Prenatal alcohol exposure alters methyl metabolism and programs serotonin transporter and glucocorticoid receptor expression in brain

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Ngai YF, Sulistyoningrum DC, O’Neill R, Innis SM, Weinberg J, Devlin AM. Prenatal alcohol exposure alters methyl metabolism and programs serotonin transporter and glucocorticoid receptor expression in brain. Am J Physiol Regul Integr Comp Physiol 309: R613–R622, 2015. First published July 15, 2015; doi:10.1152/ajpregu.00075.2015.—Prenatal alcohol exposure (PAE) programs the fetal hypothalamic-pituitary-adrenal (HPA) axis, resulting in HPA dysregulation and hyperresponsiveness to stressors in adulthood. Molecular mechanisms mediating these alterations are not fully understood. Disturbances in one-carbon metabolism, a source of methyl donors for epigenetic processes, contributes to alcoholic liver disease. We assessed whether PAE affects one-carbon metabolism (including Mtr, Mat1a, Mthfr, and Cbs mRNA) and programming of HPA function genes (Nr3c1, Nr3c2, and Slc6a4) in offspring from ethanol-fed (E), pair-fed (PF), and ad libitum-fed control (C) dams. At gestation day 21, plasma total homocysteine and methionine concentrations were higher in E compared with C dams, and E fetuses had higher plasma methionine concentrations and lower whole brain Mtr and Mat1a mRNA compared with C fetuses. In adulthood (55 days), hippocampal Mtr and Cbs mRNA was lower in E compared with C males, whereas Mtr, Mat1a, Mthfr, and Cbs mRNA were higher in E compared with C females. We found lower Nr3c1 mRNA and lower nerve growth factor inducible protein A (NGFI-A) protein in the hippocampus of E compared with PF females, whereas hippocampal Slc6a4 mRNA was higher in E than C males. By contrast, hypothalamic Slc6a4 mRNA was lower in E males and females compared with C offspring. This was accompanied by higher hypothalamic Slc6a4 mean promoter methylation in E compared with PF females. These findings demonstrate that PAE is associated with alterations in one-carbon metabolism and has long-term and region-specific effects on gene expression in the brain. These findings advance our understanding of mechanisms of HPA dysregulation associated with PAE.

Fetal alcohol spectrum disorder; prenatal alcohol exposure; methyl metabolism; developmental programming; hypothalamic-pituitary axis; glucocorticoid receptor; serotonin transporter

Fetal alcohol spectrum disorder (FASD) is a serious public health issue that is estimated to affect 9.1 individuals in every 1,000 live births in the United States (6). FASD is a nongenetic term describing the broad range of deficits in neurobehavioral and cognitive function found in children exposed to alcohol in utero. The most severe consequence of prenatal alcohol exposure is fetal alcohol syndrome (FAS), which includes characteristic facial dysmorphism, prenatal and postnatal growth retardation, and both structural and functional central nervous system abnormalities (28, 64a). Levels of alcohol exposure lower than those that result in full FAS can still result in neurocognitive, adaptive, and self-regulatory abnormalities (11, 23). Rodent models of prenatal alcohol exposure (PAE) reproduce many of the features of human FASD, including retarded prenatal and postnatal growth and development, physical malformations, central nervous system abnormalities (3, 10, 16, 40, 53, 61, 66), neurobehavioral and cognitive deficits (1, 7, 8), and increased stress system activity and depressive- and/or anxiety-like behaviors (12, 23).

Impairments in self-regulation observed in both clinical studies and animal models include long-term changes in behavioral and physiological responsiveness to stress (23). Hormones of the hypothalamic-pituitary-adrenal (HPA) axis, a critical component of the stress system, have metabolic/homostatic effects on virtually all body systems, facilitate short-term survival and recovery from challenge, and are important for normal cognitive and psychological functioning (13, 27, 39). The HPA axis is highly susceptible to early life programming and may provide a final common path for the effects of multiple early-life events (45). Clinical (21, 30, 52) and preclinical (4, 23, 33, 35, 46) studies have reported that fetal programming by alcohol results in HPA dysregulation, including both hyperresponsiveness to stressors and deficits in recovery to basal levels. While mechanisms mediating these adverse effects of alcohol are starting to be elucidated (23, 55, 59, 60, 71), the molecular pathology underlying fetal alcohol effects on the HPA axis is not fully understood.

