

Preconception Alcohol Increases Offspring Vulnerability to Stress

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The effect of preconception drinking by the mother on the life-long health outcomes of her children is not known, and therefore, in this study using an animal model, we determined the impact of preconception alcohol drinking of the mother on offspring stress response during adulthood. In our preconception alcohol exposure model, adult female rats were fed with 6.7% alcohol in their diet for 4 weeks, went without alcohol for 3 weeks and were bred to generate male and female offspring. Preconception alcohol-exposed offsprings' birth weight, body growth, stress response, anxiety-like behaviors, and changes in stress regulatory gene and protein hormone levels were evaluated. In addition, roles of epigenetic mechanisms in preconception alcohol effects were determined. Alcohol feeding three weeks prior to conception significantly affected pregnancy outcomes of female rats, with respect to delivery period and birth weight of offspring, without affecting maternal care behaviors. Preconception alcohol negatively affected offspring adult health, producing an increased stress hormone response to an immune challenge. In addition, preconception alcohol was associated with changes in expression and methylation profiles of stress regulatory genes in various brain areas. These changes in stress regulatory genes were normalized following treatment with a DNA methylation blocker during the postnatal period. These data highlight the novel possibility that preconception alcohol affects the inheritance of stress-related diseases possibly by epigenetic mechanisms.

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INTRODUCTION

The National Institute on Alcohol Abuse and Alcoholism estimated that 60% of U.S. women have at least one drink a year. Among those who drank, the prevalence of binge drinking was 15.0% among nonpregnant women and 1.4% among pregnant women. In the US, the prevalence of alcohol use disorders in women aged 18 years and older is about 4.6% (SAMHSA, 2014). A recent perspective cohort study in Britain showed nearly two-thirds of women before pregnancy reported alcohol intakes of about 2 units (16 g alcohol) per week (Nykjaer *et al*, 2014). In a study determining the prevalence of lifetime and 7-day daily alcohol consumption in a community sample of Nigerians daily alcohol use has been reported to occur in 3.6% women (Lasebikan and Gureje, 2015). Although the detrimental effects of prenatal alcohol on offspring health is known, the effects of preconception parental alcohol use on offspring is relatively unexplored. Heritability of alcohol-use disorders from paternal origin has been demonstrated (Finegersh *et al*, 2015). However, the effect of preconception drinking by the

mother on the life-long health outcome of her babies is not known, despite evidence for higher risk of having babies with lower birth weight and birth centile (Nykjaer *et al*, 2014) and increased risk for mental retardation (Roeleveld *et al*, 1992).

A rapidly accumulating body of evidence indicates that many diseases must be understood in a life-long perspective, as trajectories start at pre- or during conception and surface upon clinical detection decades later (Dover, 2009). Also, research into heritability of complex diseases has implicated transmission of epigenetic variants in the development of neuroendocrine phenotypes, including anxiety behaviors and stress responsivity (Malan-Müller *et al*, 2014). Furthermore, heritable deficiencies in specific gene function in gametes have been demonstrated (Dann *et al*, 2006).

In this study, we determined the impact of preconception alcohol drinking by the mother on pregnancy outcomes and on stress-related changes in offspring at multiple levels of analysis including endocrine, molecular, and behavioral levels using the rat as an animal model. We also investigated the epigenetic mechanisms underlying heritable stress-related phenotypes.

MATERIALS AND METHODS

Animal Feeding and Treatments

All rat studies were performed with approved protocols in compliance with Association for the Assessment and

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Accreditation of Laboratory Animal Care and the Rutgers University. Fisher-344 strain rats were obtained from Charles River (Wilmington, MA), housed in controlled conditions with a 12-h light/dark cycle at a constant temperature 22 °C throughout the study. Sixty adult female rats were assigned to one of three treatments for a period of 30 days: (1) the control group receiving rat chow and water *ad libitum* (AD; $n=18$; body weight, 128.6 ± 3.1), (2) the alcohol fed group (AF; $n=24$; body weight, 130.6 ± 2.7) receiving a liquid diet containing ethanol (6.7%), or (3) the pair fed (PF; $n=18$; body weight, 133.3 ± 3.3) group receiving an isocaloric liquid control diet (BioServ, Flemington, NJ) to match the daily intake of the rats receiving the alcohol-containing diet (Govorko *et al*, 2012). There was no significant body weight difference between these three groups in the beginning of the experiment ($F(2,57) = 0.5769$, $p < 0.05$). Blood ethanol concentrations were measured weekly using tail blood samples collected 2 h after initiation of feeding and employing an Analox AM1 Alcohol Analyzer (Analox, Lunenburg, MA). After 30 days of treatment, all animals were returned to the *ad libitum* rat chow diet. AF animals underwent behavior studies during the 42 h after completion of alcohol feeding to assess whether the animals underwent alcohol withdrawal. Two weeks after returning to AD diet, estrous cycle changes in all animals were monitored by daily vaginal smearing. During their second estrous cycle on the day of proestrus (about three weeks after alcohol withdrawal), animals were housed with an untreated male rat for breeding. The presence of vaginal plug or sperm in vaginal smears denoted the first day of pregnancy. AF, PF, and AD rats were bred to produce male offspring (AFM, PFM, and ADM) and female offspring (AFF, PFF, and ADF). Litters of each dam were normalized to eight pups (kept both sexes), to reduce confounding variables from litter size. Maternal behaviors in dams were observed on postnatal day (PND) 1–6. Most of the pups were left with their birthmother until weaning on day 23 and then housed in-group with same sex siblings until used for endocrine, molecular and behavioral studies. Cross-fostering was done with only a group of animals to check whether maternal care affects preconception alcohol-induced changes in corticosterone response to lipopolysaccharides (LPS). For this, we crossed two pups (one male and one female) from each of six AF and of six PF dams to six AD dams. The litter size of these AD dams were also maintained, like the rest of animals used in the study, to a number of eight using four of their own and four AF and PF dams. These pups were weaned at day 23 and used for LPS challenge response study on PND 60. In some non cross-fostered AD, AF and PF pups, 5-azadeoxycytidine (AZA; 5 mg/kg; Sigma-Aldrich, Ronkonkoma, NY) or saline was administered subcutaneously (5 mg/kg) in every other day between PND 1–5. These pups were weaned at day 23 and used for behavior, gene expression and methylation studies. At the end of the experiments, whole brains were collected and immediately frozen in dry ice. Prior to gene and protein measurements, whole hypothalamus, hippocampus, and amygdala were dissected from frozen brains using a rat brain atlas (Paxinos and Watson, 1982). Hypothalamus was dissected from the ventral part of a coronal section approximately Bregma -0.3 to -3.3 mm, amygdala from approximately Bregma -1.8 to Bregma -3.3 mm, and hippocampus was dissected from approximately Bregma -3.3 to -5.3 mm.

Maternal Behavior in Alcohol-Exposed Dams

Maternal behaviors in dams were observed every 3 min at PND 1–6 between 0930–1030 and 1700–1800 hours and the occurrence of the following behaviors were noted: whether the dam was physically on the pups, the occurrence of licking and grooming, and whether the dam was nursing pups and her nursing posture (arched back nursing, side nursing, or blanket nursing). Decreased time spent on pups, less licking and grooming, and less arched back nursing are indicators of poor maternal care in rats (Cameron *et al*, 2005).

