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Exposure to chronic mild stress differentially alters CRH and AVP mRNA expression in the stress-responsive neurocircuitry of male and female rats prenatally exposed to alcohol

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Abstract

Background—Prenatal alcohol exposure (PAE) results in dysregulation of the offspring hypothalamic-pituitary-adrenal (HPA) axis, increasing sensitivity to stressors and vulnerability to stress-related disorders. We have previously shown that exposure to chronic mild stress (CMS) in adulthood significantly increases anxiety-like behaviors (elevated plus maze) in PAE males and females compared to controls. To explore neurobiological mechanisms linking HPA dysregulation and altered anxiety-like behavior, we investigated neuropeptide [corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP)] expression in brain areas involved in the stress neurocircuitry of animals from this previous behavioral study.

Methods—Adult PAE, pair-fed (PF), and *ad-libitum* fed control (C) male and female offspring exposed to CMS or remaining undisturbed (Non-CMS) were terminated 30 min following behavioral testing.

Results—In the paraventricular nucleus, CMS increased CRH mRNA levels in PAE compared to PF and C males, and increased AVP mRNA levels in PAE compared to C males, with no differential effects for CRH or AVP in females. In the central nucleus of the amygdala, there was an increase in CRH mRNA expression overall, regardless of CMS condition or sex, in PAE compared to C animals. Moreover, in PF males, CMS increased AVP mRNA levels in the paraventricular nucleus, resulting in a decreased CRH/AVP ratio compared to PAE males, and decreased amygdala CRH mRNA compared to that in the Non-CMS condition.

Conclusions—CMS differentially altered central HPA peptide expression in PAE and PF animals compared to their control counterparts, with a possible shift toward greater CRH mediation of HPA regulation in PAE males, and greater AVP mediation of HPA regulation in PF males. However, changes in CRH and AVP expression do not align fully with the anxiogenic

profile observed in our previous behavior study, suggesting that other neuronal substrates and limbic forebrain regions also contribute to increased anxiety-like behavior following CMS.

Keywords

Prenatal Alcohol (Ethanol) Exposure; Hypothalamic-Pituitary-Adrenal (HPA) Axis; Corticotropin-Releasing Hormone (CRH); Arginine Vasopressin (AVP); Amygdala

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that refers to the wide range of abnormalities or deficits that can occur in children born to women who drink alcohol during pregnancy (Riley and McGee, 2005; Manning and Hoyme, 2007). Prenatal alcohol exposure (PAE) constitutes an early insult to the organism that can result in life-long consequences (Riley and McGee, 2005). The most profound effects of PAE are on the developing brain, leading to alterations in subsequent neurobiological and neurobehavioral functions. Among those, a robust phenomenon demonstrated in both clinical and animal studies is dysregulation of the offspring hypothalamic-pituitary-adrenal (HPA) axis, a key player in the stress response (Haley et al., 2006; Jacobson et al., 1999; Lee et al., 2000; Nelson et al., 1986; Ramsay et al., 1996; Weinberg et al., 2008). The survival and health of an individual requires appropriate physiological responses to stressors (i.e. environmental and homeostatic challenges). Maladaptive stress responses can culminate in homeostatic disruption and vulnerability to metabolic, cardiovascular and affective disorders (Nestler et al., 2002).

PAE programs the fetal HPA axis such that HPA tone is altered throughout life (Hellemans et al., 2010; Weinberg et al., 2008). Alcohol-exposed infants exhibit higher basal cortisol levels and increased responses to a heel-stick blood draw (Jacobson et al., 1999; Ramsay et al., 1996) and to a psychologically stressful “still-face” procedure (Haley et al., 2006) compared to non-exposed infants. Studies using animal models support and extend these findings: enhanced and/or prolonged corticosterone (CORT) and adrenocorticotropin (ACTH) responses to stressors including footshock, ether, restraint, and immune challenges, and to drugs such as alcohol and morphine, have been reported in PAE compared to control rats (Lee et al., 2000; Nelson et al., 1986; Weinberg et al., 2008). Importantly, while HPA alterations are a robust phenomenon, sex differences in patterns of response may occur depending on the stressor, time course, and hormonal endpoints measured (Weinberg et al., 2008). Moreover, pre-existing HPA abnormalities or a sensitized stress response system, may be a major contributory factor in the pathogenesis of stress-related disorders, including some forms of depression and anxiety. Thus, PAE-related HPA dysregulation may explain, at least in part, the increased propensity to depression and anxiety disorders in FASD populations (Hellemans et al., 2010). Indeed, a previous study in our laboratory (Hellemans et al., 2008) found that exposure to unpredictable chronic mild stress (CMS) in adulthood resulted in greater anxiety-like behaviors in animals prenatally exposed to alcohol than in controls during testing on the elevated plus maze, a task commonly used to measure anxiety-like behavior (File et al., 2004). Moreover, behavioral alterations occurred in a sexually dimorphic manner: following CMS, PAE males spent significantly less time in the open

arms, whereas PAE females showed a decrease in the number of total open arm entries. To explore the neurobiological mechanisms underlying a possible link between central HPA dysregulation and altered anxiety-like behavior in adulthood, the current study examined brains from adult PAE and control males and females from this previous behavioral study (Hellemans et al., 2008), focusing on changes in central HPA peptide expression.

