plus maze; HPA axis; HPG axis; sex differences; behavior; Sprague-Dawley; anxiety; fetal programming; early adversity

Address for correspondence: Kim Helleman, Ph.D., Department of Psychology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6. kim_helleman@carleton.ca.

Conflicts of Interest

The authors declare no conflicts of interest.

Introduction

Fetal alcohol syndrome (FAS) is the leading, nongenetic cause of mental retardation in the Western world.¹ However, FAS represents only the extreme end of the spectrum of effects that can occur following prenatal exposure to alcohol.^{2,3} The term *fetal alcohol spectrum disorder* (FASD) is an umbrella term that encompasses the broad range of outcomes that are observed, and includes the diagnoses of FAS, partial FAS, alcohol-related birth defects, and alcohol-related neurodevelopmental disorder.¹ Beyond the primary disabilities that occur in individuals with FASD, prenatal exposure to alcohol is often associated with a number of secondary disabilities, of which mental illness is one of the most common. In a study of 473 patients diagnosed with either FAS or fetal alcohol effects, over 90% of respondents had experienced mental health problems.⁴ Depression was documented in 52% of individuals, which is far higher than the current global prevalence of 21%.⁵ Further, the most commonly reported reason for admission at inpatient care was symptoms of depression and suicidal behaviors.⁴ Increased rates of anxiety disorders are also common in adults with FASD,^{6,7} which is not surprising given that anxiety disorders are highly comorbid with depression.^{8,9} Sex differences are also apparent in the prevalence of mental illness in FASD populations: females have higher rates of depression (50%) and anxiety (50%) than males (40% and 0%, respectively).⁷ These data are particularly relevant in light of a wealth of evidence indicating that in control populations, depression in women is up to three times more prevalent than in men.¹⁰ Despite these findings, there is a dearth of research investigating the potential biological mechanisms underlying the significantly elevated rates of mental health problems in these populations.

One of the most consistently described biological abnormalities in depressive and anxiety disorders is dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, which is typically normalized by successful antidepressant,^{11–20} or anxiolytic^{21,22} therapy. HPA hyperactivity is most often observed during an acute major depressive episode and can be manifested in a number of ways, which suggest both increased HPA drive and deficits in normal hypothalamic–pituitary regulatory mechanisms. For example, studies have reported increased basal urinary and serum cortisol levels and/or adrenal hyperplasia, as well as nonsuppression in the dexamethasone (DEX) suppression test, and blunted adrenocorticotropin (ACTH) responses to corticotropinreleasing hormone (CRH) administration, but increased ACTH responses to CRH following DEX.^{11,15,23–27} It is not known whether HPA axis abnormalities are a primary cause of depressive/anxiety disorders, represent an illness marker, or are secondary to another initiating cause. However, there is evidence that normalization of HPA disturbances may be a prerequisite for successful treatment: in patients where a neuroendocrine abnormality persists, the risk of relapse or resistance to treatment is much higher.^{25,28}

Interestingly, in parallel to findings in depressed populations, HPA axis hyperactivity and dysregulation are also observed in both animal models of prenatal alcohol exposure (PAE) and in children with FASD. PAE rats show increased HPA responses and/or delayed recovery to basal levels compared to controls in response to a variety of stressful stimuli such as restraint, noise and shake, footshock, immune challenge, and exposure to novel environments.^{29–34} Importantly, while differences in plasma corticosterone (CORT) and

ACTH are rarely evident under basal conditions,^{34–37} dysregulation of central HPA pathways may be observed under both basal and stress conditions.³⁸ Although limited human literature exists, the few published reports suggest increased HPA activity following prenatal exposure to alcohol. Two-month-old infants exposed *in utero* to alcohol or cigarettes show elevated basal cortisol levels,³⁹ and heavy drinking at conception and during pregnancy is associated with higher basal and poststress (blood draw) cortisol concentrations in 13-month-old infants.⁴⁰ Interestingly, Haley and colleagues⁴¹ found that 5- to 7-month-old fetal alcohol-exposed boys showed greater changes in cortisol than girls during a modified “still face” procedure, a paradigm used to study emotion and stress regulation, indicating that sex differences do occur in human infants, and can occur even prior to puberty. Notably, increased HPA reactivity is observed in humans and animals following other early adverse environmental experiences, including poverty, physical or sexual abuse, and increased maternal stress.^{42–46} Thus, HPA dysregulation may represent a common neuroendocrine end-point, and perhaps a common pathway, for fetal programming by early life stressors and other adverse early experiences, including PAE.

Importantly, studies using animal models have shown that HPA hyperresponsiveness in PAE offspring is often observed in a sexually dimorphic manner. HPA hyperactivity occurs primarily in PAE males in response to prolonged restraint or cold stress,^{47,48} but in PAE females in response to acute restraint, or acute ethanol or morphine challenge.^{32,33,48–50} While the mechanisms underlying the sexspecific effects of developmental insults are not yet fully known, the hypothalamic–pituitary–gonadal (HPG) axis is likely involved in mediating these differential effects of PAE. The HPA and HPG axes interact bidirectionally: the HPA hormones can inhibit HPG activity at all levels of the axis, particularly during stressful events, and in turn, the HPG hormones can differentially regulate HPA activity in males and females.^{51–53} Significantly, data suggest that the developing female HPA axis is more vulnerable to insult than that of the male,^{49,54–57} which could play a role in the significant disparity in rates of depression both globally and in FASD populations.