Anxiety-Like Behavior in Offspring

Male and female offspring at PND 90 after birth were tested for baseline anxiety-like behavior in the open field (OF) and elevated plus mazes (EPM) as previously described (Logan *et al*, 2015). The EPM consisted of a standard plus maze with four arms (each 50 cm long). The OF consisted of a square field (100 cm \times 100 cm). Behavioral testing was conducted between 1000 and 1800 hours during the animals' light cycle in dim lighting conditions with the apparatus around 100 lux. Behavior was video-recorded and videos for the EPM test were scored with Stop Watch behavioral monitoring software (CBN; Emory University) by a scorer blind to the animal treatment group. For the OF, videos were tracked by AnyMaze (Stoelting; Wood Dale, IL) tracking software.

Stress Hormone Responses to an Immune Challenge

Adult male and female offspring were tested around days 60 after birth for stress hormone (corticosterone and adrenocorticotropic hormone, ACTH) responses to lipopolysaccharide (LPS). Because sex steroids affect corticosterone response to acute stress in rats, we used female rats at the stage of diestrus (determined by vaginal smearing) when estrogen and progesterone levels are maintained in a low concentration (Kalil *et al*, 2013). Also, to avoid circadian fluctuations of hormone levels, we conducted the experiment only in the late morning. Blood samples were collected from tail vein at 0 h (basal level) before and 2 h after (stimulated level) i.p injection of 100 μ g/kg LPS. We used a 2-h period to determine the stress hormone response, since plasma corticosterone and ACTH show their maximal responses at 2 h following the LPS treatment (Govorko *et al*, 2012). All samples were run in the corticosterone ELISA (IBL, Toronto, ON) and ACTH ELISA (MyBioSource, San Diego, CA) in duplicates.

Measurement of Tissue Protein Levels

Stress regulatory protein levels were measured in brain tissue samples using ELISAs. Frozen tissue samples were quickly removed and samples homogenized in a lysis buffer (150 mM NaCl, 25 mM Tris, 1% NP-40, 5% glycerol, 1 mM PMSF, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin and 0.5 mM sodium vanadate). Homogenates were centrifuged and the supernatants used for measurement by ELISA of tissue β -endorphin (Peninsula Laboratory, San Carlos, CA), CRF (Phoenix, Burlingame, CA) and arginine vasopressin (AVP) levels (MyBioSource, San Diego, CA).

Gene Expression Analysis by qRT-PCR

Stress regulatory genes in various brain tissue samples were measured by qRT-PCR. Total RNA was isolated from tissue sample using RNeasy kit (Qiagen, Valencia, CA). The qRT-PCR conditions were as previously described (Govorko *et al*, 2012). The mRNA levels of corticotropin releasing factor (*Crf*), CRF receptor 1 (*Crfr1*), glucocorticoid receptor (*Gr*), mineralocorticoid receptor (*Mr*), proopiomelanocortin (*Pomc*), neuropeptide Y (*Npy*), *Rpl-19*, and *Gapdh* were measured using the primer sequences described in Supplementary Table S1. All runs were performed in duplicates. RT-PCR was performed using the ABI prism 7500HT sequence detection system (Applied Biosystems, Foster City, CA). Thermocycler conditions were 5 min at 95 °C, followed by 40 cycles of: 15 s at 95 °C, 30 s at 60 °C, 40 s at 72 °C. For CRF-1, 45 cycles were used. Gene expression was compared with a standard curve prepared from control samples. We have previously shown that alcohol feeding similar to the procedures used in this study produced no significant effect on the expression of house keeping genes *Gapdh*, *Rpl-19* and β -*actin* (Govorko *et al*, 2012). Similarly, in this study we found no effect of PCAE on *Gapdh* and *Rpl-19* gene expression (Supplementary Fig. S1). Because of these, we feel *Gapdh* and *Rpl-19* genes were stable during the alcohol-feeding paradigm. Hence, we presented the expression levels of hormones as ratios of both *Gapdh* and *Rpl-19*.

DNA Methylation Analysis by Methylation Specific PCR (MSPCR)

Genomic DNA was isolated by DNeasy Blood and Tissue Kit (Qiagen) from brain tissues. Samples were bisulfite treated using EZ DNA Methylation kit (Zymo, Irvine, CA). MSPCR was used to examine methylation status of the *Crfr1* and *Pomc* genes as previously described (Govorko *et al*, 2012; Wang *et al*, 2013). MSPCR was not successful to detect *Crf* gene methylation. Primers specific for methylation/unmethylation were designed using Methyl Primer Express program (Applied Biosystems; Supplementary Table S1). qPCR was used to compare the amplification using methylation specific vs unmethylation specific primers to obtain a ratio of methylation status. Prior to qPCR, pre-amplification using the same conditions and by running samples for 12 rounds, and a 2 μ l aliquot of each pre-amp sample was used for subsequent qPCR by sybr green in a total volume of 25 μ l. All wells were loaded in duplicate. Thermocycler conditions were 5 min at 95 °C, followed by 40–45 cycles of: 15 s at 95 °C, 30 s at 60 °C, 40 s at 72 °C. Standard curve methods were used for calculations of mean quantity.

Bisulfite Pyrosequencing

In order to characterize the extent of cytosine methylation of CpG dinucleotides in the *Crf*, and *Pomc* proximal promoter, we designed bisulfite sequencing primers in the CpG island. Bisulfite pyrosequencing was not successful to detect *Crfr1* gene methylation. The methods for *Crf* and *Pomc* bisulfite pyrosequencing were as described previously (Govorko *et al*, 2012; Chen *et al*, 2012). A MethPrimer program was used to assess the CpG island and bisulfite sequencing primers for

these three genes (Supplementary Table S1). Following bisulfite treatment, PCR reaction was performed with bisulfite sequencing primers using Pyromark reagents (Qiagen, Valencia, CA) as detailed in manufacturer's protocols. Pyrosequencing was carried out using a sequencing primer on a PSQ-HS-96A model pyrosequencer (Qiagen). In the study we analysed one control C in non CpG background for efficient C \rightarrow T conversion. Each CpG in the study was analysed by comparing C/T peaks. The percentage of methylation was represented using percent C remaining as C in each target CpG.

Statistical Analysis

The data shown in the figures and text are mean \pm SEM. Comparisons between groups were made using one-way analysis of variance (ANOVA) with *post hoc* analysis using the Newman Keuls posttest. Two-way ANOVA with Bonferroni *post hoc* tests were used to correct for multiple comparisons. Significance was set at $\alpha < 0.05$. F-statistics and P values of major data (Figures 1, 2, 3, 4, 5) are shown in Supplementary Table S2.

