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Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation and POMC gene expression in β-endorphin-producing POMC neurons of the hypothalamus

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Abstract

Background—Prenatal exposure to ethanol reduces the expression of hypothalamic proopiomelanocortin (POMC) gene, known to control various physiological functions including the organismal stress response. In this study, we determined whether the changes in POMC neuronal functions are associated with altered expressions of histone-modifying and DNA-methylating enzymes in POMC-producing neurons, since these enzymes are known to be involved in regulation of gene expression. In addition, we tested whether gestational choline supplementation prevents the adverse effects of ethanol on these neurons.

Methods—Pregnant rat dams were fed with alcohol-containing liquid diet or control diet during gestational days 7 and 21 with or without choline, and their male offspring rats were used during the adult period. Using double-immunohistochemistry, real-time reverse transcription polymerase chain reaction (RT-PCR) and methylation specific RT-PCR, we determined protein and mRNA levels of histone-modifying and DNA-methylating enzymes, and the changes in POMC gene methylation and expression in the hypothalamus of adult male offspring rats. Additionally, we measured the basal and lipopolysaccharide (LPS)-induced corticosterone levels in plasma by enzyme-linked immunoabsorbent assay.

Results—Prenatal ethanol treatment suppressed hypothalamic levels of protein and mRNA of histone activation marks (H3K4me3, Set7/9, acetylated H3K9, phosphorylated H3S10) increased the repressive marks (H3K9me2, G9a, Setdb1) and DNA methylating enzyme (Dnmt1) and the methyl-CpG-binding protein (MeCP2). The treatment also elevated the level of POMC gene methylation, while it reduced levels of POMC mRNA and β-EP, and elevated corticosterone response to LPS. Gestational choline normalized the ethanol-altered protein and the mRNA levels of H3K4me3, Set7/9, H3K9me2, G9a, Setdb1, Dnmt1 and MeCP2. It also normalizes the changes in POMC gene methylation and gene expression, β-EP production and the corticosterone response to LPS.

Conclusions—These data suggest that prenatal ethanol modulates histone and DNA methylation in POMC neurons that may be resulting in hypermethylation of POMC gene and

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reduction of POMC gene expression. Gestational choline supplementation prevents the adverse effects of ethanol on these neurons.

Keywords

Choline; fetal alcohol; histone modification; DNA methylation; proopiomelanocortin; stress axis

INTRODUCTION

Alcohol drinking during pregnancy is an intractable health problem worldwide and a leading cause of mental retardation and other neurological disorders in the United States (Sokol et al., 2003). Children who were prenatally exposed to alcohol most often show physiological changes and behavioral deficits in adulthood such as depression, anxiety, hyperactivity, and a reduced ability to cope with stress (Famy et al., 1998; Guerri et al., 2009; Haley et al., 2006). They also show immune and metabolic-related diseases (Arjona et al., 2006; Ting and Lautt, 2006). Hypothalamic POMC neurons are one of the major regulators of the hypothalamic-pituitary-adrenal (HPA) axis activity, immune functions and energy homeostasis (Arjona et al., 2006; Boyadjieva et al., 2009; Raffin-Sanson et al., 2003; Sarkar et al., 2007). A prenatal alcohol-exposed animal model, which mimics fetal alcohol exposure in humans, shows a decrease in the number of POMC neurons in the hypothalamus with a deficit in POMC gene expression and a decrement in the levels of its derived peptide β-endorphin (β-EP) (Govorko et al., 2012; Sarkar et al., 2007). Although the adverse effects of fetal alcohol exposure are very well documented, our understanding of how prenatal alcohol exposure causes the dysfunction of hypothalamic POMC neurons in the adult stage has not been well studied.

For example, the two epigenetic mechanisms such as histone modifications and DNA methylation have been implicated in the long-term “maternal programming” of the stress axis and in demarcating individual's susceptibility to various type of stress in adulthood (Weaver et al., 2004). Recently, epigenetic mechanisms have also been implicated in fetal programming of the hypothalamic POMC system and in altering the stress axis (Govorko et al., 2012). Abnormal changes in histone modifications and/or DNA methylation are major players in modulating gene expression and cellular functions resulting in long-lasting altered phenotypes (Vaissiere et al., 2008). For example, these changes have been associated with hyperactivity in children and with some psychiatric disorders (Mill and Petronis, 2008; Mill et al., 2008). They have been linked to the induction of an abnormal stress axis (Franklin et al., 2012; Govorko et al., 2012). Epigenetic mechanisms have also been linked to alcohol-induced fetal programming and alcohol-induced physiological and morphological abnormalities during the developmental period (Haycock, 2009; Liu et al., 2009).

Alcohol exposure exhibits wide range of conspicuous effects particularly on the brain (Harper, 2009). In the context of one carbon metabolism, alcohol inhibits folic acid absorption and decreases the availability of the methyl-donor, S-adenosylmethionine (SAM). SAM is critical for DNA methylation which is indispensable to many physiological processes including normal embryonic development and normal brain development in mammals (Kim et al, 2009; Robertson and Wolffe, 2000). SAM formation is dependent on the availability of major nutrients such as choline. Choline normal level is required for normal fetal growth and normal brain development (Zeisel, 2006). Its deficiency or abnormal level causes neurodevelopmental abnormalities such as neural tube defects in mice and in humans (Fischer et al., 2002; Shaw et al., 2004; Zeisel, 2000) and has negative effects on neuronal migration, survival and differentiation (Craciunescu et al., 2003; Zeisel, 2011). Recent studies demonstrated that gestational choline supplementation attenuates some of the prenatal or early postnatal alcohol adverse effects on learning and memory (Thomas et al.,