Fetal reprogramming of the HPA axis by ethanol may mediate the relationship between adverse or stressful early life experiences and increased vulnerability for depression and anxiety disorders in adulthood. Moreover, in parallel with the animal literature, a wealth of data suggests that women may be more susceptible to these neurobehavioral sequelae than men.¹⁶ Nonetheless, only a few studies using preclinical models have investigated the impact of PAE on depressive-like behaviors in adulthood; the few published ones report that PAE increases depressive-like behaviors such as “behavioral despair.”^{58,59} By contrast, although significantly more studies have explored the effects of PAE on anxiety-like behavior, these have yielded inconsistent results. Some studies have shown that PAE increases anxiety on the elevated plus maze (EPM),^{60,61} while others have reported decreased anxiety^{61,62} or no difference among prenatal groups.⁶³ One possible explanation for these discrepancies comes from evidence that acute stress prior to testing increases anxiety-like behaviors on the EPM in PAE rats,^{61,63,64} suggesting that prior experiences, including manipulations of the HPA axis, may be required to reveal prenatal treatment effects. This is an interesting finding, given that acute or chronic stress may precipitate depressive episodes in some populations of depressed adults,⁶⁵ and in particular, in those exposed to early life stress or adversity.^{66–68} Parallel to these findings, animals exposed to

chronic mild stress (CMS) over a 4–6 week period show a variety of symptoms analogous to those observed in depression (reviewed in Refs. 69–71.) Moreover, it is noteworthy that the experience of acute or chronic stress forms the basis of most animal models of depression.^{72–77}

The current study was undertaken to investigate whether PAE and subsequent exposure to unpredictable mild stress in adulthood leads to neurobehavioral changes that increase susceptibility to the development of anxiety/depressive-like behaviors. Male and female offspring from PAE, pair-fed (PF), and *ad libitum*-fed control (C) groups were tested in adulthood. Animals were first exposed to 10 days of CMS, using a modified version of the typical CMS procedure⁶⁹ developed in our laboratory to model milder “everyday life” stressors. We speculated that this milder and abbreviated stress regimen would prevent possible ceiling effects on HPA function, and would have greater effects on PAE than C animals, thus unmasking differences among groups. Plasma CORT levels and body weights were assessed before and after the CMS exposure period. Male and female PAE, PF, and C animals that remained undisturbed throughout the 10-day period served as a C condition, to distinguish prenatal alcohol effects from CMS-induced alterations, and to investigate whether CMS exposure is a necessary or sufficient treatment to reveal alterations in anxiety-like behavior and HPA reactivity. At the end of the CMS period, both CMS- and Non-CMS-exposed animals were tested on the EPM. Trunk blood was collected 30 min following testing, to determine CORT responses to the acute stress of EPM exposure. In addition, testosterone, estradiol, and progesterone levels were measured to compare HPG alterations among PAE, PF, and C rats under CMS and Non-CMS conditions. Our results indicate that CMS exposure is a powerful manipulation for revealing alterations in anxiety-like behavior, as well as HPA and HPG reactivity, among adult PAE animals, and demonstrate that these alterations occur in a sexually dimorphic manner.

Methods and Materials

Breeding and Animals

Female Sprague-Dawley rats (223–300 g, $N = 10$) were obtained from Charles River Laboratories (St. Constant, PQ, Canada) and male Sprague-Dawley rats (275–350 g, $N = 18$) were obtained from UBC Animal Care Centre, South Campus (Vancouver, BC, Canada). Rats were group-housed by sex and maintained on a 12:12 h light/dark cycle (lights on at 0600 h), with controlled temperature (21–22°C), and *ad libitum* access to standard lab chow (Jamieson’s Pet Food Distributors Ltd., Delta, BC, Canada) and water. One to two weeks following arrival, males and females were paired together in suspended stainlesssteel cages with mesh front and floor (25 × 18 × 18 cm). Day 1 of gestation (G1) was indicated by a vaginal plug on wax paper beneath the breeding cages, which were checked daily. Animal use and care procedures were in accordance with the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals* and the Canadian Council on Animal Care⁷⁸ guidelines and approved by the UBC Animal Care Committee, protocol #A06–0017.

Diets and Feeding

On G1, females were singly housed in polycarbonate cages (24 × 16 × 46 cm) with pineshavings bedding and randomly assigned to one of three treatment groups. The ethanol (PAE) treatment group ($n = 10$) was offered *ad libitum* liquid ethanol diet (36% ethanol-derived calories) prepared by Dyets Inc. (Bethlehem, Pennsylvania). This diet is formulated to provide adequate nutrition to pregnant rats, regardless of ethanol intake.⁷⁹ The PF group ($n = 10$) was offered a liquid control diet with maltose-dextrin isocalorically substituted for ethanol. Intake was matched to the amount consumed by an ethanol-treated partner (g/kg body weight/gestation day). Both the PAE and PF groups also had *ad libitum* access to water. The group ($n = 10$) was offered standard lab chow (Jamieson's Pet Food Distributors Ltd.) and water *ad libitum*. All animals were provided with fresh diet daily within 1.5 h prior to lights off to prevent a shift of the CORT circadian rhythms, which occurs in animals on a restricted feeding schedule, such as those in the PF group.⁸⁰ Experimental diets were continued through G21. Beginning on G22 all animals were provided with *ad libitum* access to standard laboratory chow and water, which they received throughout lactation. Pregnant dams were left undisturbed except for cage changing and weighing, which occurred on G1, G7, G14, and G21 (see Table 1). On postnatal day 1 (PN1), pups were weighed and litters randomly culled to 10 (five males and five females when possible). To maintain litter size, pups from the same prenatal treatment group born on the same day were fostered into a litter when needed. Dams and pups were weighed on PN1, PN8, PN15, and PN22 (see Table 1). On PN22, pups were ear-notched for identification and group-housed by litter and sex. On PN40, one male and one female pup from each litter were randomly assigned to either the CMS or Non-CMS condition and were pairhoused with another animal of the same sex, prenatal treatment, and stress condition. The light cycle in the colony room during the testing period was 12:12 h lights/dark (lights on at 0600 h).