Accumulating evidence suggests that the interplay between environmental factors and epigenetic processes, such as DNA methylation and chromatin modifications, could play a role in mediating the adverse effects of PAE, including its effects on HPA regulation (20, 22, 38). Alcohol-induced alterations in one-carbon metabolism provide one potential link between alcohol exposure and epigenetic processes. One-carbon metabolism generates S-adenosylmethylionine (AdoMet), a methyl donor for methylation processes, including DNA methylation. AdoMet is produced from methionine, a dietary essential sulfur-containing amino acid, and is catalyzed by methionine adenosyltransferase (encoded by liver-specific Mat1a and ubiquitous Mat2a). S-adenosylhomocysteine (AdoHcy) is formed following methyl donation by AdoMet, and AdoHcy is then converted to homocysteine through the reversible liberation of adenosine from AdoHcy. Homocysteine has two possible metabolic fates: 1) transsulfuration to cysteine, which is used for protein or glutathione synthesis; and 2) remethylation of homocysteine to methionine. This step uses the vitamin B12-dependent enzyme methionine synthase (encoded by Mtr),
which requires folate (as 5-methyltetrahydrofolate, MTHF) as a cofactor. The folate cycle generates 5-MTHF from 5,10-methylenetetrahydrofolate in a reaction catalyzed by MTHF reductase. Alcohol is known to affect one-carbon metabolism by inhibiting folate-dependent homocysteine remethylation to methionine and AdoMet synthesis (43).

Embryonic exposure to ethanol was reported to be associated with altered expression of genes in the hippocampus and prefrontal cortex involved in neurodevelopment, apoptosis, and energy metabolism (40), as well as altered methylation in the promoter regions of genes involved in the cell cycle, growth, apoptosis, and cancer (14). In addition, reduced methylation of Igf2 was rescued when alcohol-consuming dams were fed a methyl-supplemented diet (15). Hypermethylation and transcriptional silencing of the Agouti gene, as indexed by altered coat color, were observed in offspring of female mice exposed to ethanol in early gestation (31). Similarly, rat pups exposed to alcohol during the early neonatal period (third-trimester equivalent) exhibited global hypermethylation of DNA in the hippocampus and prefrontal cortex at 21 days of age, which was significantly reduced following choline supplementation (48).

These studies provide striking evidence of in utero alcohol effects on fetal and neonatal DNA methylation. Among the few studies that have begun to examine persistence of these effects as the organisms matures, long-term effects of PAE on mouse brain include changes in miRNA and gene expression, as well as differential methylation of >6,500 gene promoters (34). However, whether epigenetic mechanisms play a role in the HPA dysregulation seen in PAE offspring is unknown. The goal of the present study was to test the hypothesis that changes in epigenetic programming of gene expression contribute to the long-term molecular pathology underlying prenatal alcohol effects on HPA activity and regulation. Utilizing our well-established rat model (23), we determined the effects of PAE on methyl metabolism and programming of glucocorticoid receptor (Nr3c1), mineralocorticoid receptor (Nr3c2), and serotonin transporter (Slc6a4) gene expression, critical regulators of HPA axis function, as well as Bdnf (encodes brain-derived neurotrophic factor). Nr3c1 is well known to be susceptible to developmental programming by perinatal stressors through epigenetic mechanisms (47, 68). Nr3c2 is known to be involved in the tonic modulation of corticosterone secretion and to play a role in stress resilience (65). Altered Slc6a4 function has also been implicated in the pathology of PAE (32, 57, 71), as well as in the regulation of mood, and expression is known to be regulated by methylation (49, 50). Bdnf is an important neurotrophic factor in brain development, known to be altered by PAE (51).

