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Alcohol exposure during development: Impact on the epigenome

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Abstract

Fetal Alcohol Spectrum Disorders represent a wide range of symptoms associated with *in utero* alcohol exposure. Animal models of FASD have been useful in determining the specific neurological consequences of developmental alcohol exposure, but the mechanisms of those consequences are unclear. Long-lasting changes to the epigenome are proposed as a mechanism of alcohol-induced teratogenesis in the hippocampus. The current study utilized a three-trimester rodent model of FASD to examine changes to some of the enzymatic regulators of the epigenome in adolescence. Combined pre- and post-natal alcohol exposure resulted in a significant increase in DNA methyltransferase activity (DNMT), without affecting histone deacetylase activity (HDAC). Developmental alcohol exposure also caused a change in gene expression of regulators of the epigenome, in particular, DNMT1, DNMT3a, and methyl CpG binding protein 2 (MeCP2). The modifications of the activity and expression of epigenetic regulators in the hippocampus of rodents perinatally exposed to alcohol suggest that alcohol's impact on the epigenome and its regulators may be one of the underlying mechanisms of alcohol teratogenesis.

Keywords

Fetal Alcohol Spectrum Disorders; Epigenetics; Hippocampus

Introduction

Fetal alcohol spectrum disorders (FASD) is an umbrella term associated with the effects of *in utero* alcohol exposure, including Fetal Alcohol Syndrome (FAS) at the more severe end of the spectrum, and alcohol-related neurodevelopmental disorders (ARND) at the less severe end (Riley et al., 2011; Sampson et al., 1997). Brain damage as a result of *in utero* alcohol exposure has been studied extensively in both humans (see Riley et al., 2011, for review) and by using animal models (see Kelly et al., 2009 for a review). Neuroimaging studies in humans with FASD have demonstrated reduced volumes in the cortex,

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cerebellum, and basal ganglia (Foltran et al., 2011), along with altered functioning in a number of tasks that require inhibitory control, spatial working memory, and verbal working memory (Coles & Li, 2011).

In rodent models, the hippocampus has been shown to be particularly susceptible to alcohol-induced damage and the damage includes decreased cell number (Barnes & Walker, 1981; Bonthius & West, 1990; Bonthius & West, 1991; Miki et al., 2000; Tran & Kelly, 2003), decreased dendritic spine density (Berman et al., 1996; Tarello-Acuna et al., 2000; West, 1990), decreased neurogenesis (Hamilton et al., 2011; Klintsova et al., 2007) and changes in the electrophysiological properties of neurons (Hablitz, 1986; Swartzwelder et al., 1988; Tan et al., 1990). Importantly, behavioral deficits are often seen in tasks which require the hippocampus, indicating that the neurological changes noted above may have a functional impact. These findings suggest that the hippocampus may be very useful as a target brain region to understand the mechanism of alcohol-induced deficits in the developing brain.

Epigenetics refers to the process by which environmental events can impact gene expression. Epigenetic changes are a result of the actions of several enzymes that alter the way in which DNA and its associated proteins interact. DNA is wrapped around histone proteins, and this structure is referred to as chromatin. Chromatin structure can be dynamically regulated through protein-protein interactions (Tsankova et al., 2007). There are three main categories of epigenetic modifications: DNA methylation, chromatin modifications, and non-coding RNA expression (Hu, Wang, & Shen, 2012).

DNA methylation is the process by which methyl groups are added to CpG islands on DNA, reducing gene expression by blocking transcription factor binding, and is accomplished through the activity of DNA methyltransferase (DNMT) enzymes (Kiefer, 2007; Robertson, 2005). There are several types of DNMT enzymes, each performing a specific role in the developing brain. For example, DNMT1 is termed the “maintenance” methyltransferase, and is responsible for methylating hemi-methylated DNA during replication, while DNMT3a is responsible for *de novo* methylation (Robertson, 2002). DNMT1 is highly expressed in the mature brain even though there is little cell division. This suggests that DNMT1 is involved in processes besides the maintenance of the genome during replication (Robertson, 2002; Turek-Plawa & Jagodzinski, 2005). Conditional deletion of DNMT1 and DNMT3a causes hypomethylation in the DNA of neurons, leading to impaired long-term potentiation and synaptic function (Feng et al., 2010). Thus, disrupted DNMT activity, or expression, following early alcohol exposure, would lead to altered synaptic function.