Chronic Mild Stress Protocol

Prior to behavioral testing, animals in the CMS condition were subjected to a protocol of randomized stressors. Because animals were born over a 10-day period, and entered the experiment in a staggered fashion, the age at which the CMS procedure commenced ranged from 60 to 90 days. On day 1, blood samples for CORT were obtained from all animals (CMS and Non-CMS) via tail nick 1 mm from the tip of the tail. All animals were also weighed, handled, and moved to a new colony room in a neighboring building on day 1. CMS and Non-CMS animals were housed in different colony rooms so that Non-CMS animals were not exposed to the disturbance and stress odors of the CMS animals. On days 2–9, animals in the CMS condition were exposed to the CMS regimen and animals in the Non-CMS condition were left undisturbed in their home cages. CMS exposure occurred in a room separate from the colony room. Animals were exposed daily to two different stressors, one between 0800 h and 1200 h, and the second between 1300 h and 1600 h, with a minimum of two hours between stressors. The order and type of stressor was randomized, but all animals received the same number of exposures to each stressor over the 10-day period. Stressors included: (1) 5-min exposure to a 20 × 20 × 90-cm transparent Plexiglas platform mounted on wooden posts 90 cm high; (2) 2-h white noise (40 dB; Lafayette Instruments model #15800); (3) overnight social isolation in hanging wire mesh cages (20 × 23 × 18 cm); (4) 30-min restraint in PVC tubes (19 × 7 cm for male, 15 × 6 cm for female);

(5) 1-h exposure to a soiled cage (cagemates placed in soiled cage of another set of animals); (6) 2-h cage tilt at a 30° angle; and (7) 1-h exposure to a novel cage (cagemates rehoused in small, opaque cages (18 × 25 × 15 cm) without bedding, food, or water). On day 10, a second blood sample was obtained from CMS-exposed animals by tail nick. Both CMS and Non-CMS exposed animals were also weighed on days 4, 7, and 10 of the CMS exposure period, but other than weighing, Non-CMS animals remained undisturbed.

Behavioral Testing

One day following the end of the CMS procedure (day 11), all animals were habituated to the behavioral testing room (distinct from the stress-exposure room) for 10 min: cages were brought into the testing room, but animals remained in their cages and were not placed on the maze. Behavioral testing commenced the following day (day 12), 48 h postfinal CMS exposure.

Behavioral Apparatus—The elevated plus maze consisted of two closed arms (69 × 10.5 cm) and two open arms (69 × 10.5 cm) with a central platform (diameter 35 cm). Closed arms had walls of darkened Plexiglas 20 cm high along their length. Open arms had a 2-cm-high lip along the edges of the arms. Activity was recorded by a digital camera (Panasonic CCTV Camera, Model No. WV-BP 334) placed 130 cm above the maze. Testing took place under dim light (one 40-W and one 60-W soft white light bulb, both angled to create indirect lighting on the maze) during the light phase of circadian cycle (between 0900 h and 1400 h). The maze was cleaned with 5% acetic acid between tests. White noise (30 dB) masked extraneous background noise.

Testing—On the test day, animals were brought into the testing room in their home cages, and each pair of animals was then removed from its home cage and placed in a separate holding cage for 5 min before being placed on the maze. Animals were placed individually in the center of the maze, with head position counterbalanced between rats, and behavior was recorded for 5 min. Partial (two paws on the open arms) and full (all four paws) open arm entries, full closed arm entries, distance traveled, and percent time on open arms were recorded and analyzed using Noldus Ethovision v.3.1 software (5.994 samples/s). Frequency of rearing (rising on the hind limbs either touching or not touching a wall surface) and grooming bouts (friction on any part of the body with the paws and/or the mouth) were scored manually by an independent observer. Rearing and grooming occurred almost exclusively on the closed arms; thus, there was no score to record the location of where the behavior was performed.

Blood Sampling

Immediately following testing, animals were individually housed and left undisturbed in a quiet holding room for 30 min. Animals were then taken to a separate room and decapitated. Blood samples were centrifuged at 3200 rpm for 10 min at 0°C. Plasma was transferred into 600 µL Eppendorf tubes and stored at –80°C until assayed. CORT, testosterone (males only), estradiol, and progesterone (females only) levels were ascertained from trunk blood.

Radioimmunoassays

Corticosterone—Total CORT (bound plus free) levels were measured using a commercially available kit (MP Biomedicals, Orangeburg, New York Cat. # 07-120103). The antiserum crossreacts 100% for corticosterone. The minimum detectable corticosterone concentration was 7.7 ng/mL, and the intra- and interassay coefficients of variation were 7.1 and 7.2%, respectively.

Testosterone—Testosterone levels were determined using commercially available kit (MP Biomedicals, Cat. # 07-189102). The antiserum cross-reacts 100% for testosterone, whereas it cross-reacts with progesterone, corticosterone, and estradiol-17 β less than 0.01%. The minimum detectable testosterone concentration was 0.1 ng/mL. The intraassay coefficient of variation was 6.0%, and the interassay coefficient of variation was 7.5%.

Estradiol—Estradiol levels were determined using a commercially available kit Coat-A-Count (Los Angeles, California, Cat. # TKE21). The antiserum cross-reacts 100% for estradiol, whereas it does not cross-react with aldosterone or corticosterone. The minimum detectable estradiol concentration was 8 pg/mL. The intraassay coefficient of variation was 7.0%, and the interassay coefficient of variation was 8.1%.

Progesterone—Progesterone levels were determined using a commercially available kit (MP Biomedicals, Cat. # 07-170105). The antiserum cross-reacts 100% for progesterone, whereas it does not cross-react with aldosterone or corticosterone. The minimum detectable progesterone concentration was 0.10 ng/mL. The intraassay coefficient of variation was 6.4%, and the interassay coefficient of variation was 2.4%.

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) v.15.0 software. In each of the data sets, between-subjects factors were Sex (male or female), CMS exposure (CMS or Non-CMS), and Prenatal group (PAE, PF, or C). In addition, the within-subjects factor of Day was included to explore the change in body weight over the 10-day CMS period (1, 4, 7, and 10) and the change in CORT levels prior to (Day 1) and following CMS exposure (Day 10). Significant main effects or interactions were further explored for simple main effects: a Fisher's LSD post hoc was used for comparisons of three groups or less, and a Sydak correction for > 3 groups. In analyses that included a within-subjects factor, an estimated marginal means procedure with a Sydak correction was employed. Because the Sydak correction accurately controls for familywise α , an overall F test is not required to be significant in order to explore a priori differences between factors, that is, planned comparisons. For repeated measures analyses, the degrees of freedom (df) were corrected to more conservative values using the Huynh-Feldt epsilon ϵ^{82} to correct for any violations of the sphericity assumption.⁸¹ Outliers that were ± 2 SD from the mean were removed from analysis. Correlations between CORT, testosterone, estradiol, or progesterone (post-EPM) and behavior (% time in open arms) on the EPM were assessed using Pearson's r correlation analysis. Corrected df are reported to one decimal place. Alpha (α) was set at 0.05 for all analyses. All results are shown as means $\pm$ SEM. Hormone data for 6 rats in the female Non-CMS group were lost due to mechanical failure of our centrifuge.