RESULTS

Preconception Alcohol Drinking Affects Pregnancy Outcomes without Altering Maternal Care Behaviors

Adult cyclic female rats when given free access to 6.7% alcohol containing liquid diet and drinking water, frequently drank alcohol to achieve a blood alcohol level between 0.08 and 0.13 g/dl (Figure 1a). After 4 weeks of alcohol use, when AF rats were restricted from any alcohol they showed mild alcohol withdrawal symptoms (rigidity and agitation but no tremor or convulsion) lasting only 16 to 24 h (Supplementary Table S3). After feeding the animals a regular diet for a period of 2 weeks, the body weight of these animals were measured, and no detrimental effect of prior alcohol use on body weight was noted (Figure 1b; AF animals showed a slight but not significant increase in weight). The AF animals, like the control (PF and AD) animals, showed a normal 4-day estrous cycle (Figure 1c). However, when the AF rats were bred, about three weeks after alcohol discontinuation, they had longer gestation time in days (Figure 1d) and moderately smaller sized litters (Figure 1e). The sex ratio in litters did not differ between the treatment groups (Figure 1f and g), but the mean litter birth weight of AF dams was lower than those of AD and PF dams (Figure 1h and i). Inspection of body growth weekly during the developmental period revealed lower body weight in male and female offspring of AF dams, AFM and AFF rats, respectively (Figure 1j and k). When tested for maternal care behaviors, no differences in time spent on pups, licking and grooming, and arched back nursing were noted in AF, PF and AD dams (Supplementary Figure S2).

Preconception Alcohol Exposures (PCAE) Alter the Hypothalamic-Pituitary-Adrenal (HPA) Axis Functions and Anxiety-Like Behaviors

To investigate whether preconception alcohol feeding affected the stress axis response, we first measured the basal and LPS-induced plasma corticosterone levels. This test

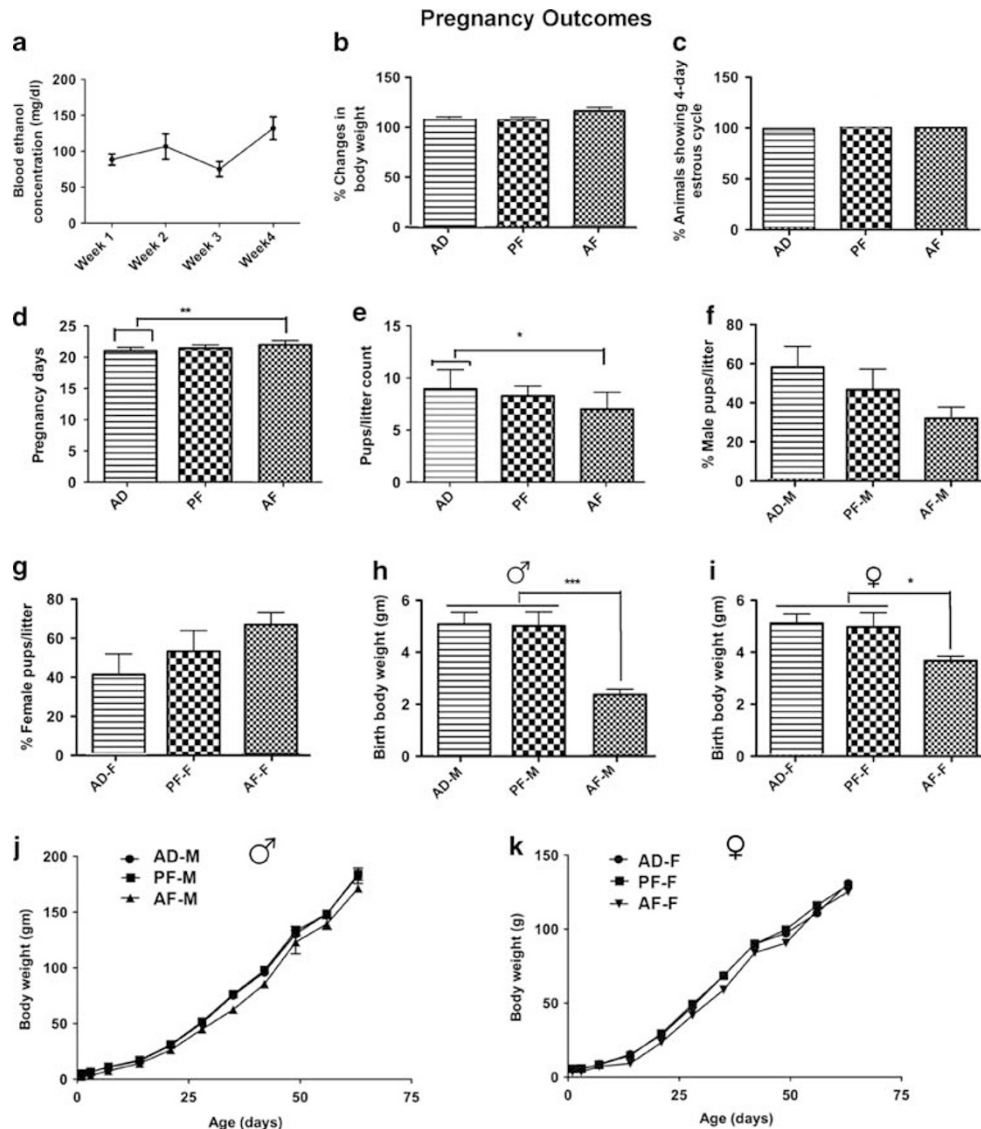


Figure 1 Effects of preconception alcohol feeding on pregnancy outcomes. Physiological parameters and pregnancy outcomes of preconception alcohol fed rats are shown. Time-dependent changes in blood concentrations of ethanol in female rats who had free access to alcohol in liquid diet for a 4-week period and were used in preconception alcohol feeding study (a). Changes in body weight before and two weeks after ethanol withdrawal (b). Estrous cycle patterns two weeks after ethanol withdrawal (c). Pregnancy outcome of the alcohol-fed rats (AF) were compared with those of pair-fed (PF) and ad lib-fed (AD) control rats by pregnancy days (d), total pups/litter count (e), % male or female pups/litter count (f and g), and birth body weight of male or female offspring pups (h and i). Data are mean \pm SEM ($n = 7$) and were compared by one-way analysis of variance (ANOVA) and Newman-Keuls post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, AF vs PF and AD. The body growth curves of male offspring (AF-M, PF-M, and AD-M) or female offspring (AF-F, PF-F, and AD-F) of dams fed with different diets (AF, PF, and AD) were shown in panels j and k, respectively. Data were analyzed by two-way ANOVA (treatment \times time) followed by the Bonferroni post-test. A significant time \times treatment effect on body growth was noted for both male ($p < 0.001$) and female offspring ($p < 0.001$).

revealed a moderate increase (not significant) in basal corticosterone levels, but a marked increase in corticosterone response to the immune challenge in both male (AFM; Figure 2a) and female offspring (AFF; Figure 2b) of AF dams. To check if the higher corticosterone response to the immune challenge in PCAE offspring might be related to altered nursing behaviors of AF dams, we determined the corticosterone response to LPS in AFM and AFF rats who were fostered to control (AD) dams during the nursing period. These data indicate that both AFM and AFF offspring who were fostered also had higher corticosterone response to the LPS challenge, suggesting the effect of PCAE

was not related to altered maternal care by AF dams (Supplementary Figure S3A and B). Hence, we used the male and female offspring of AF, PF and AD dams without any fostering in further characterization of the HPA axis functions.