Histone modifications (e.g. acetylation, methylation, phosphorylation) lead to changes in the chromatin structure which affect the ability of transcription factors to access the DNA (Tsankova et al., 2007). There are multiple forms of histone modifications (e.g. acetylation, methylation) and these modifications can occur on different histone tails and at specific amino acid residues, resulting in a complex mechanism through which gene expression can be regulated (Grant, 2001; Turek-Plewa & Jagodzinski, 2005). For example, on histone H3, lysines (K) 9, 14, 18, and 23 are most commonly regulated by acetylation/deacetylation, while on H4, acetylation occurs at lysines 5, 8, 12, and 16 (Grant, 2001; Turek-Plewa & Jagodzinski, 2005). Methylation is most likely to occur on H3K4, 9, and 27, and on H4K20. Histone tails can also be di- and tri-methylated (Grant, 2001; Turek-Plewa & Jagodzinski, 2005). Unlike DNA methylation, histone methylation is not always associated with transcriptional silencing. For example, H3K4 methylation results in increased gene transcription (Turek-Plewa & Jagodzinski, 2005). Histone acetylation occurs via the activity of histone acetyltransferases (HATs), and deacetylation is the result of histone deacetylases (HDACs). Histone acetylation leads to expansion of the chromatin structure, allowing transcription factors to access DNA. Conversely, histone deacetylation leads to a compact

chromatin structure, preventing transcription factor binding (Tsankova et al., 2007). There is incredible complexity in the regulation of the epigenome and recent research suggests that histone modifications and DNA methylation can interact (Bachman et al., 2001; Cameron et al., 1999; Hansen et al., 2010).

Development of the nervous system is a highly complex process, in which epigenetics plays an important role (Singh et al., 2009). Epigenetic regulation of imprinting control regions has recently become an area of great interest. Imprinted genes regulate fetal development and expression of these genes is modulated by DNA methylation (Haycock, 2009). Methylation occurs on either the paternal or maternal allele, leaving only one allele to be expressed. There are regions that are differentially methylated throughout development, and others that show tissue-specific differential methylation patterns (Haycock & Ramsay, 2009). Cell differentiation (e.g. hippocampal pyramidal vs. granule cell) occurs as a result of tissue-specific methylation patterns. Importantly, methylation of these imprinting control regions is affected by pre-conception (Knezovich & Ramsay, 2012) and pre-implantation (Haycock and Ramsay, 2009) alcohol exposure.

Using whole embryo culture, Liu et al. (2009) demonstrated that ethanol exposure at early neurulation caused both hyper- and hypo- methylation of specific genes, and these changes were more severe in embryos whose neural tube had failed to close. Alcohol exposure altered methylation of genes involved in neural and glial development, which would significantly alter nervous system development, and potentially impact adult nervous system function. There was no examination of tissue-specific epigenetic changes caused by alcohol exposure in this particular study.

Alcohol exposure during development has been shown to impact cell cycle regulation and nervous system growth. Hicks et al. (2010) used neural stem cells to examine the effects of ethanol on the expression of cell cycle genes, and found that ethanol increased the length of the cell cycle, and this effect was likely mediated through increased DNA methylation of cell cycle genes. Disruptions in cell cycle gene expression in development, as well as in adulthood in proliferative zones, can have a long-lasting impact on cell number, and may explain the reduction in hippocampal cell number observed in FASD models (e.g., Tran & Kelly, 2003). In another study, Zhou et al. (2011a, 2011b) examined whole embryos for changes in gene expression after alcohol exposure, and found that there was a reduction in the expression of genes related to neural development (e.g. *Ngf1*, *Ngf2*, *Sox 5*, *Sox 7*), neural growth factors (*Igf1p2*, *Efemp1*), and cell cycle regulation (*Clk1*, *Clk4*, *Ndr1*). Additionally, acute exposure to ethanol interfered with neural differentiation, locking cells in an undifferentiated state (Zhou et al., 2011). Overall, it is apparent that ethanol exposure early in development can lead to an altered methylation state, and can induce differential expression of important developmentally regulated genes. However, very few studies have focused on alcohol-induced epigenetic changes in the brain or on specific brain regions.