Results

CMS Exposure

Body Weight—Mixed factors ANOVA across the 10 sampling days indicated significant main effects of Day ($F_{(2.91,302.11)} = 78.69, P < .05$), Sex ($F_{(1,104)} = 1199.53, P < .05$), and CMS ($F_{(1,104)} = 7.32, P < .05$), as well as significant Day $\times$ Sex ($F_{(2.91,302.11)} = 50.89, P < .05$), Day $\times$ CMS ($F_{(2.91,302.11)} = 54.25, P < .05$), Sex $\times$ CMS ($F_{(1,107)} = 16.06, P < .05$), and Day $\times$ Sex $\times$ CMS ($F_{(2.91,302.11)} = 3.11, P < .05$) interactions (Table 2). Analysis of simple main effects by sex indicated a significant Day $\times$ CMS interaction in both males ($F_{(2.42,121.34)} = 21.58, P < .05$) and females ($F_{(2.00,108.13)} = 17.72, P < .05$). Post hoc analyses indicated that, whereas CMS males weighed significantly less than Non-CMS males on days 4, 7, and 10 of the CMS procedure ($P < .05$), CMS females only weighed less than Non-CMS females on day 10 ($P < 0.05$). By contrast, both males and females in the Non-CMS condition gained weight across the 10 days (all P s $< .05$). In summary, whereas body weight in CMS males decreased consistently over days, CMS females showed an attenuated decrease such that their body weight only differed from Non-CMS on the final day of CMS exposure ($P < .05$).

Plasma Corticosterone Levels Pre- and Post-CMS—Mixed factors ANOVA revealed significant main effects of Day ($F_{(1,51)} = 7.21, P < .05$) and Sex ($F_{(1,51)} = 10.71, P < .05$) for CORT levels (Table 3). As expected, basal CORT levels were higher in females than males, but surprisingly, basal CORT levels were lower on the last day of CMS compared to pre-CMS levels for females; that is, simple main effects revealed that, while basal CORT decreased over the CMS period for females (main effect of Day; ($F_{(1,25)} = 5.50, P < .05$), males did not change significantly over days (P s $> .05$). There were no significant effects of the prenatal group on CORT levels.

Elevated Plus Maze

Anxiety Measures

Figure 1A illustrates the significant overall main effect of CMS ($F_{(1,99)} = 22.92, P < .05$) on the percent time spent on the open arms. ANOVA also revealed a significant Sex $\times$ CMS interaction ($F_{(1,99)} = 4.67, P < .05$), and planned comparisons revealed that the effects of Sex and CMS exposure were in fact differential by PAE. That is, males from the three prenatal groups did not differ in the Non-CMS condition, whereas PAE males exposed to CMS spent significantly less time in the open arms compared to CMS controls (P s $< .05$). In addition, in the Non-CMS condition, PAE males spent significantly less time in the open arms compared to PAE females ($P < .05$). By contrast, there were no differential effects of prenatal group or CMS exposure among females.

Figure 1B shows that overall, CMS significantly reduced the frequency of total (full + partial) open arm entries ($F_{(1,99)} = 9.25, P < .05$). Importantly, planned comparisons revealed that this was mainly driven by a significant reduction in full open arm entries in PAE females in the CMS compared to the Non-CMS condition ($P < .05$). Although PAE males showed a significant reduction in total arm entries compared to other prenatal groups in the

CMS condition ($P < .05$), the difference from the Non-CMS PAE males did not reach significance.

Locomotor Activity Measures

Figure 2A illustrates that both Sex ($F_{(1,105)} = 19.25$, $P < .05$) and CMS ($F_{(1,105)} = 59.86$, $P < .05$) significantly influenced the frequency of total (open + closed) arm entries. Further, there were significant Sex $\times$ CMS ($F_{(1,105)} = 44.04$, $P < .05$) and Sex $\times$ CMS $\times$ Prenatal group ($F_{(2,105)} = 2.94$, $P = .05$) interactions. CMS significantly reduced total arm entries among females (P s $< .05$), but not males (P s $> .05$), and Non-CMS males made significantly fewer total arm entries than Non-CMS females (P s $< .05$). In addition, whereas there were no Prenatal group differences among males (P s $> .05$), Non-CMS PAE females made significantly more total arm entries than Non-CMS PF and C females (P s $< .05$).

There were no significant effects of Sex, Prenatal group, or CMS exposure on the total distance traveled on the EPM (Fig. 2B).

Ethological Behaviors

ANOVA revealed significant main effects of Sex ($F_{(1,108)} = 5.69$, $P < .05$) and CMS ($F_{(1,108)} = 14.04$, $P < .05$), as well as a significant Sex $\times$ CMS interaction ($F_{(1,108)} = 10.25$, $P < .05$) on the frequency of grooming bouts (Table 4). CMS males groomed significantly more than Non-CMS males ($P < .05$), while there were no effects of CMS in females. ANOVA also indicated that CMS increased the frequency of rearing ($F_{(1,108)} = 29.18$, $P < .05$); however, as with grooming behavior, this effect was only observed among males ($P < .05$; Table 4). Prenatal group did not significantly influence grooming or rearing behaviors.

Hypothalamic–Pituitary–Adrenal and Hypothalamic–Pituitary–Gonadal Reactivity to the Acute Stress of Elevated Plus Maze Testing

Corticosterone

Overall ANOVA indicated main effects of Sex ($F_{(1,99)} = 41.17$, $P < .05$) and CMS ($F_{(1,99)} = 328.20$, $P < .05$) on CORT concentration following exposure to the EPM (Fig. 3). As expected, CORT levels were significantly higher in females than males, and in CMS-compared to Non-CMS exposed animals. Simple main effects analysis of CORT levels among CMS-exposed animals indicated a significant main effect of Sex ($F_{(1,53)} = 41.92$, $P < .05$) as well as a significant Prenatal group $\times$ Sex interaction ($F_{(2,53)} = 3.33$, $P < .05$). Planned comparisons revealed that in the CMS condition, PAE females had significantly higher CORT levels than PF and C females (P s $< .05$). By contrast, there were no significant effects of Prenatal group among males or Non-CMS animals.