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2000; 2007; 2010). Moreover, choline and its derivative betaine are considered "homocysteine-lowering agents" that normalize SAM level and hence impact DNA methylation (Finkelstein, 1998; Olthof and Verhoef, 2005). Choline deficiency during critical periods of embryonic development adversely affects fetal neurogenesis (Zeisel, 2004) and modulates DNA methylation and fetal gene expression in the brain (Davison et al., 2009; Mehedint et al., 2010; Niculescu et al., 2006). Its deficiency also decreases SAM levels in peripheral organs such as the liver (Zeisel et al., 1989). On the other hand, choline supplementation in rats normalizes SAM levels, increases choline metabolites in the blood and the brain and reduces stress (Zeisel et al., 2006). Besides its role in DNA methylation, choline also plays a pivotal role in the synthesis of phosphotidylcholine to maintain the integrity of cellular membrane structure (Shaw et al., 2004). Choline has been implicated lately in altering histone methylation (Davison et al., 2009; Mehedint et al., 2010) and DNA methylation (Kovacheva et al., 2007; Niculescu et al., 2006) in a tissue-specific manner thus altering gene expression and phenotype.

Based on the beneficial effects of choline on proper physiological functioning, we rationalized that gestational choline supplementation during the period of alcohol exposure could impact fetal alcohol effects on hypothalamic POMC neurons and attenuate its adverse affects on the stress axis functions. In this study, we determined whether prenatal ethanol-induced changes in POMC gene methylation, expression and β-EP production are associated with altered expression of histone-modifying and DNA-methylating enzymes in β-EP-producing POMC neurons. Additionally, we investigated the effects of gestational choline supplementation on methylation status of histone and DNA, the methylation status of POMC gene promoter and POMC gene expression in hypothalamic POMC neurons. We also assessed its effects on plasma corticosterone levels in response to LPS in control and prenatally alcohol-exposed male offspring in the adult stage.

MATERIALS AND METHODS

Animal model

Adult male and female rats of Sprague-Dawley strain were purchased from Charles River Laboratory (Wilmington, MA) and maintained in environmentally controlled animal vivarium on a 12 h light/dark cycle (light on 0700 and light off 1900 h) at a constant temperature (22°C). Female rats were mated with experienced males, and the presence of vaginal plug in a particular day indicated mating and designated at GD1. On gestational days GD7–GD21, pregnant rats were fed daily chow ad libitum (AD), a liquid diet (BioServe Inc., Frenchtown, NJ) containing alcohol (AF) or pair-fed an isocaloric liquid control diet (PF; with the alcohol calories replaced by maltose-dextrin). The concentration of ethanol varied (1.7–5.0% v/v) in the diet for the first 4 days to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v, which provided about 35% of the total dietary calories. Some rats were fed with alcohol-containing liquid diet with 642 mg/L choline chloride (Sigma) (CAF) or isocaloric liquid diet (CPF) from gestational day 11 (GD11) until birth. Previous studies have shown that the peak blood ethanol concentration is achieved in the range of 120–150 mg/dl in pregnant dams fed with this liquid diet (Miller, 1992) and produce significant inhibitory effect on β-EP neuronal function (Sarkar et al., 2007). The choline dose is shown to be effective in altering global methylation in brain tissues (Holler et al., 1996). The day of birth was recorded as postnatal day1 (PD1). AF, PF, CAF and CPF litters were crossfostered using untreated lactating rats fed chow ad libitum (AD) to prevent any compromised nurturing by the AF lactating mother rats until postnatal day 22 (PD22) and then weaned, housed by sex, and provided rodent chow meal and water ad libitum. Litter size was maintained as 8 pups/dam. Only one pup from each litter was used in an experiment in order to prevent gene homogeneity. Male rats, 60–65 days old, were used in this study. In our

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hand although fetal alcohol exposure increases the HPA axis response to stress more in females than males, we were unable to find any sex differences in fetal alcohol-induced changes in POMC neuronal activity (Sarkar et al., 2007; Govorko et al., 2012). This might be that sex difference action of fetal alcohol might be at the levels of CRH and/or corticosterone production/secretion. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy.

Double-immunostaining for H3K4me2,3, H3K9me2, Acetylated H3K9 or pH3S10 & β-EP

Five brains from each treatment groups were cryosectioned at 20 μm in thickness and sections were placed on a prechilled slide (Superfrost plus; VWR). Brain sections were collected from plate 19 to plate 23 of the sterotaxic atlas (Paxinos and Watson, 1982) to cover the whole arcuate area of the hypothalamus, and every fifth section was used for staining peptide. Each one of these sections represents one plate in the stereotaxic atlas. A total of 5 sections of the entire arcuate area were used for each per protein determination. Brain sections were double-stained for the following antibodies; di or trimethlated H3K4 (H3K4me2,3) (1:500), dimethylated H3K9 (H3K9me2) (1:500), acetylated H3K9 (AceH3K9) (1:500), phosphorylated H3 at serine 10 (pH3S10) (1:500), methyl-CpG-binding protein (MeCP2) (1:500) and for β-EP (1:200). β-EP antibody was raised in rabbit (Bachem, San Carlos, CA). Other primary antibodies were monoclonal and raised in mouse (Abcam, Cambridge, MA). Secondary antibodies used in this study were Alexafluor 488 donkey anti-mouse (2 mg/ml; Invitrogen; NY) and AlexaFluor594 donkey anti-rabbit IgG (2 mg/ml; Invitrogen). We have also used Dnmt1 (1:100; Santa Cruz Biotechnology; CA) and Dnmt3a (1:100; Santa Cruz) antibodies raised in goat for double staining with b-EP antibody. For fluorescence labeling the goat antibodies, we used AlexaFluor488 donkey anti-goat IgG (H +L) (1:1000). Specificity of each of the primary antibodies was verified by incubating slides with excess peptide matching the primary antibody. The background fluorescence reaction was tested by incubating tissue sections without the primary antibodies and staining with the secondary antibodies. After staining, slides were mounted in DAPI (Vector Laboratories, CA) and covered with a 1mm thick coverslip (VWR). Pictures were taken on the same day using confocal microscopy and 20× objective lens (Nikon EZ-C1 3.60 build 770, Gold version). Total number of β-EP cells as well as total number of β-EP cells, located on the right and left side of the third ventricle, that are positive for H3K4me2,3, H3K9me2, AceH3K9, pH3S10, Dnmt1, Dnmt3a or MeCP2 were counted. The experimenters were blind to the experimental treatment group of the section during counting. Pictures were taken at 200 × magnification.