HPA responses are driven by neurons located in the medial parvocellular subdivision of the paraventricular nucleus (mpd PVN) of the hypothalamus. The mpd PVN contains both corticotropin-releasing hormone (CRH, the major HPA secretagogue)- and arginine vasopressin (AVP, a co-secretagogue)-expressing neurosecretory neurons, and is the final pathway that integrates multiple excitatory and inhibitory inputs from other brain areas, including the hippocampus, prefrontal cortex, and amygdala, that regulate HPA function. Together those areas form a neurocircuitry that is involved in emotional, cognitive, and behavioral responses to stress (Herman et al., 2005), and plays a role in depression and anxiety disorders (Owens and Nemeroff, 1991). The amygdala, which is important in the control in emotional and autonomic responses to stress, is rich of CRH neurons (Sah et al., 2003). Hyperactivity of these neurons increases anxiety-like behaviors (Rainnie et al., 2004). Furthermore, CRH containing neurons in the central nucleus of amygdala (CeA) project to the PVN (Dong et al., 2001; Dong et al., 2001; Prewitt and Herman, 1998) and provide stimulatory input to increase HPA drive. How PAE alters this stress-responsive neurocircuitry is not fully understood.

In the present study, we examined CRH and AVP expression in the mpd PVN of the hypothalamus, which underlies central HPA drive, as well as CRH expression in the CeA, which provides stimulatory input to the PVN and plays a key role in physiological responses to stress and pathophysiology of anxiety, in PAE and control males and females from our previous behavioral study (Hellemans et al., 2008). We hypothesized that PAE would induce central HPA dysregulation by differentially altering CRH and AVP mRNA expression in PAE and control offspring, and may do so in a stress (CMS)- and sex-dependent manner.

MATERIALS AND METHODS

The brains analyzed in this study came from C, PF and PAE animals tested previously in the elevated plus maze (Hellemans et al., 2008). Methods relating to breeding, experimental diets, CMS, and elevated plus maze testing were described in detail in our previous publication. Here we summarize these methods briefly.

Animals, Breeding, Diets, and Feeding

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

Female and male Sprague-Dawley rats obtained from Charles River Laboratories (St. Constant, PQ, Canada) and the UBC Animal Care Centre (Vancouver, BC, Canada), respectively, were adapted to the colony room prior to breeding. On gestation day (GD) 1, females were singly housed and randomly assigned to a treatment group: 1) Ethanol (PAE),

liquid ethanol diet (36% ethanol-derived calories) and water, *ad libitum*, (n=10); 2) Pair-fed (PF), liquid control diet, with maltose-dextrin isocalorically substituted for ethanol, and intake matched to the amount consumed by a PAE partner (g/kg body weight/gestation d), and water *ad libitum*; (n=10); and 3) Control (C): standard lab chow and water, *ad libitum*, (n=10). Liquid diets were prepared by Dyets Inc., Bethlehem, PA (Weinberg/Keiver high protein liquid diet – Experimental, #710324; Control, #710109). Fresh diet was provided daily within 1.5 hours prior to lights off to prevent a shift of the CORT circadian rhythm that typically occurs in animals on a restricted feeding schedule, such as the PF group (Gallo and Weinberg, 1981; Krieger, 1974). On GD21 experimental diets were replaced with standard laboratory chow. On postnatal day 1 (PN1), pups were weighed and litters were randomly culled to 10 (5 males and 5 females when possible), and dams and pups were weighed weekly until weaning on PN22. On PN40, two males and two females were chosen from each litter; one of each sex was randomly assigned to the CMS and the other to the Non-CMS condition.