We measured plasma adrenocorticotropin hormone (ACTH), a corticosterone-regulatory pituitary hormone, and the levels of ACTH regulatory hypothalamic hormones CRF, AVP, and β -endorphin in male and female offspring of AF, PF, and AD dams. These tests revealed that the ACTH response to LPS challenge was higher in AF-M and AF-F rats than those in PF-M and PF-F or AD-M and AD-F rats

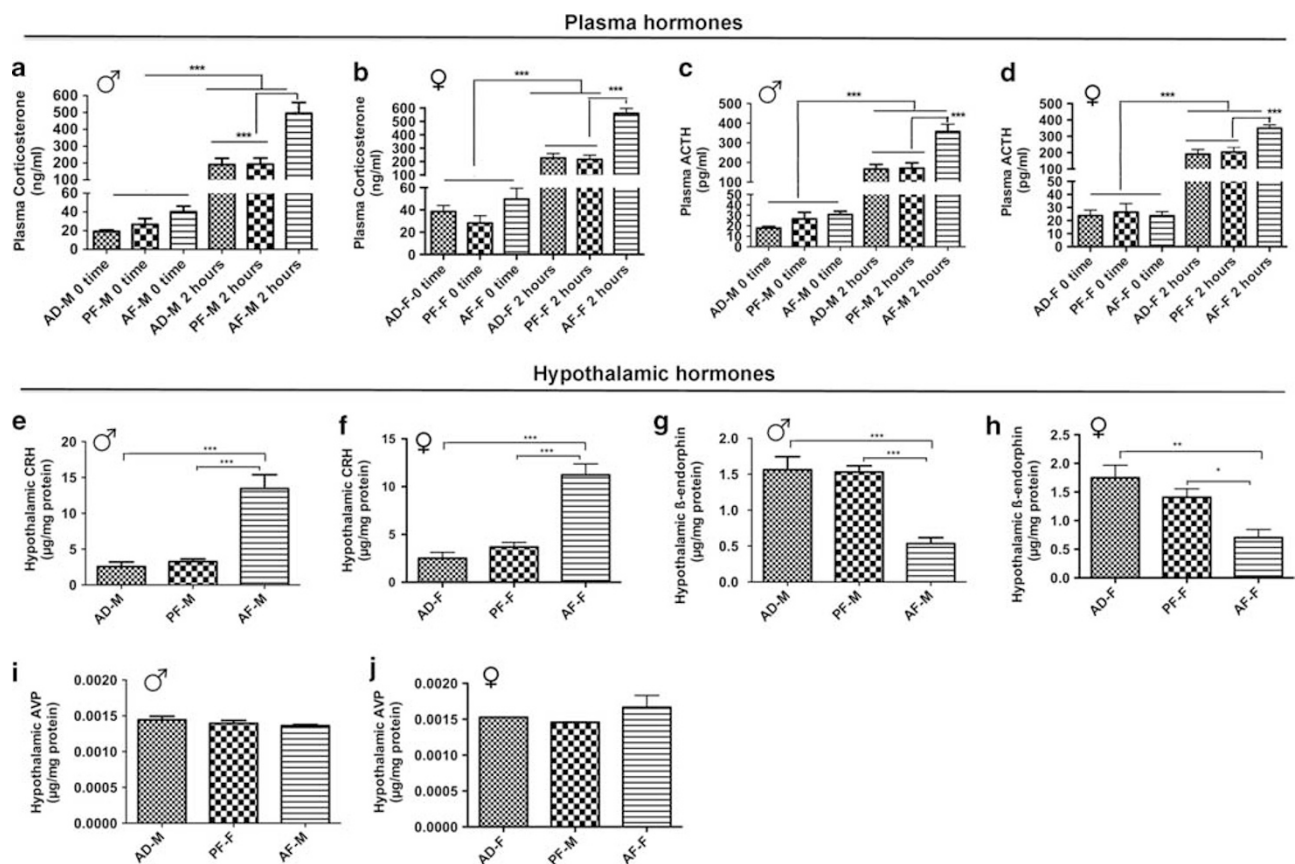


Figure 2 Consequences of preconception alcohol feeding on stress axis responses in adult male and female offspring. The stress axis function tests include measurement of plasma levels of corticosterone (a, b) and ACTH (c, d) measured before (0 time) and 2 h after LPS injection and the basal hypothalamic protein level of CRF (e, f), β -endorphin (g, h), and AVP (i, j) in adult male offspring (AF-M, PF-M, AD-M) or female offspring (AF-F, PF-F, AD-F) of dams fed with different diets (AF, PF and AD). Data are mean \pm SEM ($n = 6$). Data were analyzed using one-way ANOVA followed by the Newman-Keuls post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, AF (both M and F) vs AD and PF (both M and F) groups. (a) $P < 0.001$, within treatment group-corticosterone levels at 0 time vs 2 h post LPS.

(Figure 2c and d). Both AF-M and AF-F groups also showed higher levels of CRF (Figure 2e and f), but showed lower levels of β -endorphin in the hypothalamus compared with control groups (PF-M, PF-F, AD-M, AD-F; Figure 2g and h). Hypothalamic AVP levels between groups did not differ (Figure 2i and j). No sex differences in PCAE effects on hypothalamic peptide contents were noted.

PCAE Effects on Anxiety-Like Behavior

Next, we tested the baseline anxiety-like behaviors of PCAE animals and control offspring in the OF and EPM. We show here that the male AF-M rats showed significantly less time spent in the center zone of the OF as compared with AD-M rats, although comparisons between AD-M and PF-M were not significantly different (Figure 3a). For females (Figure 3b), the time spent in the center zone in the OF by AF-F animals did not differ from those in PF-F and AD-F rats. AF-M rats spent significantly less time in the open arms of the EPM compared with AD-M, but not PF-M, rats (Figure 3c). In addition, male AF and PF offspring made less entries into the open arms of the EPM compared with AD males (Figure 3d). In males, there were no significant differences between groups in total arm entries (Figure 3e), a measure of overall locomotor behavior in this test. For

female AF-F, PF-F, and AD-F rats, there were no significant differences in either time spent in the open arms of the EPM (Figure 3f), entries into the open arms of the EPM (Figure 3g), or total arm entries (Figure 3h).