Quantitation of gene expression of histone-modifying enzymes and DNA-methylating enzymes in the mediobasal hypothalamus by quantitative Real-time PCR

Mediobasal hypothalami were aseptically removed from the brains of experimental rats, 60–65 days old. Total RNA was extracted using Micro to Midi kit with Trizol (Invitrogen). Total RNA in each sample was quantitated and assessed for quality using the NanoDrop −1000 version 3.7 (Thermo Scientific). A ratio of A_{260}/A_{280} of ~2.0 was obtained for all RNA samples. Before RT-PCR, the RNA was treated with DNase (Qiagen, Valencia, CA) then stored in 25 μl of ultra pure DNase/RNase-free distilled water (Invitrogen). 1000 ng/μl of total RNA from each sample was converted to cDNA using GeneAmp PCR System 9700 (ABI) and using cDNA high capacity RT (ABI) and nuclease free H2O. RT-PCR conditions were 25°C for 10 minutes, 37°C for 60 minutes, 37°C for 60 minutes, 85°C for 5 minutes then kept at 4°C. After the reverse transcription reaction, qRT-PCR was performed with a total volume of 25 ml of reaction mixture. A typical reaction contains 2.5 ml of diluted cDNA, and 22.5 ml of Universal master mix, primers and nuclease free H2O. PCR conditions were 50°C for 2 mins for 1 cycle, 95°C for 10 mins, 1 cycle, 95°C for 15 secs, 40 cycles, and 60°C for 1 min, 1 cycle. qRT-PCR was performed using the ABI prism 7500 HT

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sequence detection system. GAPDH was used as the housekeeping gene. The ratio of mean quantity of gene of interest to the mean quantity of GAPDH was compared between different groups. All samples were run in duplicates and non-template controls (NTCs) were used in each run. All TaqMan primers and probes were designed by Applied Biosystem (Foster City, CA). All gene expression assays have a FAM reporter dye at the 5' end of the TaqMan MGB probe and a nonfluorescent quencher at the 3' end of the probe.

Determination of POMC gene promoter methylation by SYBR green methylation-specific (MSP) real-time PCR

DNA was extracted from the mediobasal hypothalamus (MBH) of experimental rats using the DNeasy Blood & Tissue kit and following the protocol of Qiagen (Valencia, CA). 25 mg of hypothalamic tissues was homogenized then kept in lysis buffer with 20 μl proteinase K at 56°C overnight. RNAase A (100 μg/ml, Qiagen) was added. DNA was eluted in DNase/RNAse – free H_2O, quantitated using the NanoDrop then stored at −20°C for later use. 1.5 μg of DNA extracted from each sample was treated with sodium bisulfite and converted using the EZ DNA methylation Kit protocol (Zymo Research, Orange, CA). The PCR primers were designed using the Methyl Primer Express program, version 1.0 (Applied Biosystems, Foster City, CA) or MethPrimer program (http://www.urogene.org/methprimer/index1.html) and manufactured by Sigma. The sequences of the oligos are: Methylated-5' CGTTTTAGCGGGTTTGTGTTAAC 3', forward-5' CTACAACGCAACAAACGAATCC 3', reverse-5' CGATCGGGAAGTT 3' probe; Unmethylated-5'GTGTTTTAGTGGGTTTGTGTTAATGTTAG 3', forward-5' ACTTCTACAACACAACAAACAAATCCC 3', reverse-5' GTTTTTGTATTTTTTAGGTATATTTG3' Probe. Primers were designed to be “methylation-specific” or “unmethylation-specific” with respect to the particular cytosine nucleotide in the CpG pair under analysis in POMC gene promoter. The ratios of the methylation-specific to unmethylation-specific responses were quantified by ΔCt method. A total mix of 25 μl containing the converted DNA, Syber green mix, DNAse/RNAase free water and either the methylated or unmethylated reverse and forward primers were prepared. Rat high methylated and rat low methylated DNA controls (EpigenDx, Worcester, MA) were also bisulfite converted and used for the preparation of the standard curve. The run was conducted as follows: 50°C for 2 mins 1 cycle, 95°C for 10 mins 1 cycle, 95°C for 15 secs, 58°C for 1 min and 72°C for 15 secs for 50 cycles. The dissociation stage is 95°C for 15 sec and 60°C for 1 min 1 cycle. Run for each sample was done in duplicates and non-template controls (NTCs) were used.

Determination of plasma corticosterone levels by ELISA

Rats were injected intraperitoneally with LPS (100 μg/kg body; Sigma) in saline or saline alone. After 2 hrs, trunk blood samples were collected in tubes containing ethylenediamintetracetic acid (EDTA) and centrifuged at 2,000 rpm for 15 minutes to prepare plasma samples. Plasma samples were used for determination of corticosterone levels using the rat corticosterone enzyme immunoassay following the manufacturer's instructions (Diagnostic Systems Laboratories, Brea, CA).