A majority of the research into the effects of developmental alcohol exposure on the epigenome has been focused on alcohol-related changes in the expression of single genes. However, Otero et al. (2012) recently demonstrated long-lasting increases in global DNA methylation in the prefrontal cortex and hippocampus of rats exposed to ethanol during the period equivalent to the third trimester (PD 4–9). These global effects suggest that alcohol exposure during development impacts the expression of many genes and suggest that a single gene approach will not be well suited to inform potential treatments. Furthermore, current treatments that alter epigenetic regulation have global effects, such as choline (Zeisel, 2009), a diet high in methyl donors (Shorter et al., 2012), and drugs which affect enzymatic regulation of the epigenome (e.g. HDAC inhibitors, Guan et al., 2009). In order to develop treatments, information about alcohol-induced changes in enzymatic regulation

of the epigenome will be needed. The present experiment utilized a model of FASD in which ethanol is administered throughout all three trimesters to examine long-term changes in epigenetic regulation in a specific brain region, the hippocampus. This model of FASD is likely to provide results that can be generalized to the clinical picture of FASD which is associated with prolonged alcohol exposure during development. We also look at changes in epigenetic regulators after the cessation of alcohol exposure because this is more likely the time period when any practical treatment of FASD can occur.

Materials and Methods

Subjects

Long-Evans rats were used to assess changes in epigenetic regulators in the hippocampus following alcohol exposure using a three-trimester model. The three-trimester model involves exposure to ethanol during all three trimesters of development, via intubation of the dam and intubation of the pup (described below). All subjects were housed in the animal colony of the Department of Psychology at the University of South Carolina. Temperature was maintained at 22° C with a 12h:12h light:dark cycle (lights on at 0700). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

Female Long-Evans rats were housed with breeder males overnight. The presence of sperm on a vaginal smear indicated pregnancy and was designated as gestational day (GD) 1. Pregnant dams were assigned to one of three treatment groups: ethanol treated (ET), intubated control (IC), and non-intubated control (NC). Dams were singly housed throughout pregnancy in polypropylene cages with bedding. Pup treatment was consistent with dam treatment: ET pups were born from ET dams, and ethanol administration continued until PD 10.

Dam Treatment

Ethanol-treated (ET) dams received a daily dose of ethanol (4.5 g/kg in 20 ml/kg of distilled water) using a stainless steel gavage tube throughout pregnancy (GD1 to GD 22) (Kelly & Lawrence, 2008). Food and water were available *ad libitum*. Intubated controls (IC) received an isocaloric maltose-dextrin solution (20 ml/kg) via gavage. However, IC dams were paired to an ET animal of similar weight to control for the differences in food intake due to intoxication. Non-treated control dams were handled briefly on GD 1 during weighing and were not handled throughout the remaining period of gestation. All intubations were completed between 10:00 am and 4:00 pm each day.

Pup Treatment

The day of birth (GD 23) was designated as postnatal day (PD) 1 and no treatments were given on this day. Beginning on PD 2, ethanol-treated (ET) pups (who were born to ethanol-treated dams) were intubated, using Intramedic PE 10 tubing dipped in corn oil, with 3.0 g/kg of ethanol in 27.8 ml/kg of enriched milk (West et al., 1984) between 9:00 am and 11:00 am. Two hours later, a second intubation of milk alone was given to control for differences in feeding behavior due to ethanol intoxication. IC pups (born to IC dams) were intubated daily with Intramedic PE 10 tubing, but no solutions were administered. Intubations continued through PD 10. On PD 6 or 7, NC pups (born to non-treated dams) were weighed and all animals were tattooed for identification purposes (Animal Identification & Machine Systems, Inc.). A majority of the NC pups had weights taken on PD 7, so those weights were used for analysis. Pups were housed with their dams until PD 21, at which time they were weighed, anesthetized with isoflurane via inhalation, and sacrificed to remove the hippocampus.

Blood Alcohol Concentrations (BACs)

On GD 20, a 10- μ l blood sample was obtained from ET dams via a nick to the tail 3 hours after intubation. On PD 10, 10- μ l blood samples were collected from ET pups from a small nick in the tail 2 hours after intubation. These times have been shown to be best for assessing maximum BACs (Marino et al., 2002). Both IC dams and pups received a small nick to control for the stress of the blood sampling procedure. Samples were frozen at -80°C until the time of assay and were analyzed using an enzymatic procedure (Dudek & Abbott, 1984).