METHODS

Rat model of PAE. Our well-established model of PAE (69) was used in this study. At gestational day (GD) 1, Sprague-Dawley dams were randomly assigned to one of three groups: 1) a lab chow (ad libitum-fed control, C) group that was fed a standard rodent chow diet (Rat Diet 5012; Jamieson’s Pet Food Distributors, Delta, BC, Canada), ad libitum (This group served as a reference to the standard laboratory situation.); 2) an alcohol (ethanol, E) group that was provided a liquid ethanol diet (1 kcal/ml diet, 25.1% energy from protein, 16.4% energy from fat, 23.0% energy from carbohydrate, and 35.5% energy from ethanol) ad libitum; or 3) a pair-fed (PF) group that was fed a liquid control diet with maltose dextrin isocalorically substituted for ethanol (1 kcal/ml diet, 25.1% energy from protein, 16.4% energy from fat, and 58.6% energy from carbohydrate), in the amount consumed by an ethanol-fed partner (g/kg body wt/day of gestation). Experimental diets were provided by Dyets (Bethlehem, PA). One cohort of pregnant females was assigned for assessment at GD21. Females were removed quietly and individually from the colony room, deeply anesthetized, and placed on a heating pad. An incision was made in the abdominal/pelvic cavity, the uterine horns were opened, and fetuses were exteriorized but remained attached to the placenta via their umbilical cords. Fetuses were bled via the axillary veins, blood was collected into capillary tubes, and after centrifugation, plasma from the entire litter was pooled to have sufficient quantity for analysis. Brains were then collected from two randomly chosen fetuses per litter, and a blood sample was taken from the maternal female via cardiac puncture. Because of these blood collection, pooling, and sampling procedures, we could not analyze fetal results by sex or determine fetal weights. A second cohort of pregnant females was allowed to give birth, birth weight of pups was recorded, litters were culled to 10 (5 males, 5 females when possible), and offspring were studied at postnatal day (PD) 55. Trunk blood was collected from PD55 male and female offspring following decapitation, and brains were quickly excised, immediately frozen in liquid nitrogen, and stored at −80°C until further characterization. All procedures were performed according to the NIH “Guide for the Care and Use of Laboratory Animals” and with the approval of the University of British Columbia Animal Care Committee.

Biochemical analyses. Plasma total homocysteine (tHcy), defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, and methionine were quantified by HPLC-MS/MS, as described previously (29).

Real-time PCR quantification of mRNA. Total RNA was extracted from liver, whole brain, hippocampus, and hypothalamus using the RNasey Mini Kit (Qiagen). On-column DNase I treatment was conducted to remove genomic DNA. RNA integrity was confirmed by visualization of intact 18S and 28S rRNA bands on agarose gels. RNA concentration and purity were assessed by absorbance at 260 nM and 280 nM using a NanoVue Spectrophotometer (GE Healthcare). Synthesis of cDNA was accomplished using the high-capacity cDNA reverse transcription kit, Applied Biosystems, Foster City, CA. Quantitative real-time PCR was performed using TaqMan primers/probes (Applied Biosystems), specific for each target gene in the rat and included: Cbs, Mtr, Mhfr, Mat2a, Nr3c1, Nr3c2, Slc6a4, and Bdnf. Gene expression was determined relative to 18S rRNA or Actb mRNA using the ΔΔCt method (56) and a 7500 real-time PCR system (Applied Biosystems).