Testosterone

Between-subjects ANOVA revealed a significant main effect of CMS exposure ($F_{(1,54)} = 13.82$, $P < .05$) on testosterone levels following EPM testing, but no effects of Prenatal group (Fig. 4A). CMS exposure significantly increased the testosterone response to the EPM compared to Non-CMS males ($P < .05$).

Estradiol

Figure 4B shows that previous exposure to CMS significantly lowered estradiol levels following EPM testing (significant main effect of CMS, $F_{(1,46)} = 9.34$, $P < .05$). Importantly, planned comparisons revealed that CMS selectively lowered estradiol levels in PAE and PF (P s $< .05$), but not C, females. Pearson's correlation revealed a negative correlation between CORT and estradiol levels that approached significance ($r = .25$, $P = .08$).

Progesterone

Between-subjects ANOVA revealed significant main effects of CMS ($F_{(1,46)} = 56.63$, $P < .05$) and Prenatal group ($F_{(1,46)} = 4.90$, $P < .05$), and a significant Prenatal group $\times$ CMS interaction ($F_{(1,46)} = 3.62$, $P < .05$) for progesterone levels following exposure to the EPM (Fig. 4C). Overall, CMS significantly increased progesterone levels following EPM testing. However, while PAE females had significantly higher progesterone levels than PF and C females in the CMS condition ($P < .05$), PF females had significantly lower progesterone levels than C females in the Non-CMS condition ($P < .05$). Pearson's correlation indicated a significant positive correlation between CORT and progesterone levels ($r = .70$, $P < .05$). Not surprisingly, there was also a significant negative correlation between progesterone and estradiol levels ($r = .55$, $P < .05$).

Neuroendocrine Correlates of Anxiety

Because CORT levels were, as expected, sexually dimorphic, correlations between anxiety measures and CORT were run separately by Sex. We found no significant correlations among males, but a highly significant negative correlation among females for both percent time on open arms ($r = .51$, $P < .05$) and total open arm entries ($r = .52$, $P < .05$) and CORT levels post-EPM. There were no significant correlations between testosterone, estradiol, or progesterone with percent time on the open arms.

Discussion

The results of this study reveal sexually dimorphic effects of prenatal exposure to alcohol and adult exposure to CMS on neurobehavioral measures of anxiety, and suggest that our milder, 10-day CMS regimen markedly altered HPA and HPG function. Moreover, in conjunction with PAE, CMS selectively altered anxiety and locomotor activity in the EPM in a sexually dimorphic manner: whereas PAE males exposed to CMS showed a decrease in percent time spent in the open arms, CMS exposure in PAE females reduced total open arm entries and eliminated the locomotor hyperactivity observed in the Non-CMS condition. Furthermore, in response to acute stress, PAE females exposed to CMS had elevated CORT and progesterone compared to PF and C rats, and CMS exposure selectively lowered estradiol levels in PAE and PF, but not C, females, whereas CMS elevated testosterone in males across prenatal groups. Taken together, these data support the hypothesis that adult exposure to CMS has adverse effects on behavioral and hormone reactivity in general, and in particular, appears to have the greatest effects on an already sensitized HPA axis among PAE animals, leading to behavioral and endocrine alterations suggestive of increased anxiety/depressive-like responses.

Overall, our CMS procedure produced a significant anxiogenic profile across prenatal groups. CMS exposure resulted in a significant decrease in body weight in both males and females in all prenatal treatment groups. These data support the growing body of literature indicating that exposure to persistent mild stress induces depressive-like symptoms in rodents, including slower weight gain than controls over time.^{69,70,83} It is likely that the HPA axis plays a role in this effect, as administration or genetic overexpression of CRH (a major HPA axis secretagogue) suppresses appetite and induces weight loss.⁸⁴ Consistent with our previous work,^{35,85,86} PAE did not significantly impact adult body weight in this study. However, we did find significant sex differences in the effects of CMS. For males, body weight decreased across days and did not recover by the final day of the CMS exposure, whereas females in CMS and Non-CMS conditions did not differ until the final day of CMS exposure (Day 10). These sex differences are not surprising, as previous studies found that 6 weeks exposure to CMS attenuates body weight gain in males, but not females, compared to Non-CMS-exposed controls.⁸⁷ Together these data indicate that stress exposure results in attenuated weight gain, and that females may be more resilient than males in response to chronic and varied stress exposure in this measure.

As predicted, CMS significantly influenced basal CORT levels in a sexually dimorphic manner. Overall, and consistent with previous findings,^{88–90} females had higher CORT levels than males. Moreover, basal CORT levels were altered following CMS exposure in female, but not male, rats. These data support and extend evidence that the female HPA axis is more sensitive to stress exposure than that of males.^{35,56,91} However this interaction between Sex and CMS exposure was contrary to our initial expectations, as we found a significant reduction in basal CORT levels among females following 10 days of CMS. Interestingly, we previously reported that 18 days of chronic intermittent stress resulted in elevated basal CORT levels in C males and females.⁸⁹ However, that study used a stress regimen that was almost twice as long as that used in the current study. In typical animal models of depression, CMS regimens tend to be prolonged, often on the scale of several weeks.⁷⁰ It is possible that this prolonged exposure may disrupt negative feedback such that HPA activity is elevated even under basal conditions. Indeed, we have previously reported that PAE animals have negative feedback deficits in the intermediate time domain.⁸⁶ Of relevance, we have also shown that adrenalectomy can unmask increased HPA drive and deficits in feedback regulation in PAE animals under basal conditions, even in the face of similar basal hormone levels.³⁸ Furthermore, it appears that housing condition may also modify the effects of chronic stress exposure on basal HPA regulation. While our animals were pair-housed throughout CMS exposure, except for defined periods of social isolation stress, animals in the Kim *et al.* study⁸⁹ were singly housed throughout the stress regimen. It is likely that the relative severity of the latter paradigm, which included a combination of both social-isolation housing stress and exposure to varied stressors for 18 days, was sufficient to elevate basal CORT levels, and perhaps resulted in a ceiling effect, thereby preventing observation of prenatal treatment effects. Milder stress paradigms, such as that employed in the current study, may therefore permit the investigation of HPA function in animals that are already hyperresponsive to stressors.