PCAE Effects on the Stress Regulatory Genes

To understand the cause for the elevated HPA axis functions and increased anxiety-like behaviors in PCAE offspring, we measured various regulatory genes (*Crh*, *Crhr1*, *Gr*, *Mr*, *Pomc*, and *Npy*) in the hypothalamus and/or the limbic system in these and control animals. Because we did not find sex differences in the HPA axis function and we only saw anxiety-related behavioral changes in male offspring, we focused our stress regulatory gene study only in males. We also did not measure *Avp* mRNA levels since its protein level did not change following PCAE. Measuring brain region specific changes in the regulatory genes in PCAE offspring, we found the level of *Crh* mRNA in AF-M animals was elevated in the hypothalamus (Figure 4a and b) but not in hippocampus (Supplementary Figure S4A and B) or amygdala (Supplementary Figure S4C and D), as compared with AD-M and PF-M animals. *Crhr1* mRNA levels in AF-M animals were elevated in hippocampus (Figure 4c and d) and amygdala (Figure 4e and f) but not in the hypothalamus

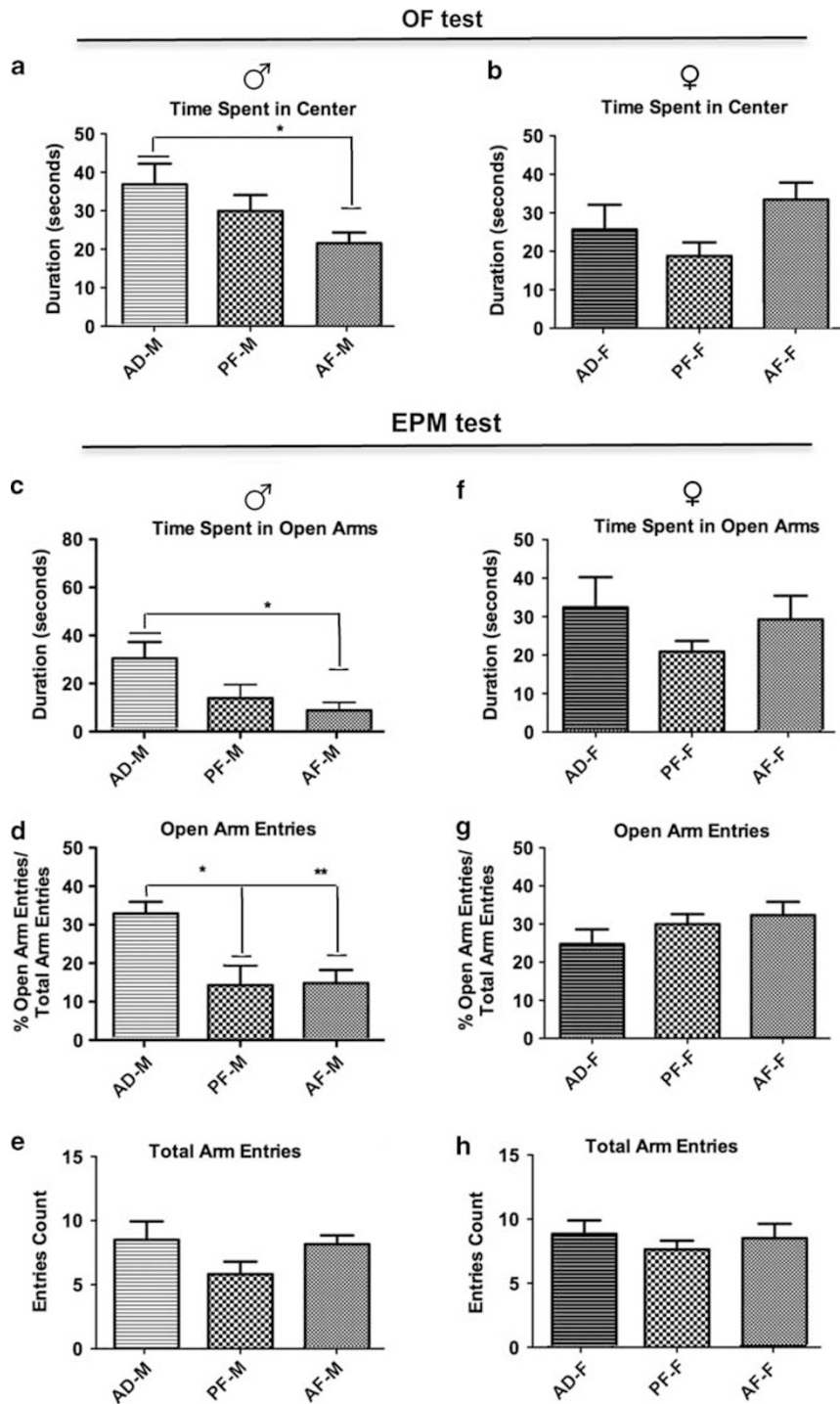


Figure 3 Consequences of preconception alcohol feeding on anxiety-like behaviors in adult male and female offspring. Adult AF-M rats spent less time in the center of the OF compared with AD-M offspring (a; $*p < 0.05$) indicating increased anxiety-like behavior in AF-M offspring. AF-F rats did not differ from PF-F and AD-F in time spent in the center zone (b; $p > 0.05$). AF-M also showed significantly more anxiety-like behavior in the EPM, since they spent significantly less time in the open arms compared with AD-M (c) and made less entries into the open arms compared with AD-M (d). In males, there were no significant differences between groups in total arm entries (e). There were no significant differences between AF-F, PF-F, and AD-F females in anxiety-like behavior in the EPM (f, g, h). Data are mean \pm SEM ($n = 7-14$; AD-M = 9, PF-M = 7; AF-M = 14) and were analyzed using one-way ANOVA and Newman-Keuls post-test. $*p < 0.05$, $**p < 0.01$, compared with the AD group.

(Supplementary Figure S4E and F). The *Pomc* mRNA level in the hypothalamus was found to be reduced in AF-M animals compared with AD-M and PF-M animals (Figure 4g and h). *Pomc* mRNA level in the hippocampus and amygdala were

not determined since their roles in the HPA regulation have not been demonstrated. The mRNA levels of *Gr*, *Mr* and *Npy* in various brain areas were similar in AF-M, AD-M and PF-M animals (Supplementary Figure S4G-T).

PCAE Affects the Methylation Status of CpG Islands in Stress Regulatory Genes

We tested whether promoter methylation status of genes are affected by the PCAE treatment. Both MSPCR assays and pyrosequencing procedures were used to examine the methylation status of HPA regulatory genes. We found methylation levels at different epigenetic sites of the *Crf* gene varied and PCAE moderately suppressed methylation of the CpG dinucleotides at -232 in the proximal promoter of *Crf* in the hypothalamus (Figure 4i). In addition, we found marked reduction in *Crf1* methylation in AF-M animals in the hippocampus (Figure 4j) and in the amygdala tissues (Figure 4k). However, we observed increased methylation of the *Pomc* gene in the hypothalamus of AF-M offspring as compared with AD-M and PF-M offspring (Figure 4l and m).