Quantification of DNA methylation by bisulfite pyrosequencing. We targeted Nr3c1 and Slc6a4 to determine whether PAE affected promoter methylation status because of the established role for DNA methylation in regulating transcriptional expression of these genes (49, 50, 68). CpG-rich regions in rat Nr3c1 and Slc6a4 were identified using Methprim (36) and the University of California, Santa Cruz genome browser (54). For Nr3c1, we analyzed the promoter region of exon 1F (corresponding to human Nr3c1 exon 1–7), which contains an NGFI-A binding site, previously shown to be important in the regulation of Nr3c1 expression in hippocampus (68). For Slc6a4, we chose to analyze a 30-bp sequence region directly upstream of a putative transcriptional start site, identified in silico and based on high-sequence conservation between the rat, mouse, and human genome, determined using EMBOSS pairwise alignment algorithms (www.ebi.ac.uk/Tools/psa/). The percent methylation at each CpG site was determined using EMBOSS pairwise alignment algorithms (www.ebi.ac.uk/Tools/psa/). The percent methylation at each CpG site was quantified by bisulfite pyrosequencing (18). DNA was extracted from whole brain, hippocampus, and hypothalamus using the DNeasy blood and tissue kit, and included RNase I treatment (Qiagen). DNA samples (200–500 ng) were bisulfite-treated using the EZ DNA methylation-direct kit (Zymo Research) and stored at −20°C until further analysis. Bisulfite-treated DNA was amplified using primers specific for Nr3c1 and Slc6a4 and HotStar Taq DNA Polymerase (Qiagen). All PCR
primers and pyrosequencing primers are listed in Supplemental Table S1. PCR products of 227 bp and 334 bp were generated for Nr3c1 and Slc6a4, respectively. The percent methylation at each CpG site was quantified using the Pyro Q-CpG software (Qiagen).

Immunoblot analysis. Hippocampi were homogenized in solubilizing buffer and homogenates (25 μg protein) resolved on 1% Tris-HCl SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Immunodetection was performed using a rabbit anti- rat NGFI-A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). Membranes were also probed with β-actin using a goat anti-human β-actin polyclonal antibody (Santa Cruz Biotechnology). Protein bands were detected, and densities were quantified using the Western Lightning CDP-Star Chemiluminescence reagent (Perkin Elmer, Mississauga, ON, Canada) and a ChemiGenius2 gel imaging system (Perkin Elmer).

Statistical analyses. For the cohort of pregnant females utilized for the GD21 measures, the effect of prenatal treatment on fetal body weight could not be assessed, as noted above. Prenatal treatment effects on one-carbon metabolism and gene expression were determined by two-way ANOVA followed by Newman-Keuls post hoc tests for multiple comparisons. For the cohort of pregnant females that gave birth, body weights of male and female offspring at PD1 and PD55 were analyzed by two-way ANOVA (prenatal group × sex) followed by Newman-Keuls post hoc test. For the PD55 offspring, two-way ANOVA identified significant interactions between sex and prenatal group. Therefore, the effect of PAE was analyzed separately by sex in the pediatric animals. For both cohorts of pregnant females, well-known sexually dimorphic effects of PAE were evident and were analyzed separately in males and females. Well-known sexually dimorphic effects of PAE on HPA activity and function have been reported (23). Significant differences in Slc6a4 and Nr3c1 individual CpG site methylation were adjusted for multiple comparisons using a Bonferroni correction. All analyses were performed using SPSS statistical software (version 18). Experimental results are expressed as means ± SE.

RESULTS

Developmental data. For both cohorts of pregnant females, alcohol intake of pregnant E dams was consistently high over the course of gestation, averaging 9.25 ± 0.30 to 11.95 ± 0.40 g/kg body wt in week 1, 13.20 ± 0.35 to 17.06 ± 0.46 g/kg body wt in week 2, and 12.80 ± 0.36–16.54 ± 0.46 g/kg body wt in week 3 of gestation. Analysis of maternal weight gain during pregnancy for dams in the second cohort showed that dams from the three prenatal treatment groups did not differ in body weight on GD1, but E and PF dams weighed significantly less than C dams from GD7 to GD21 (P < 0.05). There was no significant difference among groups for the number of live-born pups (Table 1). At birth (PD1), PF male (P < 0.01) and female (P < 0.05) pups weighed less than C pups, and there was a similar trend for reduced birth weights of PAE males (P = 0.069) and females (P = 0.064) compared with C pups. At PD55, body weights remained significantly lower in PAE and PF (P < 0.01) compared with C males. Catch-up growth occurred in females, and there were no longer significant differences in body weight among females from the three prenatal treatment groups (Table 1).