Tissue Collection and Processing

On PD 21, pups were rapidly decapitated and the brain was removed and bisected sagittally, with one hemisphere utilized for activity assays and one for expression analysis. Hemispheres were randomly alternated right and left. For activity assays, the hippocampus was removed and samples were kept at -80°C until time of assay. Hippocampal tissue was subjected to subcellular fractionation. Briefly, tissue was homogenized in ice-cold Homogenization Buffer [HB, 1.0269 g 0.32 M sucrose, 40 μ l of 500 mM stock 2 mM EDTA (Sigma), 7.44 mg 2 mM EGTA (Sigma), 47.55 mg 20 mM HEPES (Sigma)] and centrifuged at 2600 rpm for 10 min (4°C). The supernatant was removed and the pellets were washed with 500 μ l HB containing protease and phosphatase inhibitors (Sigma) and centrifuged at 500 g for 10 min (4°C). The supernatant was discarded and the remaining pellet was re-suspended in 500 μ l HB and 1000 μ l of nuclear buffer [NB; 1.8 M Sucrose in 10 mM TRIS, pH 7.4], vortexed, and spun at 105,000 g for 30 minutes at 4°C . Nuclei were extracted from the pellet by resuspending with 100 μ l 0.5M KCl (Sigma) in 10 mM TRIS with inhibitors for 30 minutes. The sample was spun at the maximum speed for 10 minutes using a microfuge. The supernatant containing the nuclear extract (NE) was removed and frozen (-80°) until time of assay.

The amount of protein in the sample used for the enzyme assays was determined using a Pierce BCA Protein Assay kit (VWR), according to the manufacturer's instructions. Samples were run in duplicate and the values averaged to determine sample protein concentration. Briefly, 25 μ l of the samples and standards to be assayed were added to a 96-well plate and combined with 200 μ l of the working reagent containing BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide) and BCA reagent B (4% cupric sulfate). The plate was incubated for 30 minutes at 37°C , read on a microplate reader at 562 nm, and compared to a standard curve to determine protein concentration.

Enzyme Assays

DNA methyltransferase (DNMT) is the enzyme that transfers a methyl group to cytosine from S-adenosyl methionine (SAM). Total activity levels of DNA methyltransferase were assessed using the EpiQuik DNMT Activity/Inhibition Activity Kit (Epigentek) according to the manufacturer's instructions. Briefly, nuclear extract samples (3 μ l) were added to a 96-well plate containing a cytosine rich DNA substrate. Samples were then incubated with a 5-methylcytosine antibody and developed using an ELISA-like colorimetric procedure. The amount of methylated DNA, which is proportional to DNMT activity, was then quantified using a microplate reader (450 nm). The amount of DNMT activity was calculated using the following formula:

$$\text{DNMT activity (OD/h/mg)} = [(\text{Sample OD}) - (\text{Blank OD})] / (\mu\text{g of protein}) * (\text{reaction time (hours)}) \times 1000$$

Histone deacetylase (HDAC) is the enzyme that transfers an acetyl group to histone proteins, and leads to gene repression. Total levels of histone deacetylase (HDAC) activity

were measured using the EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek) according to the manufacturer's instructions. Briefly, nuclear extract samples (2 μ l) were added to a 96-well plate containing a histone substrate to which HDACs in the sample bind. An antibody for acetylated histones was then used to bind the remaining substrate and developed using an ELISA-like reaction. The optical density of the samples was determined using a microplate reader (450 nm) and compared to a standard curve to determine the amount of non-deacetylated substrate, which is inversely proportional to HDAC activity in the sample. The amount of HDAC activity was calculated using the following formula: $[\text{OD (control-blank)} - \text{OD (sample-blank)}] / \text{reaction time} * \text{sample dilution}$.

RNA Isolation

Brains were flash frozen and stored at -80°C until time of assay. Hemispheres were sliced coronally in 1mm thick sections (plates 49 to 79, Paxinos & Watson, 2005). Sections containing the hippocampus were placed onto a freezing platform and two 1mm micropunches were taken containing the hippocampus and placed into lysis buffer [QiagenAllPrep DNA/RNA/protein kit] containing β -mercaptoethanol (Sigma). Tissue punches were homogenized by sonication and RNA isolated as per instructions (Qiagen ALL Prep DNA/RNA/Protein Kit). RNA purity was checked by ratio of 260/280nm absorbance. cDNA was produced via reverse transcription of 5 μ g of RNA using iScripttm cDNA Synthesis Kit (Bio-Rad #170-8890) with a 20 μ l reaction volume containing: 4 μ l 5x iScript reaction mix, 1 μ l iScript Reverse Transcriptase, 10 μ l Nuclease free H₂O and 5 μ l RNA. Reverse transcription occurred under the following conditions: 5 minutes at 25°C , 30 minutes at 42°C , 5 minutes at 85°C and then held at 4°C until used in quantitative real time PCR (qtPCR).