Administration of a DNA Methylation Blocker Normalizes Stress Response and Anxiety-Like Behaviors

To further determine the role of epigenetic programming, we tested the effects of AZA, a DNA methylation blocker (22) on PCAE-associated changes in the stress response, anxiety-like behavior and gene expression levels. For the stress test, we found increased ACTH and corticosterone responses following LPS challenge in saline-treated AF-F animals as compared with those in saline-treated PF-F and AD-F animals, whereas LPS-induced ACTH and corticosterone levels in AZA-treated AF-F animals were not different from those in AZA-treated AD-F and PF-F animals (Figure 5a–d). In the case of anxiety-like behavior in the OF test, we found significant effects of AZA. AF-M animals, when treated with saline, spent a reduced amount of time in the center zone compared with AD-M but not PF-M animals, but they spent a similar amount of time in the center as AD-M and PF-M controls when treated with AZA (Figure 5e). In the EPM test, saline, but not AZA-treated AF-M rats, spent significantly less time in the open arms of the EPM compared with similarly treated AD offspring (Figure 5f). In addition, saline- but not AZA-treated AF-M and PF-M, made less entries into the open arms of the EPM compared with similarly treated AD-M rats (Figure 5g). Nonetheless, comparison between AZA-treated AF, PF and AD animals showed no differences in the entries into the open arms indicating AZA treatment normalized EPM behavior. For the gene expression measurements, we also found that AZA was effective in preventing PCAE induced changes in *Crf* (Figure 5i and j) and *Pomc* gene expression (Figure 5m and n) in the hypothalamus and normalized

the PCAE-induced changes in *Crf1* levels in the amygdala (Figure 5q and r). For methylation study, we found that AZA treatment reduced methylation in all genes in all groups studied (Figure 5k, l, o, p and s). It is interesting to note that the effect of AZA on expression seems to be only on hypermethylated promoters as it was observed with *Crf* in control samples (Figure 5i and j) and with *Pomc* in AF samples which have reduced expression (Figure 5m and n). AZA treatment did not elicit any increased expression of *Pomc* in control samples and *Crf* in AF samples even they have reduced level of methylation.

DISCUSSION

In the present study, we demonstrate chronic alcohol exposure in female rats that elevates blood alcohol level equivalent to human binge drinking level (NIAAA, 2016) for a period of four weeks produces no effect on the reproductive cycle or body weight after 3 weeks of alcohol withdrawal but significantly affects the pregnancy outcomes including longer gestation time, smaller size litters and lower birth weight of the litter. In addition, despite these PCAE dams showing normal maternal care behaviors their litter show reduced body growth throughout the nursing period.

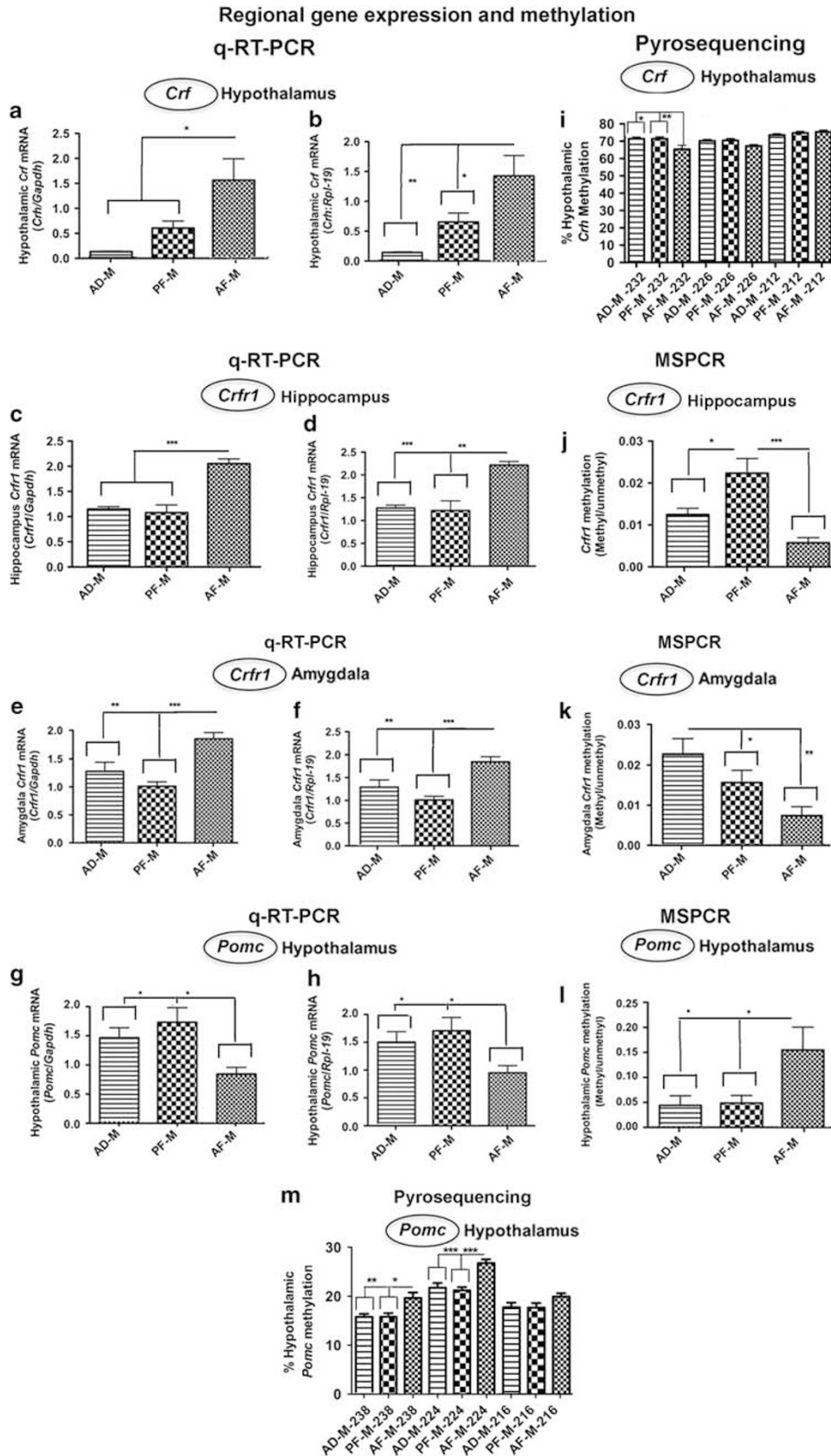
Data presented here show that both male and female PCAE offspring had increased corticosterone and ACTH responses to the LPS challenge during the adult period. Interestingly, similar stress hyperresponse to the LPS challenge has been reported in fetal alcohol exposed rat offspring (Govorko *et al*, 2012). This identifies a similarity between PCAE offspring and fetal alcohol exposed offspring. The higher stress hormone response to the immune challenge in adulthood in PCAE offspring persisted even after maintaining them under the maternal care of a control foster dam, suggesting the possibility that the stress abnormality is not caused by altered maternal care but possibly due to inheritance of alcohol marks influencing stress axis functions.