Alcohol intake during pregnancy affects one-carbon metabolism in dams and fetuses and alters Slc6a4 mRNA expression in fetal brains. At GD21, E dams had higher plasma total homocysteine (P < 0.05) and methionine (P < 0.001) concentrations than C and PF dams, as typically occurs with chronic ethanol intake (43) (Fig. 1, A and B). Maternal alcohol consumption had no effect on plasma total homocysteine concentrations in GD21 fetuses (Fig. 1C). However, plasma methionine concentrations were higher in E compared with C fetuses (P < 0.01) (Fig. 1D), and this was accompanied by alterations in one carbon metabolism enzyme gene expression in whole brain: lower (P < 0.05) Mtr and Mat2a mRNA in brains from E and PF compared with C fetuses was found (Fig. 1E).

We further determined whether PAE affected Nr3c1, Nr3c2, Bdnf, and Slc6a4 mRNA levels in whole brain from GD21 fetuses. Lower (P < 0.05) Slc6a4 mRNA was found in E compared with PF fetuses, although there were no differences between E and C (Fig. 2A), and we found no effect of PAE on Nr3c1, Bdnf, or Nr3c2 mRNA levels (Fig. 2, B–D).

PAE alters expression of one-carbon metabolism gene expression in hippocampus but not hypothalamus at PD55. We then studied the effects of PAE on methyl metabolism gene expression in the adult brain, specifically in the hippocampus and hypothalamus, two regions critically important in the regulation of behavior, learning, and memory, as well as neuroendocrine and autonomic activity, among other functions (25). At PD55, we found tissue-specific effects of PAE on one-carbon metabolism gene expression. In the hippocampus, Mtr mRNA levels were lower (P < 0.05) in E compared with C males, and Cbs mRNA levels were lower (P < 0.01) in both E and PF compared with C males (Fig. 3A). In contrast, higher (P < 0.05) Mtr, Mat2a, and Mthfr mRNA levels were found in the hippocampus from E compared with C females (Fig. 3B), and levels of Cbs mRNA were higher in hippocampus from E and PF compared with C females. No effects of PAE on one-carbon metabolism enzyme gene expression were detected in the hypothalamus of either male or female offspring (Fig. 3, C and D).

Effects of PAE on Slc6a4, Nr3c1, Bdnf, and Nr3c2 in hippocampus and hypothalamus at PD55. Epigenetic programming by PAE could result in long-lasting changes in behavioral, cognitive, and physiological function. Therefore, we determined whether the PAE-induced decrease in Slc6a4 mRNA expression observed in whole brain from GD21 fetuses persisted into adulthood. Interestingly, in hippocampus, we found higher (P < 0.05) Slc6a4 mRNA in E compared with C in males (Fig. 4A), whereas in females, we found lower (P < 0.05) Nr3c1 mRNA in E compared with PF (Fig. 4B), but no significant differences between E and C females. Similar to what we observed in whole brain from GD 21 fetuses, we found no effect of PAE on Bdnf in males or Nr3c2 mRNA levels in males or females in hippocampus (Fig. 4, C and D). However, Bdnf mRNA levels were higher (P < 0.05) in PF compared with C females (Fig. 4C).

In the hypothalamus, Slc6a4 mRNA levels were lower (P < 0.05) in E and PF males and E females compared with C male and female counterparts (Fig. 4E). There was no effect of PAE on Nr3c1 or Bdnf mRNA from either male or female offspring.
and no effect on \(Nr3c2\) mRNA from males (Fig. 4H). However, \(Nr3c2\) mRNA levels were lower \((P < 0.05)\) in E compared with PF females, and E did not differ from C females (Fig. 4H).