Chronic Mild Stress, Elevated Plus Maze testing, and Sampling and Tissue Collection

Because the breeding period extended over several weeks, and animals entered the experiment in a staggered fashion, the age at which the CMS procedure commenced ranged from PN60 – PN90. CMS comprised 10 consecutive days of exposure to stressors, 2 different stressors/day, one between 0800 and 1200 hr, and the second between 1300 and 1700 hr, 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. On day 1 of CMS, all animals were weighed, and moved to designated CMS or Non-CMS colony room. Animals underwent the CMS regimen in a room separate from the colony room, or remained undisturbed (Non-CMS condition) except for routine husbandry. Stressors included: (1) 5-min exposure to a 20 × 20 × 90 cm Plexiglas platform mounted on wooden posts 90 cm high; (2) 2 hour white noise (40 dB; Lafayette Instruments model #15800); (3) overnight social isolation in hanging cages (20 × 23 × 18 cm) with wire mesh front and floor; (4) 30 min restraint in PVC tubes (19 × 7 cm for male, 15 × 6 cm for female); (5) 1 hour exposure to a soiled cage (cagemates placed in soiled cage of another pair of animals); (6) 2 hour cage tilt at a 30° angle; (7) 1 hour exposure to a novel cage (cagemates rehoused in small, opaque cages (18 × 25 × 15 cm) without bedding, food, or water); (8) Tail nick: a cut was made 1 mm from the tip of the tail. Tail nick occurred as the stressor on the morning of Day 1 (pre-CMS) and Day 10 (post-CMS), and the blood samples were used for analysis of plasma CORT levels (plasma CORT data were published previously, Hellemans et al., 2008). One day following the end of the CMS period (day 11), all animals (N=10 per prenatal treatment group, CMS condition and sex) were habituated to a new room (distinct from the stress-exposure room) for 10 min. On the following day, 48 hours post-CMS exposure, all animals were exposed to the elevated plus maze for a 5 min test, decapitated 30 min later in a separate room, and trunk blood collected. Brains were rapidly removed and immediately frozen on dry ice, then stored at –80 °C until sectioning. For the present study, 6–8 brains per prenatal treatment group, CMS condition and sex were randomly selected for analysis.

In situ Hybridization

Brain Preparation—Brains were sectioned into 20 µm coronal sections. Frozen sections were thaw-mounted onto precleaned colorfrost/plus microscope slides (Fisher Scientific, Whitby, ON, Canada) and stored at -80 °C.

Oligonucleotide Probes and Labeling—Oligonucleotide probes were used to measure CRH and AVP mRNA. Probes were synthesized at the Oligonucleotide Synthesis Laboratory, University of British Columbia as follows: antisense CRH (5'-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3') (Jingami et al. 1985; Young et al. 1986a), antisense AVP (5'-GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG-3') (Ivell and Richter 1984; Young et al. 1986b). Sense oligos for CRH and AVP mRNA were used as negative controls. Probes were 3' tail labeled with ³⁵S-dATP (Amersham Biosciences, Piscataway, NJ, USA) using terminal deoxytransferase (New England Biolabs Inc., Pickering, ON, Canada) as per supplier protocol. Probes were purified using Roche DNA G-25 Sephadex Columns (Roche Scientific, Indianapolis, IN., USA). 1 M dithiothreitol (DTT) was added to prevent oxidation.

Hybridization with Oligonucleotides—Sections were thawed (20 min) and went through prehybridization as follows: formalin (30 min), 1 × PBS (10 min) twice, 0.1M triethanolamine-hydrochloride - 0.9 % NaCl + 0.25 % acetic anhydride (10 min), 2 × SSC (5 min), dehydrated through a graded series of ethanol concentrations, chloroform (5 min) followed by 100 % ethanol, and then air-dried. Probe activities for this study were 1 × 10⁶ / slide for CRH and AVP. Hybridization buffer (50 % formamide, 3 × SSC, 1 × Denhardt's solution, 100 µg/ml yeast tRNA, 25 mM sodium phosphate buffer (pH 7.4), 10 % dextran sulphate, 55 mM DTT, 30 % deionized water) was applied; sections were covered with hybrislips (Sigma-Aldrich Canada Ltd., St Louis, ON, Canada), and incubated overnight at 40 °C in 50 % formamide humidified containers. Hybrislips were removed and slides were washed in 2 × SSC (20 min) twice, 2 × SSC/0.01 M DTT (45 °C, 20 min), 1 × SSC (45 °C, 15 min), 1 × SSC/50 % formamide (45 °C, 30 min), 1 × SSC (10 min), 0.5 × SSC (10 min). Sections were dipped briefly in water five times then plunged into 70 % ethanol (5 min), then air dried overnight. Sections for CRH and AVP mRNA were exposed to Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY, USA). All slides were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H₂O) and exposed for 20 days for CRH in the PVN, 40 days for CRH in the CeA; 24 hours for AVP in the PVN, in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak fixer at 14 °C, then counterstained with Cresyl Violet. Coverslips were mounted with Permount (Fisher Scientific Ltd., Nepean, ON, Canada).