Statistical analysis

Statistical analysis of data was performed using Graph Pad Prism software version 4.0 (LA Jolla, CA). For immunohistochemistry, qRT-PCR and ELISA data, the mean values were calculated and analyzed between all groups using one-way analysis of variance (ANOVA) with Student Neuman-Keuls post hoc test. All results are presented as standard error of the mean (SEM). $P<0.05$ was considered as significant.

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RESULTS

Effects of gestational choline on protein levels of histone-modifying enzymes, DNA methylating enzymes, methylbinding protein MeCP2 and β-EP in neurons producing β-EP in the hypothalamus

We assessed the effects of maternal nutrient supplementation with choline upon fetal alcohol exposure on protein levels of histone-modifying enzymes and DNA-methylating enzymes in β-EP neurons of the hypothalamus of AF and controls (PF and AD) offspring during the adult period. We found that fetal alcohol exposure reduced the activation mark H3K4me2,3 methylation as demonstrated by showing a reduced number of β-EP neurons positive for this mark, when compared to PF and AD ($F=4.88$; dfs=4, 20; $P<0.05$) (Fig. 1A & B).). On the other hand, fetal alcohol exposure increased the level of the repressive mark, H3K9me2 in β-EP neurons as compared to AD ($F=4.88$; dfs=4, 20; $P<0.01$) and PF rats ($F=4.88$; dfs=4, 20; $P<0.05$). Gestational choline supplementation normalized the methylation levels of H3K4me2,3 ($F=4.88$; dfs=4, 20; $P<0.01$) and H3K9me2 ($F=11.71$; dfs=4, 20; $P<0.05$) to levels comparable to that of controls (Fig. 1A–D). We also determined the effects of fetal alcohol exposure on other activation marks such as acetylated H3K9 (Fig. 1 E & F) and phosphorylated H3S10 (Fig. 1G & H). We found that both marks were reduced in AF rats compared to AD and PF rats. Fetal alcohol reduced the levels of these histone marks in AF rats compared to AD (AceH3K9; $F=5.390$; dfs=4, 20; $P<0.05$), (pH3S10; $F=9.326$; dfs=4, 20; $P<0.01$) and PF rats (AceH3K9; $F=5.390$; dfs=4, 20; $P<0.05$),(pH3S10; $F=9.326$; dfs=4, 20; $P<0.05$). Unlike H3K4me2,3 and H3K9me2, gestational choline supplementation did not reverse alcohol effects on AceH3K9 or pH3S10 in CAF compared to AF rats ($P>0.05$).

We also determined the effects of fetal alcohol exposure with or without choline supplementation on protein levels of key enzymes regulating DNA methylation in β-EP neurons such as Dnmt1 and Dnmt3a and on protein levels of the methyl-CpG-binding protein (MeCP2). As shown in Fig. 2, fetal alcohol exposure increased the protein levels of both Dnmt1 and Dnmt3a as compared to AD (Dnmt1; $F=7.768$; dfs=4, 20; $P<0.01$), (Dnmt3a; $F= 4.719$; dfs=4, 20; $P<0.05$) and PF rats (Dnmt1; $F=7.768$; dfs=4, 20; $P<0.001$), (Dnmt3a; $F=4.719$; dfs=4, 20; $P<0.01$).. Choline supplementation completely reversed alcohol effects on proteins levels of Dnmt1, but not Dnmt3a, in CAF rats compared to AF ($F=7.768$; dfs=4, 20; $P<0.01$) (Fig. 2A–D). Similarly, gestational choline supplementation normalized MeCP2 levels in β-EP neurons of CAF rats ($F=3.249$; dfs=4, 20; $P<0.05$) (Fig. 2E & F).

To assess the physiological consequences of supplemental gestational choline, we compared the total number of β-EP neurons between different groups in the adult stage. Fetal alcohol exposure decreased β-EP immunoreactivity in AF rats compared to controls ($F=6.618$: dfs=4, 53; $P<0.05$). Choline treatment normalized β-EP count to a level comparable to that of controls ($F=6.618$; df=4, 53; $P<0.001$) (Fig 2G & H).

Effects of gestational choline on gene expression of histone-modifying enzymes, DNA methylating enzymes and methylbinding protein MeCP2 in the mediobasal hypothalamus

We next determined the effects of fetal alcohol exposure with or without gestational choline supplementation on mRNA levels of Set domain histone lysine methyltransferase (Set7/9) that catalyzes the methylation of H3K4, G9a and histone-lysine methyltransferase Set domain bifurcated 1 (Setdb1) that regulate methylation of H3K9. We also determined the mRNA levels of Dnmt1, Dnmt3a, and MeCP2 in adult male rat offspring. Fetal alcohol exposure decreased the mRNA levels of Set7/9 ($F=6.084$; dfs=4, 53; $P<0.05$) and increased the mRNA level of G9a ($F=18.96$, f=4, $P<0.01$) and Setdb1 ($F=11.07$; dfs=4, 53; $P<0.05$) (Fig. 3A–C). Choline supplementation reversed the alcohol effect on all these genes in CAF

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rats compared to AF (Set7/9; F=6.084; dfs=4, 53; P<0.05); (G9a; F= 18.96, ; dfs=4, 45; P<0.001), (Setdb1; F=11.07; dfs=4, 53; P<0.001). Choline treatment also suppressed G9a mRNA levels in CPF rats compared to AD (F= 18.96; dfs=4, 45; P<0.01) and PF rats (F= 18.96; dfs=4, 53; P<0.001).. In addition to its effect on histone methylating genes, fetal alcohol exposure also increased mRNA levels of the Dnmt1 (F=11.07; dfs=4, 53; P<0.05) but not Dnmt3a (F=10.65; dfs=4, 57; P>0.05) (Fig. 3D,E).. It also increased mRNA levels of MeCP2 (F=14.68; dfs=4, 45; P<0.01) (Fig. 3F). Choline supplementation normalized Dnmt1 and MeCP2 levels in AF (Dnmt1; P<0.05, MeCP2; P<0.001) and CAF rats (Dnmt1; P<0.001, MeCP2; P<0.01) compared to AF and controls, but it increased Dnmt3a level in both CAF (F=10.65; dfs=4, 57; P<0.01) and CPF rats (F=10.65, ; dfs=4, 57; P<0.001) compared to AD and PF rats.