PAE alters NGFI-A expression but not \(Nr3c1\) exon 1F promoter methylation in hippocampus of females at PD55. Given our finding of lower \(Nr3c1\) mRNA in the hippocampus from E compared with PF females in adulthood and the

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**Fig. 1.** Prenatal alcohol exposure and one carbon metabolism in dams and fetuses at gestational day 21. Plasma total homocysteine (A, C) and methionine (B, D) concentrations in dams and fetuses, respectively. E: one carbon metabolism enzyme gene expression in whole brain from fetuses at gestational day 21. Values shown are expressed as means ± SE; \(n = 6\) group. \(*P < 0.05, **P < 0.01, ***P < 0.001\). C, control; PF, pair-fed; E, ethanol.

**Fig. 2.** Prenatal alcohol exposure and Slc6a4, Nr3c1, Bdnf, and Nr3c2 expression in whole brain from fetuses at gestational day 21. A: Slc6a4 mRNA. B: Nr3c1 mRNA. C: Bdnf mRNA. D: Nr3c2 mRNA. Values shown are expressed as means ± SE; \(n = 6\) group. \(*P < 0.05\).
important roles for DNA methylation and NGFI-A in regulating hippocampal expression of *Nr3c1* (68), we quantified the methylation status of the *Nr3c1* exon 1F promoter and assessed NGFI-A protein expression. *Nr3c1* has multiple first exons, and methylation of an NGFI-A binding site in the exon 1F promoter region is known to be susceptible to programming by perinatal stressors in humans and rodents (42, 47, 68). We found no significant effect of PAE on the methylation status of the *Nr3c1* exon 1F promoter region in hippocampus from PD55 females (Fig. 5A). Interestingly, however, we found lower (*P < 0.05*) NGFI-A protein expression in hippocampus from E compared with PF females, although E did not differ significantly from C females (Fig. 5B). 

**PAE alters Slc6a4 methylation at PD55.** Alterations in Slc6a4 promoter methylation have been implicated in mood disorders in humans (5, 14, 49, 50). Sequence comparison between mouse, rat, and human identified a highly conserved region, 130 bp upstream of the putative transcriptional start site of *Slc6a4*, which is also in close proximity to region of the human *SLC6A4* promoter analyzed by others (14, 50). We found lower (*P = 0.05*) methylation at CpG site 15 and site 23 in the hypothalamus from PD compared with C males at PD 55; there was no significant effect of PAE on *Slc6a4* methylation (Fig. 6A). In contrast, for females, we found higher (*P < 0.001*) *Slc6a4* mean methylation in the hypothalamus of E compared with PF females (Fig. 6B).

**DISCUSSION**

The present study demonstrates that PAE alters one-carbon metabolism in dams and fetuses at the end of gestation, and it has long-term and region-specific effects on gene expression in the brain, with the most significant effects observed for *Slc6a4*. These data support and extend previous findings identifying a role for disturbances in serotonin metabolism and the differential regulation of genes important in HPA function in the phenotype associated with PAE (32, 35, 57).
study, we found that PAE was associated with changes in \(Nr3c1\) mRNA expression that were sex- and brain region-specific, with lower \(Nr3c1\) mRNA levels found in hippocampus from E relative to PF females. Interestingly, we found this was accompanied by lower NGFI-A protein levels but not by changes in \(Nr3c1\) exon 1F promoter methylation. Given that NGFI-A is an important transcriptional regulator in the hippocampus (67), our finding that NGFI-A protein expression is affected by PAE suggests that NGFI-A regulation of gene expression may contribute to the mechanisms underlying the PAE phenotype.