Densitometric Analysis

In situ signals were visualized with a Q-imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope. Images were captured using Northern Elite 6.0v (Empix Imaging Inc., Mississauga, ON, Canada) and semiquantitative densitometric analyses were performed using Image J 1.33v software (National Institutes of Health,

Bethesda, MD). The mean optical density (OD) of hybridization signal, corrected by background subtraction, was taken at 120 μm intervals under dark-field illumination. Semiquantitative densitometric analysis for CRH and AVP in the mpd PVN was described previously (Lan et al., 2006). Semiquantitative densitometric analysis of CRH mRNA in the CeA was assisted by redirected sampling of dark-field autoradiographic images aligned to corresponding Nissl stained sections. The CeA was measured using a fixed oval (Width = 0.45; Height = 0.71; scale 300 pixels/inch) (Lan et al., 2009a). Background signal was measured over a region immediately lateral to each side of the area of interest. The corrected grey levels from both sides of two sections of each region were averaged to obtain a mean corrected grey level of the 4 measurements for each animal.

Statistical Analyses

Data were analyzed using Statistical Package for the Social Sciences (SPSS) v.15.0 software (Chicago, IL, USA). In each of the data sets, between-subjects factors were prenatal treatment (C, PF, PAE), CMS Condition (CMS, Non-CMS) and Sex (Male, Female). To test the *a priori* hypothesis that PAE would induce central HPA dysregulation by differentially altering CRH and AVP mRNA expression in PAE and control offspring, and may do so in a stress (CMS)- and sex-dependent manner, planned comparisons using a Šidák correction were carried out to examine: 1) PAE effects (ie., PAE compared to PF or C, our two procedural controls) on neuropeptide expression overall, or under non-CMS or CMS conditions in males or females; 2) CMS effects (ie., non-CMS compared to CMS condition) on neuropeptide expression within PAE, compared to that within controls (PF or C), in males or females. Because the Šidák correction accurately controls for family-wise α , an overall F test is not required to be significant in order to explore *a priori* differences between factors using planned comparisons, and the planned comparisons need not be orthogonal (Cardinal and Aitken, 2006; Myers and Well, 2003; Day and Quinn, 1989). Statistical significance was set at $P < 0.05$.

RESULTS

Maternal and offspring body weight data have been reported previously (Hellemans et al., 2008). Briefly, PAE and PF dams weighed less on gestation days 7–21 and throughout lactation compared to C dams ($P_s < 0.05$). Male PAE and PF weighed less than C offspring on PN 1 and 7, while female PAE and PF offspring weighed less than C on PN 1 only ($P_s < 0.05$). Both PAE and PF offspring showed catch up growth by weaning.

In the mpd PVN, prenatal treatment and CMS differentially altered CRH mRNA in PAE compared to PF and C males

Planned comparisons revealed that CMS increased CRH mRNA in PAE compared to PF and C males ($P < 0.05$), and selectively increased CRH mRNA in PAE males compared to their Non-CMS counterparts ($P < 0.05$) (Figure 1A; Prenatal treatment X Condition [$F(2,43) = 3.47$, $P < 0.05$; Prenatal treatment $\times$ Sex [$F(2,43) = 6.28$, $P < 0.01$]). There were no effects of CMS on CRH mRNA levels in PF or C males compared to their Non-CMS counterparts, and no differential effects of prenatal treatment or CMS on CRH mRNA expression in female offspring.

In the mpd PVN, prenatal treatment and CMS differentially altered AVP mRNA in PAE and PF compared to C males

While effects of prenatal treatment and CMS on AVP mRNA levels failed to reach significance in the ANOVA, planned comparisons revealed that PAE males had significantly higher AVP levels than C males following CMS exposure (PAE CMS > C CMS, $P < 0.05$). As well, CMS selectively increased AVP levels in PF males ($P < 0.05$) compared to that in their Non-CMS counterparts (Figure 1B).

There were no differential effects of prenatal treatment or CMS exposure on AVP mRNA expression in female offspring.

CMS selectively decreased the CRH/AVP ratio in the mpd PVN in PF males

ANOVA failed to reveal significant main effects or interactions. Planned comparisons showed that CMS selectively decreased the CRH/AVP ratio in PF males ($P < 0.05$), such that under CMS conditions, PF males had a *lower* CRH/AVP ratio than PAE males ($P < 0.05$) (Figure 1C).

There were no differential effects of prenatal treatment or CMS exposure on CRH/AVP ratio in female offspring.