We also show male PCAE offspring spend significantly less time in the center zone of the OF and in the open arms of the EPM compared with AD controls, indicating increased anxiety-like behavior in PCAE males. Notably, effects of the maternal pair feeding (PF) condition on male offspring were also observed in the behavior studies, as PF males showed increased anxiety in the EPM compared with AD males and a modest trend for increased anxiety in the OF. The PF condition is included in alcohol-exposure studies as a control for diet and handling factors involved in alcohol administration. Thus, we cannot say conclusively that the anxiety-like behavior observed in AF males is due specifically to

Figure 4 Effects of preconception alcohol drinking on expression and methylation of stress regulatory genes in various brain areas. Adult AF-M rats showed tissue specific changes in mRNA expression, as determined by q-RT-PCR, of *Crf* (a, b), *Crf1* (c–f) and *Pomc* (g, h) in the hypothalamus, hippocampus, and/or amygdala. AF-M rats had increased *Crf* mRNA compared with PF-M and AD-M rats in the hypothalamus (a, b). In addition, AF-M rats had increased *Crf1* mRNA compared with PF-M and AD-M rats in the hippocampus (c, d) and amygdala (e, f). *Pomc* mRNA levels in the hypothalamus were lower in AF-M compared with AD-M and PF-M offspring (g, h). AF-M rats also showed differential methylation patterns of *Crf* (i), *Crf1* in hippocampus (j) and amygdala (k) and *Pomc* (l, m) genes in hypothalamus. AF-M rats had suppressed CRF methylation in the hypothalamus (i) of the CpG dinucleotides at -232 in the proximal promoter as determined by pyrosequencing methods. AF-M rats also showed a reduction in *Crf1* methylation in the hippocampus (j) and amygdala (k) as determined by MSPR assay. In contrast, the methylation of the POMC gene was increased in the hypothalamus of AF-M as compared with AD-M and PF-M (l, m) as determined by MSPR assay and or pyrosequencing assay. Data are mean \pm SEM ($n=7$, except $n=12$ in AF group shown in i) and were compared by a one-way ANOVA followed by the Newman-Keuls post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, group with this symbol vs AF-M.

PCAE, as the PF condition also had a moderate anxiogenic effect, and the effects of preconception maternal diet and stress may have contributed to the observed increases in

anxiety-like behavior in male AF offspring. However, it is also a possibility that the overlapping behavioral effects observed in AF and PF male offspring do stem from distinct



causes, but manifest in a similar manner in behavior. Similar to our findings, other studies utilizing pair feeding or sham pair feeding controls in fetal alcohol exposure models have observed effects of the PF conditions on both the HPA axis and the neuroimmune system that overlap with the effects observed in AF conditions (Boschen *et al*, 2016; Glavas *et al*, 2007). In addition, preconception stress and diet alterations have been shown to affect offspring (Leshem and Schulkin, 2012; Li *et al*, 2010; McGowan *et al*, 2008). However, there was no effect of the PF condition on other measures of stress vulnerability, including stress-related gene expression, protein levels, immune stress response, or gene methylation (discussed below), as PF animals did not differ from AD control animals in these studies. Thus, the driving force for the majority of phenotypes observed in our studies can be concluded to be due specifically to preconception maternal alcohol, and not due to other factors such as maternal stress or nutrition.

Interestingly, both male and female AF offspring show increased CORT and ACTH levels in response to LPS, whereas, only male AF offspring, and PF offspring to some extent, show increased anxiety behavior. The reason for the observed sex differences in behavior are likely due to inherent sex differences in rats in these behavioral tests, as female rats, in comparison with males, have been shown to be less susceptible to anxiety behavior in the EPM and OF tests (Blizard *et al*, 1975; Osborn *et al*, 1998; Johnston & File, 1991). Thus, it is possible that these behavioral tests were not sufficiently stressful enough to assay anxiety-like behavior in females, and future studies utilizing different behavioral measures of anxiety are needed to explore the behavioral effects of PCAE in females. Similarly, fetal alcohol exposure studies have found sex differences on the effects of prenatal alcohol exposure on behavior, with males being more susceptible to fetal-alcohol-induced anxiety and depression in certain behavioral tests (Hellemans *et al*, 2010). Moreover, although increased corticosterone signaling is associated with increased anxiety-like behavior in the EPM and OF, behavior in these tests are likely a manifestation of effects on multiple hormonal and neural substrates. The OF and EPM tests are also known to be heavily regulated by the GABAergic and serotonergic systems (Osborn *et al*, 1998; Tu *et al*, 2014), which were not investigated in these studies and might be explored in future studies using our PCAE model. Finally, another factor in the observed sex difference in behavior might be the modulating effects of ovarian hormones on behavioral testing, as, in the LPS study, females were only used in diestrus, whereas in the behavior studies, estrus cycle was not restricted to diestrus, and some females were likely in estrus and proestrus during testing. It is possible, variations in ovarian hormones may have altered the effects of preconception alcohol on behavior in female offspring. For these reasons, the effects of preconception alcohol on hormonal response to stress and anxiety-like behavior might be divergent, as was observed in our studies.

Enhanced ACTH and corticosterone responses to stress in rats are known to result from increased CRF and/or AVP levels in the hypothalamus and possibly by impaired negative-feedback control by GR, MR and CRFR1 receptors in the limbic areas and by the POMC-derived peptide β -endorphin in the hypothalamus that functions to terminate stress responses (McEwen *et al*, 2015; Sarkar and Zhang,

2013). In addition, anxiety-related behavior is regulated by *Crfr1* in the amygdala (Gilpin *et al*, 2015) and NPY in the hypothalamus and limbic areas (Sotnikov *et al*, 2014). The data of the present study show that in PCAE offspring increased levels of hypothalamic CRF and reduced levels of hypothalamic POMC/ β -endorphin; identifying a dysregulation in the functions of two key regulatory neurotransmitters of the HPA axis. PCAE offspring also showed increased levels of *Crfr1* mRNA levels in the amygdala and hippocampus. Thus, PCAE offspring have significant alterations in the neuroendocrine system regulating HPA axis function.

DNA methylation is known to inhibit gene transcription by interfering with the recruitment of transcription factors or by recruiting methylated-DNA binding proteins that decrease transcription efficiency (Vaissière *et al*, 2008). We showed here that PCAE induces long-lasting hypermethylation of the *Pomc* gene, while causing hypomethylation of *Crf* and *Crfr1* genes. This inference is based on the findings that the percentage of the cytosine methylation of CpG-rich sites adjacent to the gene transcription start site for *Pomc* was higher, whereas for *Crf* and *Crfr1* was lower in PCAE animals than in controls. Methylation of the proximal region of the promoter often correlates closely with a negative effect on gene expression (Vaissière *et al*, 2008). Moreover, deletions or mutations in these promoter regions cause a significant decrease in gene expression for *Pomc*, *Crf* and *Crfr1* (Chen *et al*, 2012; Sotniko *et al*, 2014; Ehrlich *et al*, 2010). Hence, increased DNA methylation in the key epigenetic island in the promoter regions of the *Pomc* gene could have impaired the function of the hypothalamus resulting in reduced expression levels of this gene, as has been demonstrated in the PCAE offspring. Conversely, reduced DNA methylation in the proximal region of *Crf* and *Crfr1* promoters in key HPA regulatory brain tissues could have resulted in the increased expression levels of these genes in PCAE offspring.