It is possible that the differential expression of \(Nr3c1\) and NGFI-A protein levels in PAE compared with control animals may result from differential effects of alcohol on cellular differentiation and the epigenome of neurons in the hippocampus. Sexually dimorphic effects of PAE on HPA activity and regulation are well known. While hyper-responsiveness to stress is a robust phenomenon, PAE males and females may show different patterns of response depending on the nature of the stressor, the time course, and the hormonal endpoints measured (23). With acute or short-duration stressors, corticosterone, and/or adrenocorticotropic hormone (ACTH) re-
responses may be greater in PAE females than males or occur only in PAE females. By contrast, HPA hyperresponsiveness is often observed in PAE males or in both male and female PAE offspring in response to stressors of greater intensity or duration (69). Although we did not directly assess HPA responsiveness in the present study, greater corticosterone and/or ACTH responses to stressors, as well as deficits in recovery to basal levels following stress have been demonstrated in numerous clinical (21, 30, 52) and preclinical (4, 23, 33, 35, 46) studies, as noted. Moreover, offspring of dams that were part of the same breeding that produced the PD55 animals for the present study were tested in another study for stress and anxiety-like behavior (elevated plus maze, EPM) in adulthood, following exposure to 10 days of chronic mild stress or under baseline (non-CMS) conditions (24). We found that PAE males and females both showed increased anxiety-like behavior (albeit with somewhat different behaviors). However, while corticosterone responses to the EPM were greater in animals from all prenatal groups in the CMS compared with the non-CMS condition, corticosterone responses to the EPM were higher in E than in PF and C animals. Our current findings suggest that alterations in levels of Ntr3c1 and NGFI-A protein could play a role in the altered HPA function of PAE animals. However, we make these conclusions with caution. Because levels of Ntr3c1 mRNA and NGFI-A protein expression in E females were significantly different from those of PF but not significantly different from those of C females, it is possible that different mechanisms mediate the differential findings in E and PF offspring, i.e., direct effects of alcohol vs. effects of restricted feeding and/or mild stress in PF dams (see further discussion below).

Serotonin (5-HT) plays a key role as a neurotrophic factor during brain development in addition to its role as an important neurotransmitter in both the developing and mature brain (70). Prenatal alcohol exposure has been reported to alter development and function of the serotonergic system, resulting in reduced density of 5-HT neurons and concentrations of 5-HT and 5-HT reuptake sites, and impaired development of 5-HT1A receptors in several brain areas (17, 63). Moreover, there is a bidirectional regulatory relationship between 5-HT and the HPA axis (44). We have shown that PAE animals exhibit increased hypothermic and anxiolytic responses and blunted ACTH responses to 8-OH-DPAT, a 5-HT1a receptor agonist, compared with PF and C animals (26), and that E females show alterations in 5-HT1A receptor levels in the hippocampus that are estrous cycle stage-dependent (62). Furthermore, it has been reported that the short allele of the SLC6A4 promoter variant, 5HTTLPR, increases the probability of neonatal irritability and stress responsiveness in rhesus monkeys exposed prenatally to alcohol and that this gene-environment interaction may affect psychosocial development (58). In the present study, we found brain region-specific effects of PAE on Slc6a4, with higher Slc6a4 mRNA expression in the hippocampus of PAE males, but lower Slc6a4 mRNA expression in the hypothalamus of PAE males and females. Interestingly, this latter finding was accompanied by greater mean methylation of Slc6a4 in the hypothalamus of PAE compared with control females.

Adverse effects of PAE on Slc6a4 expression suggest the possibility that disturbances in serotonin signaling may play a role in the phenotype associated with PAE. This is relevant not only to HPA dysregulation but also in relation to the high incidence of mental health problems in individuals with FASD, a finding supported by preclinical studies (23). Our data showing disturbances in serotonin signaling in the hippocampus and hypothalamus, brain regions critical in HPA activity and regulation, provide molecular evidence to support the suggestion that altered HPA-5-HT interactions could play a role in both behavioral and neurobiological outcomes in FASD. However, caution must be exercised in the interpretation of our findings, as the overall level of Slc6a4 methylation was low (1–5%), and the biological relevance of such small changes as it pertains to regulating gene expression remains to be determined. Further
studies are required to elucidate the potential role for altered serotonin transport in PAE pathophysiology.