Effects of gestational choline on POMC gene promoter methylation and POMC mRNA expression in the hypothalamus

We determined the effects of gestational choline supplementation during the period of alcohol exposure on the changes in methylation status of POMC gene promoter and POMC gene expression. In order to characterize the extent of cytosine methylation of CpG dinucleotides in the 5' CpG island of POMC gene promoter, we designed one set of primers specific to either the methylated or the unmethylated state of the CpG sites adjacent to the POMC gene transcription start site. TaqMan methylation-specific real-time PCR, with the probes derived from the sequence in the region −81 to −154, identified significant increase in cytosine methylation between AF and control animals (Fig. 4A). Choline supplementation normalized fetal alcohol effect on methylation status of these two sites in POMC gene promoter in AF rats. Interestingly, these CpG sites reside in the binding site of transcription factors essential of transcriptional activation. Normalization of POMC gene promoter methylation with choline supplementation correlated with normalization of POMC mRNA levels in the hypothalamus of CAF rats compared to AD, PF (P<0.01) and CAF (P<0.001) (F=9.122; dfs=4, 53) (Fig. 4B). Overall, our data indicate that fetal alcohol exposure resulted in POMC gene hypermethylation in adulthood and gestational choline normalized POMC gene promoter methylation and POMC gene expression in adult male rats.

Effects of gestational choline on plasma corticosterone levels

We determined the basal and LPS-induced corticosterone levels in choline supplemented (CAF and CPF) or non-supplemented alcohol-fed (AF) and control-fed rats (AD and PF). Gestational choline attenuated the stress hyperresponsiveness in AF rats (Fig. 5), suggesting the possibility that normalization of POMC gene methylation and expression resulted in the normalization of β-EP peptide production from POMC.

DISCUSSION

In this study we determined whether gestational choline could counteract fetal alcohol effects on histone marks and DNA methylation and normalize POMC gene methylation and expression in β-EP-producing POMC neurons. We showed here that gestational choline supplementation altered the expression patterns of various histone modifying genes and DNA methylating genes and normalized fetal alcohol-altered POMC gene expression and β-EP production in the hypothalamus in the adult stage. Choline attenuated alcohol effects on the activation mark H3K4me2,3 and the repressive mark H3K9me2. Choline also reversed alcohol repression of Set7/9 expression that controls H3K4 methylation. Choline suppressed alcohol activational effect on G9a and Setdb1 that regulate H3K9 methylation. The nutrient also normalized fetal alcohol induced changes in mRNA and protein levels of Dnmt1 and MeCP2 and increased Dnmt3a expression. Additionally, it normalized fetal alcohol-induced abnormalities in POMC gene methylation, POMC mRNA expression and β-EP peptide

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production. Choline treatment also reduced corticosterone hyperresponse to LPS in fetal alcohol exposed rats. These results suggest that choline supplementation was able to prevent alcohol-induced epigenetic modifications of POMC gene leading to normalization of POMC gene expression and its control of the stress axis function.

Alcohol ingestion is known to inhibit folic acid absorption and reduces methionine synthase ability to convert homocysteine to methionine and SAM that are critical for methylation processes during development (Wang et al., 2009). Fetal alcohol exposure is also known to cause DNA hypomethylation (Garro et al., 1991). Hence, the possibility is raised that alcohol-induced DNA hypomethylation is induced by low SAM availability and possibly by a deficiency of one of its methyl donors, choline, and could be compensated by supplementation of choline. Behavioral deficits and hyperresponses to stress have been reported in rodents which were exposed to alcohol during embryonic development (Berman and Hannigan, 2000; Boyadjieva et al., 2009; Govorko et al., 2012; Hellemans et al., 2008). So, we determined whether gestational choline supplementation could mitigate alcohol effects on the stress axis by altering the expression of one of its regulators POMC gene by modulating histone marks and DNA methylation in this gene. We showed here that gestational choline supplementation modified the levels of methylation marks H3K4me2,3 and H3K9me2, altered the expression of histone modifying enzymes such as Set7/9, G9a and Setdb1 and altered the expression of Dnmt1 and MeCP2 in the mediobasal hypothalamus where the POMC neurons are localized and/or in POMC neurons. These changes in histone modification and DNA methylation were associated with normalization of fetal alcohol altered POMC gene methylation and expression. Although these findings are correlative in nature, yet they indicate that methylation status in POMC neurons was altered by alcohol exposure that was normalized following choline supplementation. Choline deficiency has previously been shown to alter the activity of G9a in neural progenitor cells. It decreased H3K9me1,2 levels and increased the expression of genes involved in neurogenesis in mice fetal hippocampus (Mehedint et al., 2010). In our study, we found that gestational choline increased H3K4me2,3 methylation, decreased H3K9me2 methylation in β-EP-producing POMC neurons in AF rats as compared to AD and PF rats. Gestational choline did not significantly alter alcohol effects on other histone marks such as H3K9 acetylation or H3S10 phosphorylation. This could be explained by the fact that histone-modifying enzymes that acetylate H3K9 or phosphorylate H3S10 do not require methyl group for their activity. It could also suggest that other factors and/or their complex interactions with chromatin or chromatin-modifying factors could be involved.