Overall, PAE animals had higher CRH mRNA expression than C animals in the CeA

Planned comparisons indicated that there was an increase in CRH mRNA expression overall, regardless of CMS condition or sex, in PAE compared to C animals ($P < 0.05$). By contrast, PF males but not females showed significantly lower CRH mRNA following CMS compared to their Non-CMS counterparts ($P < 0.05$; non-significant trend for a main effect of Sex X Condition interaction ($F(1,74) = 3.74$, $P = 0.057$).), although levels were not significantly different from those of controls (Figure 2).

There were no differential effects of CMS exposure on CeA CRH mRNA expression in female offspring.

DISCUSSION

In the present study, we tested the hypothesis that PAE would induce central HPA dysregulation by differentially altering CRH and AVP mRNA expression in PAE and control offspring, and may do so in a stress- and sex-dependent manner. We found significant CMS-induced increases in CRH and AVP mRNA levels in the hypothalamus of PAE compared to PF and/or C males, but not females, and an increase in CRH mRNA expression overall in the CeA, regardless of CMS condition or sex, in PAE compared to C animals. In addition, we found effects related to pair-feeding: CMS selectively decreased the PVN CRH/AVP ratio in PF males, such that under CMS conditions, PF males had a lower CRH/AVP ratio than PAE males. PF males also had decreased CRH mRNA levels in the CeA in the CMS compared to the Non-CMS condition. Thus, CMS differentially altered central HPA peptide expression in PAE and PF animals compared to their control counterparts, with a possible shift toward greater CRH mediation of HPA regulation in PAE males, and greater AVP mediation of HPA regulation in PF males. However, it appears that

changes in central CRH and AVP expression do not align fully with the anxiogenic profile observed in our previous behavior study, suggesting that other neuronal substrates and limbic forebrain regions also contribute to the increased anxiety-like behavior following CMS.

Differential PVN CRH and AVP mRNA expression in PAE and PF males was revealed by CMS

Our findings are consistent with previous studies from our laboratory and others, which have suggested sexually dimorphic effects of PAE on dysregulation of HPA activity, and have indicated a key role for CRH in the increased HPA drive in PAE males and females (Lee et al., 1990; Redei et al., 1993; Gabriel et al., 2005; Glavas et al., 2007). However, we are the first to examine the possible role of AVP in HPA regulation in PAE compared to PF animals. Importantly, the differences in mRNA expression levels observed likely reflect prior effects of prenatal treatment and adult stress on baseline HPA activity rather than effects of the acute stressor imposed on the termination day, as a relatively long time frame (hours rather than minutes) is needed for the increase in mRNA expression following acute stress (Herman et al., 1992; Ma et al., 1999; Rivier and Lee 1996). Our novel data suggest differentially mediation of HPA activity in PAE and PF males, with a greater role for CRH in PAE and a greater role for AVP in PF males. Furthermore, CMS selectively increased AVP mRNA levels in PF but not PAE males compared to those of their non-CMS counterparts, resulting in a lower CRH/AVP ratio. Of note, we interpret the CRH/AVP ratio data with caution, as expression of CRH is significantly higher than that of AVP by densitometric analyses. Nevertheless, the ratio does provide an additional index of the balance between the two secretagogues and provides additional insight into effects of prenatal treatment and stress in adulthood on HPA activity and regulation. These data support and extend our previous findings demonstrating that: 1) PAE animals had higher CRH mRNA and lower AVP mRNA levels (a *higher* CRH/AVP ratio) than controls following adrenalectomy (removal of the CORT feedback signal, Glavas et al., 2007), and that under intact condition, PAE males exhibited a blunted AVP mRNA response to restraint stress (Lan et al., 2006); and 2) PF males showed a greater AVP mRNA increase than PAE males following adrenalectomy, and CORT replacement failed to normalize the elevated AVP mRNA levels in PF, but not PAE, males and females (Glavas et al., 2007). Together, these novel findings provide evidence that the balance between CRH- and AVP-mediated effects differs in PAE and PF animals, and thus the underlying mechanism(s) of HPA hyperresponsiveness differ rather than occurring along a continuum of effects on the same pathway (Glavas et al., 2007).

Effect of prenatal alcohol exposure on CeA CRH mRNA expression

While the PVN is the final pathway that integrates multiple excitatory and inhibitory inputs from other brain areas to regulate HPA function, the role of the amygdala in providing input to the PVN to modulate the stress response is critical. Indeed, dysregulation of amygdala CRH has been linked to a number of mood disorders (Cullen et al., 2013; Davis, 1992; Flandreau et al., 2012; McEwen et al., 2012; Rainnie et al., 2004). CRH neurons in the CeA are involved in control of behavioral and autonomic responses to stress, and hyperactivity of these neurons has been shown to induce increased levels of anxiety-like behaviors (Rainnie

et al., 2004). While our finding that PAE animals overall had significantly higher CeA CRH mRNA levels than controls is consistent with the increased anxiety-like behavior observed in our previous study (Hellemans et al., 2008), it is likely that other neural substrates and limbic forebrain areas contributed to those behavioral alterations.