The finding that pair-feeding resulted in effects on one-carbon metabolism and gene expression that were in some cases, similar to those of PAE animals, and in some cases unique, is not entirely surprising. While pair-feeding is the accepted procedure to separate nutritional effects of alcohol from its direct effects, pair-feeding is at best an imperfect control procedure. The pair-feeding procedure can only control for the reduced intake of the alcohol-consuming dams, but can never control for the secondary nutritional effects of alcohol, such as absorption and utilization of nutrients. Moreover, the pair-feeding procedure is stressful to the dam and, thus, may constitute a mild form of prenatal stress for the fetus. In contrast to alcohol-consuming dams that consume their diet ad libitum, PF dams get a reduced ration of food, and are, therefore, hungry. They typically consume their entire daily ration within a few hours of presentation, remaining deprived for the rest of the 24-h period until the next feeding. For animals on a “meal-feeding” schedule, such as this, there are numerous consequences, including metabolic effects (9), as well as phase shifts in circadian periodicities of behavioral and physiological outcomes, including HPA activity (19), as food rather than light becomes the dominant cue in the environment.

Of relevance to the present study, both prenatal stress and maternal undernutrition can affect hormonal and behavioral development of offspring and program the fetal HPA axis, such that activity and regulation are altered in adulthood (41). Together, these findings suggest several possible explanations for our data: 1) Effects of caloric or growth restriction, rather than a specific effect of PAE, may explain some of the changes observed in PAE fetuses/offspring; 2) PAE and pair-feeding could act through similar mechanisms (e.g., effects on the HPA axis as a final common pathway) to influence outcomes in E and PF offspring; 3) similar outcomes in PAE and PF offspring may be mediated by different mechanisms (e.g., effects of ethanol on cell-signaling pathways vs. prenatal stress or metabolic effects of pair-feeding) rather than occurring along a continuum of effects on the same pathway; and/or, 4) given its complex nature, pair-feeding may be mediated by mechanisms that result in unique effects on offspring. Further investigation is needed to understand fully the mechanisms that underlie our current findings in PAE and PF offspring.

In conclusion, this is the first study to examine long-lasting changes of prenatal alcohol exposure on one-carbon metabolism in the brain and the role of DNA methylation in regulating expression of genes involved in HPA function. We report for the first time that PAE is associated with altered plasma levels

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**Fig. 6.** Prenatal alcohol exposure and Slc6a4 methylation in hypothalamus from male and female offspring at postnatal day 55. A: methylation status of individual CpG sites and the mean methylation of all sites in Slc6a4 in hypothalamus from male offspring. B: methylation status of individual CpG sites and the mean methylation of all sites in Slc6a4 in hypothalamus from female offspring. Values are shown as means ± SE; n = 6/group *P < 0.05, **P < 0.01.
of methyl metabolites, altered one-carbon metabolism enzyme gene expression in liver and brain, and changes in the expression of two genes central to HPA axis function. These findings provide molecular evidence that supports and significantly extends previous studies on the effects of prenatal alcohol exposure on gene expression and epigenetic mechanisms, and advances our understanding of the pathophysiology of FASD. Also, these findings have important implications for the development of novel treatments and interventions for children prenatally exposed to alcohol.

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DISCLOSURES

A. Devlin serves as a consultant for Nestle Health Science-Pamlab, Inc. No conflicts of interest, financial or otherwise, are declared by the other authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.F.N., D.C.S., and R.O. performed experiments; Y.F.N., D.C.S., R.O., J.W., and A.M.D. analyzed data; Y.F.N. prepared figures; Y.F.N. drafted manuscript; S.M.I., J.W., and A.M.D. conceived and designed research; S.M.I., J.W., and A.M.D. approved final version of manuscript; J.W. and A.M.D. interpreted results of experiments; J.W. and A.M.D. edited and revised manuscript.

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