Substantial evidence has been accumulated in the recent years that there is an interplay between histone modifications and DNA methylation in regulation of gene expression. For example, H3K9 methylation affects DNA methylation by recruiting methyl-binding proteins such as MBDs or MeCP2 and other chromatin-modifying factors to the promoter of a specific gene resulting in gene repression (Guibert et al., 2009; Vaissiere et al., 2008). MeCP2 is abundantly expressed in the hypothalamus and acts both as a repressor or an activator of gene expression (Chahrour et al., 2008). It has also been implicated in the regulation of stress and behavior (Fyffe et al., 2008). In our study, we found that gestational choline normalized the alcohol-induced changes in protein and gene levels of histone-modifying enzymes, DNA-methylating enzymes and the methylbinding protein MeCP2 in the mediobasal hypothalamus except for Dnmt3a. Dnmt3a gene is located very close to POMC gene on chromosome 6. Govorko et al. (2012) demonstrated that fetal alcohol exposure did not alter the methylation status of Dnmt3a promoter compared to POMC gene promoter in the hypothalamus. Thess data support our Dnmt3a gene expression data where the later was unaffected by alcohol exposure. This suggests that fetal alcohol effect on POMC neurons is gene specific. The upregulation of Dnmt3a expression in CAF and CPF rats is interesting. This upregulation could be explained by the necessity of Dnmt3a activity

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in the adult stage for de novo methylation that is required for normal cellular functioning in response to alcohol exposure. Another possibility is that the observed upregulation of Dnmt3a expression could be an adaptive response of the system to the abundance of the nutrient choline. The quantitative real-time-PCR analysis confirmed a decrease in the expression of Dnmt1 in the mediobasal hypothalamus by choline supplementation. It also reduced the expression of G9a and Setdb1 that methylate the repressive mark H3K9. These data indicate that choline supplementation has the ability to normalize ethanol-induced changes in histone-modifying enzymes and DNA-methylating enzymes in POMC neurons.

In a MeCP2 knockout mouse model, gestational choline supplementation attenuated some of the behavioral and anatomical changes that are observed in a mutant mouse model of Rett Syndrome (Ward et al., 2008; Zeisel, 2011). In our study, MeCP2 gene expression was decreased upon choline supplementation and this decrease correlated with normalization of POMC gene expression and β-EP peptide production. Further studies are needed to determine whether MeCP2 binds to POMC gene promoter and whether this binding regulates POMC gene expression.

Histone modifications and DNA methylation are key epigenetic mechanisms that are essential for neuronal development and gene expression regulation (Izzo and Schneider, 2011; Robertson, 2002). The changes in histone methylation that we found in β-EP-producing POMC neurons positively correlated with changes in Dnmt1 expression but negatively correlated with changes in POMC gene expression in the mediobasal hypothalamus. Kovacheva et al. (2007) demonstrated that choline-supplemented embryos had an attenuated expression of Dnmt1 in the brain with a hypermethylation of its promoter. In our study, fetal alcohol exposure modulated histone and DNA methylation, and choline reversed alcohol effect. Although these findings are correlative in nature, they could suggest that choline has a beneficial effect in attenuating alcohol adverse effects on several parameters related to histone and DNA methylation in POMC neurons. Possibly, fetal hypomethylation upregulated the expression and activity of histone-modifying enzymes and DNA-methylating enzymes in these neurons. As a compensatory mechanism, this upregulation resulted in a state of hypermethylation in POMC neurons of adult exposed offspring. Thus, gestational choline supplementation from GD11–21 normalized the methylation status and prevented hypomethylation in the fetus and hypermethylation in the adult stage.

Several studies demonstrated that gestational choline attenuates prenatal and postnatal effects of alcohol exposure and induces long-lasting beneficial effects on behavior, memory, attention and learning in the adult exposed offspring (Thomas et al., 2000; 2007; 2010). Moreover, it plays a critical role in modulating histone and DNA methylation in the brain (Mehedint et al., 2010). In the context of POMC system, fetal alcohol exposure has been shown to decrease the expression and function of this gene. (Kuhn and Sarkar, 2008; Sarkar et al., 2007). The normalization of POMC gene expression that we detected upon choline supplementation paralleled the normalization of POMC gene promoter methylation in the hypothalamus. These data suggest that choline, as a major methyl group for the formation of SAM, compensated the lack of CH3 induced by fetal alcohol exposures and normalized POMC gene promoter methylation, POMC gene expression and β-EP peptide production.

It has been shown previously that transplantation of β-EP neurons into the paraventricular nucleus of the hypothalamus or administration of the opioid peptide β-EP into the third ventricle reduces the LPS- increased plasma corticosterone levels in fetal alcohol exposed animals (Boyadjieva et al., 2009). Hence, we rationalized that if choline supplementation normalizes POMC gene expression and β-EP production, it may normalize the LPS-induced corticosterone levels in fetal alcohol exposed animals and attenuate the adverse

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ramifications of β-EP peptide reduction on the stress axis. The decrease in corticosterone levels that we found in male rats upon choline supplementation could suggest that choline attenuated the adverse effect of alcohol on POMC gene expression. This normalization of POMC gene expression restore the inhibitory effect of its derived peptide β-EP on the stress axis resulting in attenuation of the corticosterone hyperresponsiveness in response to immune challenge in alcohol-exposed rats.

In conclusion, our study is the first to report that gestational choline altered alcohol-induced epigenetic changes such as histone marks in β-EP-producing POMC neurons and DNA methylation in hypothalamic POMC gene promoter of the adult offspring and normalized the stress response to immune challenge in alcohol-exposed rats. It would be important to investigate in the future the behavioral aspect of choline supplementation in AF rats. Further studies are also needed to confirm that choline is a safe choice to mitigate the effects of fetal alcohol exposure on the regulation of stress axis in the adult stage.