CMS exposure also significantly increased anxiety measures in the EPM, and importantly, did so in a Sex- and Prenatal group-specific manner. As noted, while effects of PAE on

anxiety-like behaviors on the EPM have been somewhat inconsistent,^{60–63} increased anxiety-like behaviors have been observed following exposure to acute mild stressors such as social isolation, vehicle injection, and open-field exposure.^{61,63,64} Data from the current study extend these findings to show that CMS sensitizes the HPA response to acute stressors, and that this sensitization is increased by PAE. Importantly, CMS exposure was critical in revealing the effects of PAE on behavior in the EPM. Moreover, while both males and females showed increased anxiety, this was manifest in a sexually dimorphic manner: PAE males exposed to CMS showed a significant decrease in percent time in the open arms, whereas PAE females showed a decrease in the number of total open arm entries. These data are intriguing, as they suggest that the neurobiological alterations induced by CMS and PAE may manifest themselves differentially, depending on sex, and highlight the importance of testing both males and females and utilizing a range of outcome measures in studies that examine the influence of PAE and/or CMS on neurobehavioral measures of anxiety. These findings support and extend our previous work demonstrating that the behavioral and neuroendocrine effects of PAE may be observed differentially in male and female offspring.⁵³

Notably, our results reveal that sex differences in PAE and CMS effects on anxiety-like behavior can be related to alterations in HPA reactivity. All animals exposed to the CMS treatment had significantly elevated CORT levels compared to non-CMS exposed rats following testing on the elevated plus maze. These data also support previous findings from our lab and others indicating PAE both increases basal HPA tone,^{32,38,92,93} and results in hyperresponsiveness to acute and/or chronic stressors.^{34,37,94,95} Importantly, this is strongly reminiscent of the HPA hyperactivity and increased HPA drive that are key features of depression and anxiety disorders (e.g., References 96 and 97). This is in line with prior evidence indicating that chronic intermittent stress leads to elevated adrenal weights in PAE, but not PF or C, females.⁸⁹

Our finding that the percentage of time spent on the open arms is negatively correlated with CORT supports previous work in this area,⁹⁸ indicating a positive relationship between anxiety-like behavior and HPA reactivity. We extend this finding to show that frequency of open arm entries is also negatively correlated with CORT. Importantly, this relationship was found only in females, further supporting the notion that the neural alterations induced by CMS and PAE may manifest themselves differentially, depending on sex. Indeed, consistent with previous findings from our laboratory,⁶¹ we found that CORT levels following EPM testing were significantly higher in PAE females compared to their PF and C counterparts, while there were no effects of prenatal treatment in males. The combination of CMS and PAE may thus lead to a unique HPA axis profile in females such that they display blunted basal CORT activity, but hyperresponsivity following acute stress. This is an important finding in the context of the literature on early life adversity and endocrine regulation. For example, Fisher and colleagues^{99,100} found that early adversity (i.e., neglect), a younger age at first foster placement, and a higher number of foster placements results in blunting of the daily cortisol rhythm, with atypically low morning cortisol levels. These children show the least resilience in the face of subsequent stressful events, and indeed, are often hyperresponsive to acute stressors or challenges despite the overall flattening of the circadian rhythm. It is possible that in our model, increased HPA tone and increased

responsiveness to the CMS protocol may result in an overall blunting of basal hormone levels and/or the HPA circadian rhythm. Moreover, a comparable sex difference has been reported in clinically depressed populations: In response to stress, depressed women, but not men, show an elevated cortisol response to a negative event.¹⁰¹ In contrast to the depression literature, there is a dearth of knowledge about the HPA activity of adults with FAS/FASD. Future studies are required to investigate fully whether differences in basal and stress-induced HPA reactivity exist, and if so, whether they might play a role in mediating the comorbid diagnosis of FASD and depression.

Our results also reveal critical effects of sex, CMS condition, and prenatal group on locomotor activity on the EPM. CMS exposure reduced total arm entries in females, but not males, and non-CMS females were hyperactive compared to non-CMS males. Importantly, whereas we saw no prenatal differences in activity among males, PAE females in the non-CMS condition made more total arm entries than their C counterparts. These data support previous findings that PAE females, but not males, are hyperactive compared to controls on the EPM.⁶¹ Interestingly, CMS exposure prior to EPM testing significantly attenuated this locomotor hyperactivity. This finding is noteworthy, as it parallels symptoms of both depression¹⁰² and FASD.^{103,104} Because total distance traveled did not differ between sexes and CMS conditions, or among prenatal groups, these activity differences are not likely a result of differences in body size or a reduction in exploratory behavior.

Our results support previous findings that prenatal stress does not alter the frequency of grooming behaviors on the EPM.¹⁰⁵ However, males, but not females, groomed more on the EPM following CMS exposure, suggesting that CMS differentially alters the response of males and females to a novel environment. Although the interpretation of grooming behavior is not well understood, one explanation is that grooming represents a form of displacement behavior, which is considered a reliable, ethological indicator of increased emotional or physiological arousal in both rodents and in humans.^{106,107} CMS exposure also significantly increased the frequency of rearing behavior among males, which may be a consequence of a reduction in time spent on the open arms, as animals reared mostly in the closed arms. We did not observe any prenatal treatment effects on rearing, despite prior evidence from our lab indicating that PAE males rear significantly less than PF and C following CRH administration.⁶⁴ One possible explanation for this discrepancy is that the CMS exposure used in the current study may be a more severe stressor than acute CRH administration, thus resulting in a ceiling effect on this measure, and thereby preventing the observation of prenatal effects. Consequently, our data further support the use of the CMS regimen in the investigation of depressive/anxiety-like behaviors.