Real-time PCR

For each sample, SYBR Green real-time qtPCR was conducted in triplicate in 96-well plates using a MyiQSingle-Color Real-Time PCR detection System (Bio-Rad #170-9770). PCR reaction volume of 20 μ l was prepared as follows: 10 μ l 2x SyBr green Mix, 0.4 μ l 10 μ M Forward Primers (MECP2 -5'-GGAAGTCTGGCCGATCTGCTGGAAAGTA-3'; DNMT1-5'-CCAGATACCTACCGGTTATTCG-3'; DNMT3a-5'-CTGAAATGGAAAGGGTGTTTGGC-3'; Cyclophilin-5'-ATAAGGGTTCCCTCCTTTCAC-3'), 0.4 μ l 10 μ M Reverse Primers (MECP2-5'-CACCTGAACACCTTCTGATGCTGCTGCC-3'; DNMT1-5'-TCCTTTAACTGCAGCTGAGGC-3'; DNMT3a-5'-CCATGTCCCTTACACACAAGC-3'; Cyclophilin-5'-TCTCTCCGTAGATGGACTTG-3'), 8.2 μ l water and 1 μ l of reverse transcribed samples. Primers were obtained from Integrated DNA Technologies. Real-time qtPCR was performed with a 2 step amplification with melting curve under the following conditions: MeCP2: 3 minutes at 95°C , $40 \times 95^{\circ}\text{C}$ for 10s followed by 65°C for 45s, 1 minute at 95°C , 1 minute at 65°C , $60 \times 65^{\circ}\text{C}$ for 10s; DNMT1: 3 minutes at 95°C , $40 \times 95^{\circ}\text{C}$ for 10s followed by 59°C for 45s, 1 minute at 95°C , 1 minute at 59°C , $72 \times 59^{\circ}\text{C}$ for 10s; DNMT3a: 3 minutes at 95°C , $40 \times 95^{\circ}\text{C}$ for 10s followed by 56°C for 45s, 1 minute at 95°C , 1 minute at 56°C , $78 \times 56^{\circ}\text{C}$ for 10s. Cyclophilin, a housekeeping gene, was used to normalize gene expression.

Data Analysis

All data, except for quantitative RT-PCR, were analyzed using SPSS. The level of significance was $p < .05$ except when controlling for family-wise error rate. A 3 (treatment) $\times$ 2 (sex) design was used in this study, with data analyzed using ANOVA with Tukey post-hoc tests, when necessary. Data from males and females were combined if there was no main effect of sex, or an interaction with treatment. Outliers were discarded if they were

more than 2 standard deviations from the mean. All data from two animals were discarded from analysis because they were outliers on multiple dependent measures.

For quantitative RT-PCR data, the CT (crossing threshold) values for each sample were obtained and efficiency (E) was calculated according to the method of Pfaffl (2001). Relative expression ratios (R) were calculated using E and ΔCT (control-sample) values in the following equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}(\text{control-sample})}}}{(E_{\text{reference}})^{\Delta CT_{\text{reference}(\text{control-sample})}}}$$

where the target genes were MeCP2, DNMT1, and DNMT3a, and the reference gene was cyclophilin (Pfaffl, 2011). The following comparisons were made: ET (sample) to NC (control), ET (sample) to IC (control), and IC (sample) to NC (control). For the control values, the average Ct was used in the equation. The ratio between treatment groups was calculated to determine the fold-change in expression normalized to the reference gene.

Results

Dam Body Weights

A repeated-measures ANOVA was used to assess dam body weights across gestation with treatment as the between-subjects factor and day as the within-subjects factor. NC dams were not weighed daily, so they were excluded from analysis. There was a significant main effect of day on weight, $F(2, 35) = 364.06$, $p = 0.001$ such that body weight increased over days of pregnancy. There was no main effect of treatment or interaction between treatment and day on dam body weights, demonstrating that the ethanol intubation and exposure did not alter body weights differentially between IC and ET dams (data not shown).

Pup Body Weights

Body weights of the pups are shown in Table 1. Separate ANOVAs were conducted to assess body weights for PD 2, 7, 10, and 21, using Tukey post-hoc tests when necessary. On PD 2 and 10, the data from the IC and ET groups were analyzed since the NC group was not weighed on these days. On PD 7 and 21, the data from all three groups were analyzed. On postnatal day 2, there was a significant effect of treatment, $F(1, 36) = 15.89$, $p < 0.001$, where ethanol-exposed pups weighed significantly less than IC pups. There was a significant effect of treatment on PD 7 weights, $F(2, 47) = 22.36$, $p < 0.001$, with ethanol-exposed animals weighing less than both IC and NC pups (p 's < 0.001) and no differences in body weight between IC and NC pups. There was no difference between males and females in PD 7 body weights, nor was there an interaction between the sex and treatment. On postnatal day 10, ethanol-exposed animals weighed significantly less than IC animals ($F(1, 36) = 11.54$, $p < 0.01$). However, on PD 21, there was no difference in weights due to treatment ($F(2, 49) = 1.60$, $p = 0.21$) or sex ($F(1, 49) = 1.10$, $p = 0.30$) (see Table 1) demonstrating that the body weight differences observed at earlier ages were transient.