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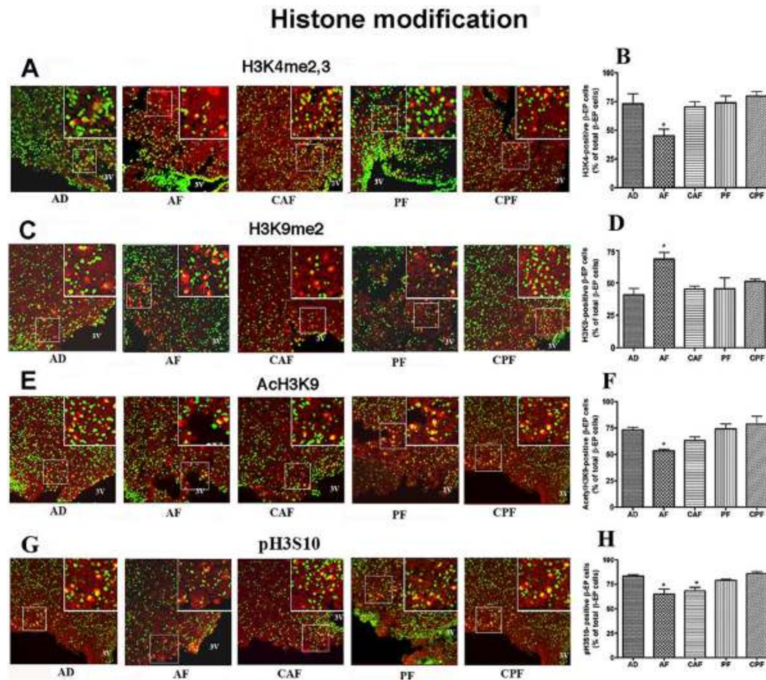

Figure 1.

Effects of gestational choline with or without ethanol on histone modifying enzyme proteins levels in β-endorphin (β-EP) neurons in the arcuate nucleus of the hypothalamus of male PND60 offspring. Pregnant rats were fed *ad lib* rat chow (AD), alcohol-containing liquid diet (AF) or alcohol and choline-containing liquid fed (CAF), or pair-fed isocaloric liquid diet without (PF) or with choline (CPF). Histone modifying enzyme proteins in β-EP neurons are measured using double-labeled Immunohistochemistry. Changes in the number of β-EP cell-positive to H3K4m2,3 (**A, B**), H3K9me2 (**C, D**), AcetylH3K9 (**E,F**) and pH3S10 (**G,H**) in the hypothalamus (histone proteins shown in green and β-EP shown in red). Representative photographs show the double-labeled cells (**A, C, E & G;** red and green combined), in each treatment group, and histograms (**B, D, F & H**) show the mean ± SEM values of the percentage of β-EP cells that were double-labeled. Magnification 200×. N = 5. H3K4me2,3 (*$p < 0.05$, compared to the rest). H3K9me2 (*$p<0.05$, compared to the rest). AceH3K9 (*$p<0.05$, compared to rest except CAF). pH3S10 (*$p<0.05$, compared to the rest except AF or CAF). Data were analyzed using one-way analysis of variance (ANOVA) followed by Student Neuman-Keuls post-hoc test.

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Figure 2.

Effects of gestational choline with or without ethanol on the protein level of DNA methyltransferases, MeCP2 and β-EP in cells containing β-EP in the arcuate nucleus of the hypothalamus of male PND60 offspring treated similarly as in Fig. 1. Changes in the number of β-EP neurons positive to Dnmt1 (**A, B**), Dnmt3a (**C, D**), MeCP2 (**E,F**) and the number of β-EP neurons **(G,H)** in the hypothalamus (Dnmts and MeCP2 shown in green and β-EP shown in red). Representative photographs show the double-labeled cells (**A, C, E & G**), indicated by arrows, in each treatment group, and histograms (**B, D, F & H**) show the mean ± SEM values of the percentage of β-EP cells that were double-labeled. N = 5. Dnmt1 (**p* <0.05, compared to the rest). Dnmt3a, (**p*<0.05, AF compared to AD and PF;[a], p<0.05, PF compared to CPF). MeCP2 (**p*<0.05, compared to the rest). β-EP (**p* <0.05, compared to the rest;[a], p<0.05, compared to AD). Data were analyzed using one-way ANOVA followed by Student Neuman-Keuls post hoc test.

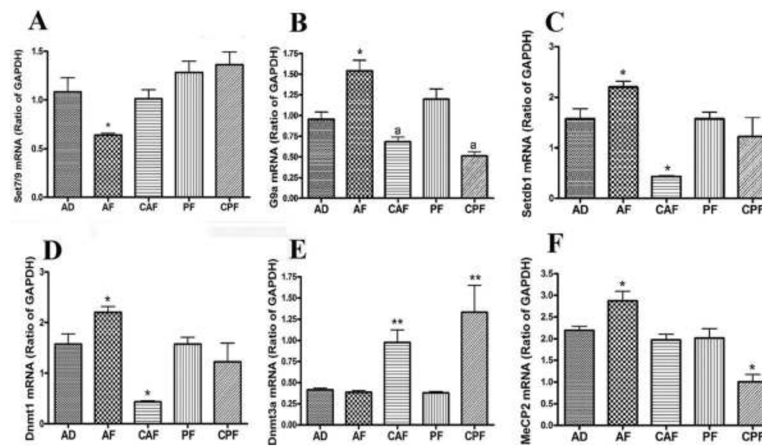

Figure 3.