Our study is one of the first to examine the combined effects of prenatal ethanol and adult CMS exposure on HPG reactivity to acute stress. The finding that males previously exposed to CMS showed elevated testosterone levels compared to nonstressed controls following testing on the EPM was unexpected. While acute stress has been shown to elicit a small and transient increase in plasma testosterone levels,^{94,108,109} chronic stress typically suppresses testosterone secretion,¹¹⁰ perhaps as a result of increased HPA activity. Therefore, we hypothesized that CMS-exposed animals would show blunted testosterone responses relative to controls. However, the finding that basal CORT levels were not elevated following the

CMS exposure period suggests that the relatively short duration of stress exposure in our study might not have elicited the alterations in CORT levels observed in longer stress regimens.^{91,111} The finding that prenatal groups did not differ in testosterone levels following exposure to the acute stress of EPM exposure was also somewhat unexpected, as we have shown previously that PAE leads to a blunted testosterone response to restraint stress compared to that in PF and C males.⁹⁴ In view of data indicating that HPA hyperresponsiveness in PAE animals may be manifested differently depending on the nature and intensity of the stressor, the time course, and the hormonal endpoint examined,^{35,95} it is possible that the relatively mild and short-duration stressor (5-min exposure to EPM) in the present study compared to the more severe (30-min restraint stress) stressor in the Lan *et al.* study⁹⁴ accounts for the differences in results. More work is needed to resolve this issue fully.

CMS exposure also differentially altered HPG activity in females, resulting in significantly lower estradiol levels in PAE and PF compared to C females following EPM testing. These data are consistent with previous findings from our lab^{94,112} and others.^{31,113} Specifically, PAE reduces the activity of the HPG axis, suppressing the secretion of sex steroids, including estradiol. The suppression of sex steroids is thought to be due to the decreased hypothalamic gonadotropin-releasing hormone (GnRH) content found in PAE females,¹¹⁴ resulting in decreased secretion of luteinizing hormone, and in turn, lower estradiol release.¹¹⁵ Although we did not specifically examine the stage of estrous cycle in this study, we can hypothesize that estrous stage would significantly influence behavior on the EPM as well HPA/HPG reactivity. Specifically, we have reported previously that, despite the fact that PAE typically suppresses HPG activity, PAE and PF animals show increased basal and stress estradiol levels compared to C females during proestrus compared to other phases of the cycle, and basal estradiol and CORT are positively correlated.¹¹⁶ Moreover, these hormonal changes were related to central alterations at the level of the hippocampus, as hippocampal mineralocorticoid receptor (MR) mRNA levels were significantly decreased, and glucocorticoid receptor mRNA increased, during proestrus in PAE compared to PF and C females.¹¹⁷ These findings suggest a role for estradiol in mediating the increased HPA tone and deficits in HPA feedback regulation observed in PAE females, and would predict that females in the present study would show greater anxiogenic effects during proestrus than their PF and C counterparts.

CMS also elevated progesterone levels overall, but resulted in significantly greater progesterone elevations in PAE compared to C CMS females. Importantly, progesterone levels were also positively correlated with CORT levels in our females. A previous study investigating the effect of restraint stress on gonadal hormones revealed that both females¹¹⁸ and males^{119,120} exposed to stress exhibited higher progesterone levels than nonstressed rats. Interestingly, the latter study showed that the stress-induced progesterone may be adrenal and not gonadal in origin.¹¹⁹ Currently, neither the neurobehavioral consequences, nor mechanisms of increased adrenal secretion of progesterone in response to stress is known. However, progesterone is a precursor to many neurosteroids that modulate the HPA axis.^{121–123} Thus, future studies are required to investigate the neurobehavioral consequences of increased progesterone release, and the interactions of estradiol and progesterone in response to stress among PAE animals.

Although there are no studies to our knowledge that examine the effects of both PAE and CMS on alterations of the HPG axis, chronic stress can cause HPG dysregulation and desynchronization of the estrous cycle in females.^{87,91,124} It is possible that the combination of PAE and CMS exacerbates this desynchronization, causing a disruption in the estrus cycle as well as the interaction of HPG and HPA hormones. This could have far-reaching effects on reproductive function, including the onset of puberty and reproductive aging.^{125,126} Indeed, as noted earlier in this chapter, basal CORT levels are higher, and hippocampal MR mRNA levels are lower, in PAE compared to PF and/or C females in proestrus, when estradiol levels are high.^{116,117} Together with data from the present study, these findings suggest that differential HPA sensitivity to gonadal hormone fluctuations across the estrous cycle, and in particular, increased sensitivity to the stimulatory effects of estradiol, in PAE compared to C females may play a role in the HPA hyperresponsiveness observed. Future studies will explore this issue further by examining how ovariectomy, with or without estradiol and progesterone replacement, differentially influences CORT levels among CMS-exposed animals.

Taken together, findings from this article provide further evidence that CMS leads to neurobehavioral alterations indicative of increased anxiety and depression: almost every variable that was examined was altered by CMS exposure. We extend these data to show that CMS may selectively increase anxiety-like behaviors and alter both HPA and HPG reactivity in PAE compared to C offspring. These findings support the hypothesis that fetal programming of HPA activity by PAE can permanently sensitize neuroadaptive mechanisms that mediate responses to stress, resulting in hyperreactivity to subsequent, even mild, stressful life events.^{127–129} Ultimately, repeated stress exposure may result in a maladaptive cascade of events, which may underlie increased vulnerability to depression and anxiety disorders. These neurobehavioral alterations could play a role in the abnormally high rate of mental illness in FASD populations. Furthermore, our results provide strong support that neurobiological differences in the propensity to depression and anxiety disorders exist between sexes. In due course, this may lead to the identification of novel therapeutic targets to improve the quality of life for those who are affected by FAS/FASD and mood disorders.

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Figure 1.

Effect of prenatal treatment, sex, and chronic mild stress (CMS) on (A) percent time spent in the open arms (%), and (B) frequency of total (full + partial) open arm entries on the elevated plus maze (EPM). *Black bars* represent prenatal alcohol exposure (PAE), *hatched bars* represent pair-fed (PF), *white bars* represent control (C) animals. (*) Denotes significantly different from the Non-CMS group, $P < .05$. (#) Denotes significantly different from other prenatal groups, $P < .05$. (^) Denotes significant differences between sexes, $P < .05$. n 's = 7–10 in each condition.

Figure 2.