BACs

Blood alcohol concentrations were analyzed using Students *t*-tests. Male and female pups were compared to examine possible sex differences. There was no significant difference in the BACs of male and female pups, $t(14) = -1.79$, $p = 0.10$. The male and female pups were combined and compared with ET dams. There was no significant difference between pup and dam BACs, $t(28) = 0.45$, $p = 0.66$, demonstrating that peak BACs were similar across

development. The mean BACs ($\pm$ SEM) were 385.79 ($\pm$ 49.92) and 361.26 ($\pm$ 25.42) for dams and pups, respectively.

Measures of Epigenetic Regulation

There were no significant differences in the amount of protein among groups. DNMT activity was analyzed using a 3 (treatment) $\times$ 2 (sex) ANOVA. There was a main effect of treatment, $F(1, 40) = 10.88$, $p = 0.002$, where ethanol exposed animals had a significant increase in DNMT activity compared to both the IC and NC control group (see Figure 1). There was no effect of sex, $F(1, 40) = 0.059$, $p = .81$. Additionally, there was no interaction between sex and treatment, $F(1, 40) = 3.00$, $p = 0.091$. These results represent an almost 2-fold increase in DNMT activity in juveniles exposed to alcohol during perinatal development compared to control animals.

HDAC activity was analyzed using a 3 (treatment) $\times$ 2 (sex) ANOVA. There was no significant effect of treatment, $F(1, 50) = 0.87$, $p = 0.77$, or sex, $F(1, 50) = 0.04$, $p = 0.84$ (see Figure 2). Furthermore, there was no interaction between sex and treatment, $F(1, 50) = .50$, $p = .48$.

Gene expression

Using the method of Pfaffl (2001), changes in gene expression were determined, and expressed as an average fold-change with standard error of the means (SEMs) when comparing two treatment groups, see Table 2. Developmental alcohol exposure caused a decrease in expression of DNMT1, DNMT3a, and MeCP2, compared to the NC group, while causing an increase in expression compared to the IC group.

Discussion

The present study provides further evidence that epigenetic modifications likely occur as a result of perinatal alcohol exposure, and thus may be a mechanism through which ethanol can alter the developing brain. DNMT activity was elevated in the hippocampus eleven days after cessation of alcohol treatment. There was no observed change in HDAC activity. These findings are consistent with previous research demonstrating an increase in global DNA methylation in the prefrontal cortex and hippocampus following postnatal alcohol exposure (Otero et al., 2012). However, it should be noted that the study by Otero and colleagues (2012) used a different model of developmental alcohol exposure than the present study, and epigenetic changes are likely to differ based on the model of alcohol exposure. Furthermore, the expression of three genes involved in epigenetic regulation were examined, with ethanol exposure causing a decrease in expression of MeCP2, DNMT1, and DNMT3a, compared to the non-treated group and an increase in expression compared to the intubated control group. The intubated control group had decreased expression of these enzymes relative to the non-treated group suggesting an impact of stress on these measures. The divergence between findings on DNMT activity and levels of DNMT1 and DNMT3a suggest that ethanol impacts factors that result in increased activity independent of levels and that there are likely other epigenetic regulators impacted by ethanol. Overall, our results confirm the hypothesis that developmental alcohol administration has a long-lasting impact the factors that regulate the epigenome, and further stress the need for more research in this field.

Developmental exposure to ethanol did not alter HDAC activity in the hippocampus. This was surprising because there is evidence that histone acetylation and DNA methylation interact to facilitate gene repression (Fuks et al., 2001; Tsankova et al., 2007). There is also evidence that one of the ways in which DNMTs lead to gene repression is through the recruitment of methyl CpG binding domain proteins (MBDs), which in turn recruit HDACs