Effect of pair-feeding on central CRH and AVP mRNA expression was revealed by CMS

CMS exposure significantly decreased CeA CRH mRNA levels in PF males compared to their Non-CMS counterparts, and resulted in a lower CRH/AVP ratio in the PVN, supporting our suggestion that AVP-mediated alterations play a larger role in HPA regulation in PF than in PAE males. That pair-feeding resulted in specific effects on peptide expression is not entirely surprising. While pair-feeding is typically considered a nutritional control group, it only controls for the reduced food intake of the PAE dam, and can never control for alcohol's effects on the gut, on absorption and utilization of nutrients, etc (Weinberg, 1984). Furthermore, pair-feeding is a treatment in itself. Because PF dams receive a restricted ration of food, they are hungry and typically consume their entire ration within a few hours of presentation; effectively, they are on a "meal-feeding" schedule. This combination of factors – hunger due to the reduced ration plus a disordered pattern of eating – results in mild stress in the PF dams and thus, some level of mild prenatal stress for the PF offspring (Weinberg, 1985; Weinberg, 1984). It is possible that the mechanisms underlying HPA dysregulation in PF animals are also due to fetal programming, as both mild undernutrition and mild prenatal stress may serve as early life adversities for PF offspring.

Possible mechanisms linking central HPA peptide expression and anxiety-like behavior in females

The finding of no significant main effects of PAE or CMS exposure on PVN CRH and AVP expression in female offspring is somewhat unexpected, as following elevated plus maze testing, we found increased CORT and increased anxiety-like behavior in these same PAE CMS females compared to their non-CMS counterparts (Hellemans et al., 2008). It is possible that while CRH levels were not altered in PAE females, they may be more sensitive to the central or behavioral effects of CRH, and/or to circulating CORT levels, compared to controls. Other mechanisms also likely play a role in the observed behavioral and hormonal responses of PAE females. For example, we have previously shown a shift in the balance of the two glucocorticoid receptors (lower mineralocorticoid receptor and higher glucocorticoid receptor mRNA levels in the hippocampus, Sliwowska et al., 2008), as well as greater adrenal sensitivity to estradiol (Lan et al., 2009b) in PAE females, suggesting greater potentiation of HPA activity in PAE than control females. Moreover, analysis of c-fos expression in the brains of these same animals demonstrated increased neural activity within the central and lateral amygdala in response to elevated plus maze testing in PAE compared to control females, regardless of CMS conditions (Raineke et al., 2014). The neural networks that modulate HPA activity and anxiety-like behavior in PAE females remain to be fully elucidated.

Summary and Conclusions

In summary, our noval data demonstrated that CRH mRNA expression was upregulated in PAE animals in the hypothalamus and amygdala in a stress- and sex- dependent manner.

Furthermore, while stress also increased hypothalamic AVP mRNA levels in PAE compared to C males, CMS selectively increased AVP mRNA levels in the hypothalamus, and decreased CRH mRNA levels in the amygdala in PF males. Thus, the balance between CRH- and AVP-mediated effects following stress was differentially altered in PAE and PF animals. These data support and extend previous findings suggesting a possible shift toward greater CRH mediation of HPA regulation in PAE males, and greater AVP mediation of HPA regulation in PF males following stress. However, these changes in CRH and AVP expression do not align fully with the anxiogenic profile observed in our previous behavior study, suggesting that other neuronal substrates and limbic forebrain regions also contribute to increased anxiety-like behavior following CMS in PAE males and females.