Effect of prenatal treatment, sex, and chronic mild stress (CMS) on (A) frequency of total arm entries, and (B) total distance traveled (cm) on the elevated plus maze (EPM). *Black bars* represent prenatal alcohol exposed (PAE), *hatched bars* represent pair-fed (PF), *white bars* represent control (C) animals. (*) Denotes significantly different from the Non-CMS group, $P < .05$. (#) Denotes significantly different from other prenatal groups, $P < .05$. (^) Denotes significant differences between sexes, $P < .05$. n 's = 7–10 in each condition.

Figure 3.

Effect of prenatal treatment, sex, and chronic mild stress (CMS) on plasma corticosterone levels (µg/dL) 30-min post-elevated plus maze (EPM) testing. *Black bars* represent prenatal alcohol exposed (PAE), *hatched bars* represent pair-fed (PF), *white bars* represent control (C) animals. (*) Denotes significantly different from the Non-CMS group, $P < .05$. (#) Denotes significantly different from other prenatal groups, $P < .05$. n 's = 7–10 for each condition.

Figure 4.

Effect of prenatal treatment and chronic mild stress (CMS) on plasma levels of (A) testosterone, (B) estradiol, and (C) progesterone (ng/mL) post-elevated plus maze (EPM) testing in male (A) and female (B and C) rats. *Black bars* represent prenatal alcohol exposed (PAE), *hatched bars* represent pair-fed (PF), *white bars* represent control (C) animals. (*) Denotes significantly different from the Non-CMS group, $P < .05$. (#) Denotes significantly different from other prenatal groups, $P < .05$. n 's = 7–10 for each condition.

TABLE 1

Developmental Data from Ethanol (prenatal alcohol exposed), Pair-Fed, and Control Dams and Offspring from Birth to Weaning

Pregnancy outcome variable	Prenatal treatment group		
	Prenatal alcohol exposed	Pair-fed	Control
Number of pregnant dams	14	14	13
Maternal death/illness	0	0	0
Perinatal death	0	0	0
Length of gestation (d)	23.1 ± 0.1	22.6 ± 0.1	22.6 ± 0.2
Dam weight (g)			
GD1	275.4 ± 2.5	268.7 ± 3.2	278.7 ± 6.3
GD7	279.5 ± 3.5 ^a	264.9 ± 3.1	316.5 ± 6.0
GD14	307.3 ± 4.6 ^a	284.7 ± 4.3	353.0 ± 5.8
GD21	364.2 ± 8.7 ^a	353.1 ± 8.1	437.1 ± 9.5
LD1	307.2 ± 4.1 ^a	289.5 ± 4.0	347.0 ± 8.4
LD7	353.4 ± 5.2 ^a	340.5 ± 4.5	370.5 ± 6.3
LD14	378.4 ± 6.3 ^b	362.5 ± 4.2	381.3 ± 6.3
LD21	357.6 ± 7.4 ^c	334.8 ± 5.1	352.5 ± 5.8
Litter size	14.4 ± 0.1	15.9 ± 0.5	14.4 ± 0.9

Offspring weight (g)						
	Males			Females		
	PA E	PF	C	PA E	PF	C
PND1	6.0 ± 0.1 ^a	5.6 ± 0.2	6.5 ± 0.2	5.8 ± 0.1 ^a	5.3 ± 0.2	6.2 ± 0.2
PND7	16.6 ± 0.6 ^a	17.0 ± 0.7	18.4 ± 0.5	16.2 ± 0.6	16.3 ± 0.8	17.4 ± 0.5
PND14	35.8 ± 1.3	35.1 ± 1.0	35.7 ± 0.8	34.7 ± 1.1	33.5 ± 1.3	34.2 ± 0.7
PND21	54.7 ± 1.9	54.7 ± 1.6	57.8 ± 1.7	52.5 ± 2.2	52.6 ± 1.7	53.6 ± 2.0

Abbreviations: PAE, prenatal alcohol exposed; PF, pair-fed; C, control; GD, gestational day; LD, lactation day.

^aPAE = PF < C;

^bPAE < C;

^cPAE = C > PF.

TABLE 2Effect of Chronic Mild Stress on Body Weight in Male and Female Rats^{*}

	Males		Females	
	CMS	Non-CMS	CMS	Non-CMS
Day 1	484.76 ± 5.50	491.29 ± 1.13	300.60 ± 1.13	301.43 ± 0.79
Day 4	468.72 ± 5.59 ^a	495.03 ± 1.16	292.47 ± 1.09	302.87 ± 0.90
Day 7	486.35 ± 6.75 ^a	502.07 ± 1.17	293.87 ± 1.03	305.30 ± 0.75
Day 10	492.37 ± 6.39 ^a	527.83 ± 1.22	291.37 ± 1.07 ^a	313.03 ± 0.76

^{*}Data represent body weight (g) in chronic mild stress (CMS) ($n = 60$) or Non-CMS ($n = 60$) animals.^aDenotes significantly different from Non-CMS condition, P s < .05.

TABLE 3Effect of Chronic Mild Stress and Sex on Plasma Corticosterone (ng/mL) ^{*}

	Males	Females
Day 1	0.91 ± 0.24	2.74 ± 0.56 ^a
Day 10	0.77 ± 0.05	1.43 ± 0.40 ^{a,b}

^{*}Data represent mean basal plasma corticosterone (µg/dL) in chronic mild stress (CMS) males (*n* = 30) or females (*n* = 30).

^aDenotes significantly different across days of CMS exposure; *P* s < .05.

^bDenotes significantly different between sexes; *P* s < .05.

TABLE 4

Effect of Chronic Mild Stress, Prenatal Group, and Sex on Elevated Plus Maze Behaviors in Male and Female Rats*

	Males		Females	
	CMS	Non-CMS	CMS	Non-CMS
Rearing	12.5 ± 0.65 ^a	8.72 ± 0.46	11.59 ± 0.60	9.35 ± 0.44
Grooming	3.23 ± 0.27 ^a	1.55 ± 0.22	1.86 ± 0.23	1.72 ± 0.24

Abbreviations: CMS, chronic mild stress; EPM, elevated plus maze.

*Data represent behavior scored during EPM testing in animals tested after 10 days of CMS ($n = 30$) or left undisturbed (Non-CMS; $n = 30$).

^aDenotes significantly different from Non-CMS; P s < .05.