(Jones et al., 1998). We found increased DNMT activity in hippocampus, but did not observe a related increase in global HDAC activity. However, it may be that ethanol alters the activity of specific subtypes of the HDAC enzyme which were not detected in this study. Furthermore, specific cell types from the hippocampus were not isolated and epigenetic changes could well be tissue- and/or cell type-specific. In line with this hypothesis, MacDonald and Roskams (2008) demonstrated that HDAC 1 and HDAC 2 are expressed in distinct cell types of the developing rodent brain. Specifically, HDAC 1 is expressed in neural stem cells and glia. However, HDAC 2 is expressed in neuroblasts and neurons, but not in glia. A final possibility is that alcohol exposure did alter HDAC activity but this alteration did not persist to the time of the assay. The null findings for HDAC in the current study may be due to differential expression of the HDAC subtypes and differential expression of HDAC in various cell types or due to lack of persistent effect on this enzyme's activity. However, it is also possible that the brain region that was examined in this study does not show any changes in HDAC activity as a result of alcohol exposure during development.

The recruitment of HDACs to methylated DNA is a two-step process requiring MBDs, such as MeCP2, followed by HDAC recruitment. We demonstrated down-regulation of MeCP2 expression in ethanol-exposed animals compared to the non-treated controls concomitant with no changes in HDAC activity. Because of the down-regulation of MeCP2 seen in the intubated control group relative to non-treated controls, the difference between the ethanol-exposed and non-treated animals may include some stress effect in addition to ethanol and so care should be taken in the interpretation. Nevertheless, the current findings suggest that ethanol may interfere with DNMT recruitment of MBDs, and thus HDACs in the hippocampus. Reductions in MeCP2 result in Rett syndrome, an autism spectrum disorder, which is characterized by severe cognitive and behavioral impairments (Chapleau et al., 2009; Percy et al., 2011). Interestingly, MeCP2 deficiency as seen in Rett syndrome results in reduced spine densities in area CA1 of the hippocampus (Belichenko et al., 2009) similar to those reported in FASD (Berman et al., 1996; Tarello-Acuna et al., 1990). Thus, increased DNMT activity and decreased MeCP2 expression may produce a specific mis-expression pattern that impacts neuronal differentiation in complex ways.

Importantly, impaired neurological function can be caused by *both* increases and decreases in MeCP2 expression, suggesting that tight regulation of this protein is crucial to normal development. Furthermore, MeCP2 expression is regulated by microRNA 132 (miR132). A recent study found that levels of this microRNA are relatively low before birth, and do not rise to significant levels until shortly after birth in rodents, suggesting that this microRNA may play an important role in neuronal maturation (Klein et al., 2007). Importantly, alcohol exposure during the third trimester in rodents produces the most severe structural and functional impairments (e.g., Tran and Kelly 2003). MeCP2 has also been shown to regulate brain-derived neurotrophic factor (BDNF) expression (Klein et al., 2007) in an activity-dependent fashion (Chen et al., 2003). Decreased hippocampal BDNF mRNA levels (Caldwell et al., 2008) and TrkB receptor expression (Moore et al., 2004a; Moore et al., 2004b) are observed following developmental alcohol exposure, suggesting the observed decrease in MeCP2 may be involved in alcohol-induced reductions in BDNF expression, although more research will be needed to determine if this is indeed the case.

Using qRT-PCR, we found that alcohol caused a decrease in the expression of MeCP2, DNMT1, and DNMT3a, when compared to the non-treated control group. In contrast, alcohol caused an increase in expression compared to the intubated control group. This is not surprising, as stressors are well known to affect epigenetic regulation, especially when they occur in the perinatal period (Cameron et al., 2008; Gudsruk & Champagne, 2011; Szyf et al., 2007; Zhang et al., 2010). What these data suggest is that the stress of the

intubation procedure affects expression of genes involved in epigenetic regulation. It will be essential as this field of study advances to control for the stress induced by alcohol administration procedures.

Treatment approaches for FASD are still very much in their formative stage. The results of our and other's research demonstrate that there are changes in the epigenome and in the regulators of the epigenome in the brain following developmental alcohol exposure and that these epigenetic changes are global and long-lasting. At this point, the ability to alter the epigenome in people is focused on changing epigenomic enzymes or changing the methyl content of the diet. Altering the epigenetic regulation of single genes in humans is not currently possible and pinpointing single genes that are responsible for complex functions such as cognition is simply not feasible at this time. As a result, translation of the current findings in animal models of FASD must focus on global epigenetic changes and the possibility of either preventing or reversing these changes. Future research on the impact of alcohol exposure during development on the epigenome must focus on the eventuality of being able use these findings for the benefit of individuals with FASD.