We further confirm the role of promoter methylation in suppressing the gene expression as well as altered behavior by treating the rat offspring with AZA, a blocker of the regulators of gene promoter methylation, DNA methyltransferases (Vaissière *et al*, 2008). PCAE saline-treated animals, and PF saline-treated animals to some extent, showed increased anxiety-like behavior in the EPM and OF compared with AD offspring. However, comparison between AZA-treated AF, PF and AD animals showed no differences in behavior, indicating AZA treatment normalized anxiety-like behavior. Aza treatment also reduced the promoter methylation of *Pomc* and *Crf* in the hypothalamus. Aza treatment normalized *Pomc* expression in hypothalamus of AF rat offspring, whereas Aza treatment increased the expression of *Crf* in control rats but did not produce any effect in AF rats. These results show although Aza reduces methylation, its effect on expression seems to be specific to only hypermethylated promoters as it was observed with *Crf* in control samples and with *Pomc* in AF samples, which have reduced expression. Aza treatment did not elicit any increased expression of *Pomc* in control samples and *Crf* in AF samples as they have reduced levels of methylation. We and others previously reported that hypermethylated promoters recruit methyl CpG binding proteins to repress the transcription of *Pomc* and tumor suppressor genes

Stress-normalization by a methylation blocker

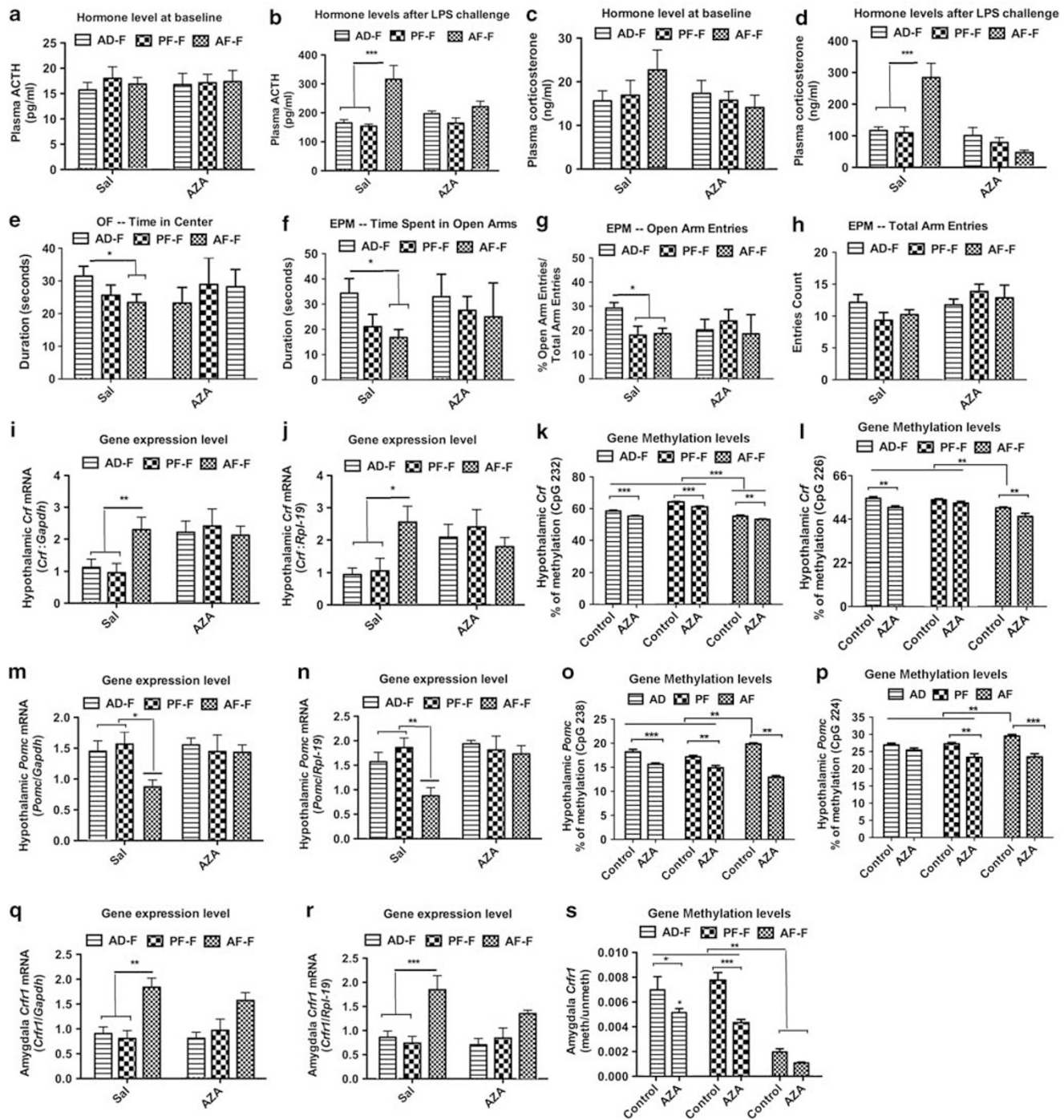


Figure 5 Suppression of DNA methylation prevents preconception alcohol effect on stress response, anxiety behaviors and stress regulatory gene expression. Treatment of AZA prevents the stress response (a, b, c, d), anxiety behaviors measured in OF (e) and in EPM (f, g, h), and the regulatory genes expressions (i, j, m, n, q, r), and methylation (k, l, o, p, s) in AF-M or AF-F rats. Data are mean \pm SEM ($n = 8$, except $n = 18$ in all groups shown in f-h) and were analyzed by two-way ANOVA (alcohol \times drug treatment) followed by Bonferroni post-test comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with saline-treated but differentially fed offspring for the gene measurements. * $p < 0.05$, compared with saline-treated AFM for the gene measurements.

(Gangisetty *et al*, 2014; Lopez-Serra *et al*, 2008). In our experiments, Aza elicited its effect on stress regulatory genes by reducing their methylation and increasing expression. Hence, Aza effects on stress regulatory genes may be mediated by reducing the affinity of MeCP2. MeCP2 is also known to recruit histone modifying enzymes such as HDACs

(Jones *et al*, 1998; Wade *et al*, 1999) and HMTs (Fujita *et al*, 2003; Fuks *et al*, 2003). Hence, these epigenetic modifiers may also have roles in regulation of these HPA axis genes. It has been reported that early stress induced *Crf* expression was associated with increased H3 acetylation and reduced level of cytosine methylation at its promoter in hippocampus

(Wang *et al*, 2014). Rats exposed to chronic mild stress associated with increased expression of *Crf1* showed reduced levels of H3K9 trimethylation at the promoter in hypothalamus (Wang *et al*, 2014). Hence, further investigation is needed to evaluate whether, in addition to DNA methylation, other histone code modifications are involved in mediation of PCAE effects on the HPA axis. Also, in this study AZA treatment was given during postnatal period. Therefore, it is difficult to determine whether the effects of AZA are due to reversal of heritable epigenetic marks or simply inhibition of DNA methylation during postnatal development.

In summary, we have shown that maternal alcohol consumption three weeks prior to conception confers stress axis functional abnormalities in rat offspring. Alterations in stress axis functions were observed on multiple levels of analysis. It should be emphasized that rats were fed with alcohol three weeks, equivalent to five rat reproductive cycles, prior to conception. Thus, the effects we observed in the offspring were not due to a direct effect of alcohol on the offspring rather the effect was possibly inherited from the mother following alcohol abuse and passed on to the offspring. The results presented here suggest the intriguing, and potentially alarming, possibility that exposure to alcohol produces transmissible long-lasting epigenetic changes that result in profound alterations to the physiology of the offspring. In this regard, it is interesting to note that increased anxiety in humans is often correlated with alcohol consumption and that alcohol use/abuse does run in families (Kendler *et al*, 2015; Sjoerds *et al*, 2013; Morean *et al*, 2009). These human data tie the data of our present study in animal model in a translational way.

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