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References

- Cardinal, RN.; Aitken, MRF. ANOVA for the Behavioural Sciences Researcher. London, UK.: Lawrence Erlbaum Associates; 2006.
- Cullen CL, Burne TH, Lavidis NA, Moritz KM. Low dose prenatal ethanol exposure induces anxiety-like behaviour and alters dendritic morphology in the basolateral amygdala of rat offspring. *PLoS One*. 2013; 8:e54924. [PubMed: 23383000]
- Davis M. The role of the amygdala in fear and anxiety. *Annu Rev Neurosci*. 1992; 15:353–375. [PubMed: 1575447]
- Day RW, Quinn GP. Comparisons of treatments after an analysis of variance in ecology. *Ecological Monographs*. 1989; 59:433–463.
- Dong HW, Petrovich GD, Swanson LW. Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Brain Res Rev*. 2001a; 38:192–246. [PubMed: 11750933]
- Dong HW, Petrovich GD, Watts AG, Swanson LW. Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *J Comp Neurol*. 2001b; 436:430–455. [PubMed: 11447588]
- File SE, Lippa AS, Beer B, Lippa MT. Animal tests of anxiety. *Curr Protoc Neurosci*. 2004; Chapter 8(Unit 8.3)
- Flandreau EI, Ressler KJ, Owens MJ, Nemeroff CB. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. *Psychoneuroendocrinology*. 2012; 37:27–38. [PubMed: 21616602]
- Gabriel KI, Glavas MM, Ellis L, Weinberg J. Postnatal handling does not normalize hypothalamic corticotropin-releasing factor mRNA levels in animals prenatally exposed to ethanol. *Brain Res Dev Brain Res*. 2005; 157:74–82. [PubMed: 15939087]
- Gallo PV, Weinberg J. Corticosterone rhythmicity in the rat: interactive effects of dietary restriction and schedule of feeding. *J Nutr*. 1981; 111:208–218. [PubMed: 7463165]
- Glavas MM, Ellis L, Yu WK, Weinberg J. Effects of prenatal ethanol exposure on basal limbic-hypothalamic-pituitary-adrenal regulation: role of corticosterone. *Alcohol Clin Exp Res*. 2007; 31:1598–1610. [PubMed: 17760789]
- Haley DW, Handmaker NS, Lowe J. Infant stress reactivity and prenatal alcohol exposure. *Alcohol Clin Exp Res*. 2006; 30:2055–2064. [PubMed: 17117971]

- Hellemans KG, Sliwowska JH, Verma P, Weinberg J. Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neurosci Biobehav Rev*. 2010; 34:791–807. [PubMed: 19545588]
- Hellemans KG, Verma P, Yoon E, Yu W, Weinberg J. Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. *Ann N Y Acad Sci*. 2008; 1144:154–175. [PubMed: 19076375]
- Herman JP, Ostrander MM, Mueller NK, Figueiredo H. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry*. 2005; 29:1201–1213. [PubMed: 16271821]
- Herman JP, Schafer MK, Thompson RC, Watson SJ. Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. *Mol Endocrinol*. 1992; 6:1061–1069. [PubMed: 1324419]
- Ivell R, Richter D. Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc Natl Acad Sci U S A*. 1984; 81:2006–2010. [PubMed: 6326097]
- Jacobson SW, Bihun JT, Chiodo LM. Effects of prenatal alcohol and cocaine exposure on infant cortisol levels. *Dev Psychopathol*. 1999; 11:195–208. [PubMed: 16506530]
- Jingami H, Mizuno N, Takahashi H, Shibahara S, Furutani Y, Imura H, Numa S. Cloning and sequence analysis of cDNA for rat corticotropin-releasing factor precursor. *FEBS Lett*. 1985; 191:63–66. [PubMed: 3876950]
- Krieger DT. Food and water restriction shifts corticosterone, temperature, activity and brain amine periodicity. *Endocrinology*. 1974; 95:1195–1201. [PubMed: 4426285]
- Lan N, Hellemans KG, Ellis L, Viau V, Weinberg J. Role of testosterone in mediating prenatal ethanol effects on hypothalamic-pituitary-adrenal activity in male rats. *Psychoneuroendocrinology*. 2009a; 34:1314–1328. [PubMed: 19410376]
- Lan N, Yamashita F, Halpert AG, Ellis L, Yu WK, Viau V, Weinberg J. Prenatal ethanol exposure alters the effects of gonadectomy on hypothalamic-pituitary-adrenal activity in male rats. *J Neuroendocrinol*. 2006; 18:672–684. [PubMed: 16879166]
- Lan N, Yamashita F, Halpert AG, Sliwowska JH, Viau V, Weinberg J. Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal function across the estrous cycle. *Alcohol Clin Exp Res*. 2009b; 33:1075–1088. [PubMed: 19382903]
- Lee S, Imaki T, Vale W, Rivier C. Effect of prenatal exposure to ethanol on the activity of the hypothalamic-pituitary-adrenal axis of the offspring: Importance of the time of exposure to ethanol and possible modulating mechanisms. *Mol Cell Neurosci*. 1990; 1:168–177. [PubMed: 19912768]
- Lee S, Schmidt D, Tilders F, Rivier C. Increased activity of the hypothalamic-pituitary-adrenal axis of rats exposed to alcohol in utero: role of altered pituitary and hypothalamic function. *Mol Cell Neurosci*. 2000; 16:515–528. [PubMed: 11085885]
- Ma XM, Lightman SL, Aguilera G. Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology*. 1999; 140:3623–3632. [PubMed: 10433220]
- Manning MA, Hoyme H. Fetal alcohol spectrum disorders: a practical clinical approach to diagnosis. *Neurosci Biobehav Rev*. 2007; 31:230–238. [PubMed: 16962173]
- McEwen BS, Eiland L, Hunter RG, Miller MM. Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology*. 2012; 62:3–12. [PubMed: 21807003]
- Myers, JL.; Well, AD. *Research Design and Statistical Analysis*. Mahwah, NJ.: Lawrence Erlbaum Associates; 2003.
- Nelson LR, Taylor AN, Lewis JW, Poland RE, Redei E, Branch BJ. Pituitary-adrenal responses to morphine and footshock stress are enhanced following prenatal alcohol exposure. *Alcohol Clin Exp Res*. 1986; 10:397–402. [PubMed: 3019174]
- Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. *Neuron*. 2002; 34:13–25. [PubMed: 11931738]
- Owens MJ, Nemeroff CB. Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev*. 1991; 43:425–473. [PubMed: 1775506]
- Prewitt CM, Herman JP. Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. *J Chem Neuroanat*. 1998; 15:173–185. [PubMed: 9797074]