Conclusion

Epigenetic regulation of gene expression has been shown to play a role in developmental processes, and recent research demonstrates epigenetic modifications in the etiology of FASD, using animal models. Importantly, very few studies have focused on alcohol-induced epigenetic changes in the brain, let alone specific brain regions. Moreover, research into long-term epigenetic changes caused by developmental alcohol exposure is very much needed. In the central nervous system, hypermethylation of the DNA in the hippocampus and prefrontal cortex have been demonstrated. In this paper, DNMT activity is shown to be increased in the hippocampus of ethanol-exposed rats a considerable time after the end of ethanol exposure during the period equivalent to all three trimesters in the human. This suggests long-lasting changes in the regulation of the epigenome in brain which would explain the long-lasting and extensive behavioral deficits seen in both animal models of FASD and the human condition. Findings focusing on global changes in the epigenome will be useful in guiding treatment approaches to FASD.

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Abbreviations

ARND	alcohol-related neurodevelopmental disorder
BAC	blood alcohol concentration
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
CT	crossing threshold
DMR	differentially methylated region
DNMT	DNA methyltransferase
ET	ethanol-exposed group

FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
GD	gestational day
HAT	histone acetyltransferase
HDAC	histone deacetylase
IC	intubated control group
MeCP2	methyl CpG binding protein 2
MBDs	methyl binding domains
NC	non-treated control group
PD	postnatal day
PFC	prefrontal cortex

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Highlights

- 1** We review recent research demonstrating epigenetic modifications in animal models of FASD.
- 3** DNMT activity is shown to be increased in the hippocampus after the cessation of ethanol exposure.
- 4** There are no changes in HDAC activity in the hippocampus after the cessation of ethanol exposure.

Figure 1. Average DNA methyltransferase activity ($\pm$ SEM) in the hippocampus

Data are collapsed across sex and error bars represent SEM. The four-pointed star indicates a significant difference ($p < 0.05$) between the ethanol-exposed group and the intubated control group. The five-pointed star indicates a significant difference between the ethanol-exposed group and the non-treated control group.

Figure 2. Average Histone Deacetylase (HDAC) activity ($\pm$ SEM) in the hippocampus
Data are collapsed across sex and error bars represent SEM. There were no significant differences among any treatment groups.

Table 1

Mean body weight (g; $\pm$ SEM) in all subjects.

Groups	N	Weight on PD 2	Weight on PD 7	Weight on PD 10	Weight on PD 21
NC*	18	-	15.1 $\pm$.4 ^b	-	48.1 $\pm$ 1.0
Males	9	-	15.7 $\pm$.5	-	49.9 $\pm$ 1.3
Females	9	-	14.5 $\pm$.5	-	46.3 $\pm$ 1.4
IC	17	6.8 $\pm$.1 ^a	13.9 $\pm$.4 ^a	19.9 $\pm$.4 ^a	47.6 $\pm$ 1.7
Males	8	7.2 $\pm$.2	14.5 $\pm$.4	20.5 $\pm$.4	49.9 $\pm$ 1.0
Females	9	6.6 $\pm$.2	13.5 $\pm$.5	19.3 $\pm$.7	45.6 $\pm$ 3.0
ET	22	6.1 $\pm$.1	11.2 $\pm$.4	17.3 $\pm$.6	46.1 $\pm$ 1.8
Males	11	6.2 $\pm$.2	11.2 $\pm$.7	17.2 $\pm$ 1.0	46.6 $\pm$ 2.8
Females	11	6.0 $\pm$.2	11.3 $\pm$.6	17.3 $\pm$.7	45.6 $\pm$ 2.4

^a indicates that IC pups weighed significantly more than ET pups, $p < 0.001$, on that day.

^b indicates that NC pups weighed significantly more than ET pups, $p < 0.001$, on that day.

Note: Body weights were not taken from NC pups on all days in order to minimize handling.

Table 2

Alcohol-induced changes in gene expression using quantitative RT-PCR expressed as average fold changes $\pm$ SEM.

Comparison	N	MeCP2	DNMT1	DNMT3a
ET to IC	9	0.933 $\pm$ 0.109	0.844 $\pm$ 0.082	1.014 $\pm$ 0.198
ET to NC	6	-1.322 $\pm$ 0.154	-1.016 $\pm$ 0.098	-1.877 $\pm$ 0.366
IC to NC	6	-1.475 $\pm$ 0.189	-1.232 $\pm$ 0.124	-1.925 $\pm$ 0.236

Note: Values indicate relative expression ratios of the target gene (using cyclophilin as the reference gene) and positive numbers indicate a relative increase in expression and negative numbers indicate a relative decrease in expression.