Effects of gestational choline with or without ethanol on the mRNA level of histone-modifying enzymes, DNA methylating enzymes and methylbinding protein MeCP2 gene in the mediobasal hypothalamus of male PND60 offspring treated similarly as in Fig. 1. Changes in mRNA levels of Set7/9 **(A),** G9a (B), Setdb1 (C), Dnmt1 (**D**), Dnmt3a (**E**) and MeCP2 (**F**). Data presented are mean ± SEM. N=5–9. Set7/9 (*p <0.05, compared to the rest). G9a (*p<0.05, compared to the rest;[a], p<0.05, compared to the rest except CAF or CPF). Setdb1 (*p<0.05, compared to the rest). Dnmt1 (*p<0.05, compared to the rest). Dnmt3a (**p<0.01, compared to the rest except CPF or CAF). MeCP2 (*p<0.05, compared to the rest). Data were analyzed using one-way ANOVA followed by Student Neuman-Keuls post hoc test.

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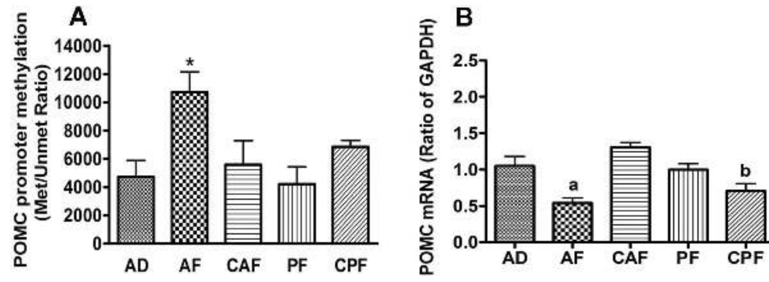

Figure 4.

Effects of gestational choline on methylation of POMC gene promoter (A) and POMC mRNA levels (B) in the mediobasal hypothalamus of male PND60 offspring treated similarly as in Fig. 1. Rats were injected intraperitoneally with LPS (100 μg/kg body) in saline or saline alone 2 hrs prior to blood sampling for corticosterone measurements. Data presented are mean ± SEM. N = 7–8. *$p<0.05$, compared to the rest.[a], p<0.05, compared to the rest except CPF.[b], $p<0.05$, compared to the rest AF. Data were analyzed using one-way ANOVA followed by Student Neuman-Keuls post hoc test.

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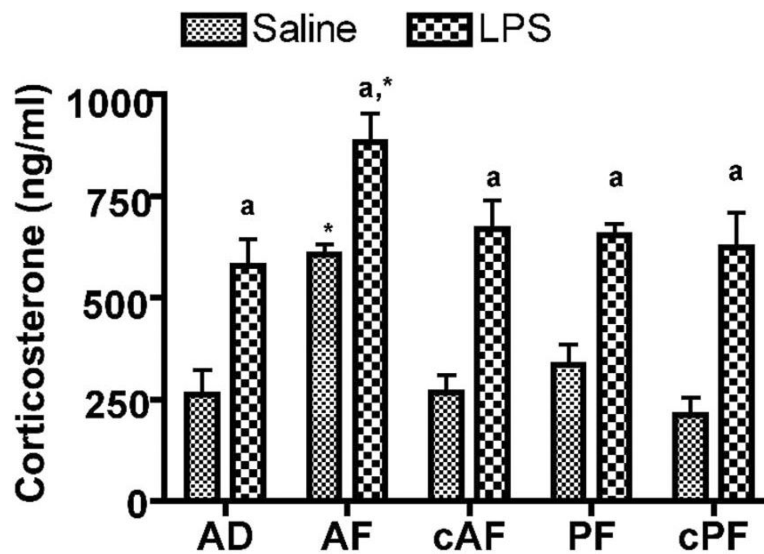

Figure 5.

Effects of gestational choline on basal (saline) and lipopolysaccharide (LPS)-stimulated corticosterone levels in plasma of male PND60 offspring treated similarly as in Fig. 1. Data presented are mean ± SEM. N = 7–8. *$p<0.05$, compared to the rest.[a], $p<0.05$, compared with similarly treated control. Data were analyzed using one-way analysis of variance (ANOVA) with Student Neuman-Keuls post hoc test.

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Table 1

Changes in protein levels of histone-modifying enzymes, DNA mcthylating enzymes and methylbinding protein MeCP2 in β-endorphin positive cells

Target proteins	AD	AF	PF	CAF	CPF
H3K4me2,3	0	↓	0	0	0
H3K9me2	0	↑	0	0	0
AceH3K9	0	↓	0	0	0
pH3S10	0	↓	0	↓	0
Dnmt1	0	↑	0	0	0
Dnmt3a	0	↑	0	0	↑*
MeCP2	0	↑	0	0	0

Arrows shows no change (0), increase (↓) or decrease (↓) levels compared to AD (↓ or ↑) or PF(↑ *)

H3K4me2,3 = Trimethylated histone H3 at lysine 2 and 3 (activation mark)

H3K9me2 = Dimethylated histone H3 at lysine 2 (repressive mark)

AceH3K9 = acetylated histone H3 at lysine 9 (activation mark)

pH3S10= phosphorylated histone H3 at serine 10 (activation mark)

Dnmt1 = Maintenance methyltransferase (DNA methylation)

Dnmt3a= De novo methyltransferase (DNA methylation)

MeCP2 = Methyl-CpG-binding protein 2 (Transcriptional repression or activation)

Set7/9 = Set containing domain lysine methyltransferase (modify H3K4me1, me2, me3; Transcriptional repression)

G9a = Lysine methyltransferase (modify H3K4me1, me2; Transcriptional repression)

Setdb1 = Set containing domain lysine methyltransferase (modify H3K4me1, me2, me3; Transcriptional repression)