- Raineki C, Hellemans KG, Bodnar T, Lavigne KM, Ellis L, Woodward TS, Weinberg J. Neurocircuitry underlying stress and emotional regulation in animals prenatally exposed to alcohol and subjected to chronic mild stress in adulthood. *Front Endocrinol (Lausanne)*. 2014; 5:5. [PubMed: 24592255]
- Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A. Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. *J Neurosci*. 2004; 24:3471–3479. [PubMed: 15071094]
- Ramsay DS, Bendersky MI, Lewis M. Effect of prenatal alcohol and cigarette exposure on two- and six-month-old infants' adrenocortical reactivity to stress. *J Pediatr Psychol*. 1996; 21:833–840. [PubMed: 8990727]
- Redei E, Halasz I, Li LF, Prystowsky MB, Aird F. Maternal adrenalectomy alters the immune and endocrine functions of fetal alcohol-exposed male offspring. *Endocrinology*. 1993; 133:452–460. [PubMed: 8344191]
- Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med (Maywood)*. 2005; 230:357–365. [PubMed: 15956765]
- Rivier C, Lee S. Acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotropin-releasing factor and vasopressin. *Brain Res*. 1996; 726:1–10. [PubMed: 8836539]
- Sah P, Faber ES, Lopez De Armentia M, Power J. The amygdaloid complex: anatomy and physiology. *Physiol Rev*. 2003; 83:803–834. [PubMed: 12843409]
- Sliwowska JH, Lan N, Yamashita F, Halpert AG, Viau V, Weinberg J. Effects of prenatal ethanol exposure on regulation of basal hypothalamic-pituitary-adrenal activity and hippocampal 5-HT1A receptor mRNA levels in female rats across the estrous cycle. *Psychoneuroendocrinology*. 2008; 33:1111–1123. [PubMed: 18672336]
- Weinberg J, Sliwowska JH, Lan N, Hellemans KG. Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *J Neuroendocrinol*. 2008; 20:470–488. [PubMed: 18266938]
- Weinberg J. Effects of ethanol and maternal nutritional status on fetal development. *Alcohol Clin Exp Res*. 1985; 9:49–55. [PubMed: 3887968]
- Weinberg J. Nutritional issues in perinatal alcohol exposure. *Neurobehav Toxicol Teratol*. 1984; 6:261–269. [PubMed: 6514087]
- Young WS 3rd, Mezey E, Siegel RE. Quantitative in situ hybridization histochemistry reveals increased levels of corticotropin-releasing factor mRNA after adrenalectomy in rats. *Neurosci Lett*. 1986a; 70:198–203. [PubMed: 3490635]
- Young WS 3rd, Mezey E, Siegel RE. Vasopressin and oxytocin mRNAs in adrenalectomized and Brattleboro rats: analysis by quantitative in situ hybridization histochemistry. *Brain Res*. 1986b; 387:231–241. [PubMed: 3828759]

Figure 1.

Effect of prenatal treatment and chronic mild stress (CMS) on CRH (Panel A), AVP (Panel B) mRNA levels, and CRH/AVP ratio (Panel C) in the mpd PVN in the male (left) and female (right) offspring. Solid bars represent Non-CMS condition, and hatched bars represent CMS condition. (*) Denotes significant differences from non-CMS group. (#) Denotes significant differences from PF and C males. (&) Denotes significant differences from C males. (^) Denotes significant differences from PF males, $P_s < 0.05$

A **Figure 2.**

Effect of prenatal treatment and chronic mild stress (CMS) on CRH mRNA levels in the central nucleus of amygdala (CeA) in the male (left) and female (right) offspring. Overall PAE > C, $P < 0.05$; (*) Denotes significant differences from non-CMS group, $P < 0.05$