REGULAR RESEARCH ARTICLE

Postnatal Ethanol Exposure Activates HDAC-Mediated Histone Deacetylation, Impairs Synaptic Plasticity Gene Expression and Behavior in Mice

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Abstract

Background: Alcohol consumption during pregnancy is widespread and contributes to pediatric neurological defects, including hippocampal and neocortex dysfunction, causing cognitive deficits termed fetal alcohol spectrum disorders. However, the critical mechanisms underlying these brain abnormalities remain poorly described.

Methods: Using a postnatal ethanol exposure (PEE) animal model and pharmacological, epigenetic, synaptic plasticity-related and behavioral approaches, we discovered a novel persistent epigenetic mechanism of neurodegeneration in neonatal hippocampus and neocortex brain regions and of cognitive decline in adult animals.

Results: PEE, which activates caspase-3 (CC3, a neurodegeneration marker), enhanced histone deacetylase (HDAC1–HDAC3) levels and reduced histone 3 (H3) and 4 (H4) acetylation (ac) in mature neurons. PEE repressed the expression of several synaptic plasticity genes, such as brain-derived neurotrophic factor, C-Fos, early growth response 1 (Egr1), and activity-regulated cytoskeleton-associated protein (Arc). Detailed studies on Egr1 and Arc expression revealed HDAC enrichment at their promoter regions. HDAC inhibition with trichostatin A (TSA) before PEE rescued H3ac/H4ac levels and prevented CC3 formation. Antagonism/null mutation of cannabinoid receptor type-1 (CB1R) before PEE to inhibit CC3 production prevented Egr1 and Arc loss via epigenetic events. TSA administration before PEE prevented postnatal ethanol-induced loss of Egr1 and Arc expression and neurobehavioral defects in adult mice via epigenetic remodeling. In adult mice, 3-day TSA administration attenuated PEE-induced behavioral defects.

Conclusions: These findings demonstrate that CB1R/HDAC-mediated epigenetic remodeling disrupts gene expression and is a critical step in fetal alcohol spectrum disorder-associated cognitive decline but is reversed by restoration of histone acetylation in the brain.

Key Words: Neurodegeneration, epigenetics, FASD, developmental brain, histones
Significance Statement

Fetal exposure to alcohol is associated with congenital disabilities and causes a range of developmental, cognitive, and behavioral abnormalities, which can appear at any time during development and last a lifetime. Postnatal ethanol exposure (PEE) during the active synaptogenesis period causes neurodegeneration in neonatal mice and impairs cognitive function in the adult. However, mechanisms have not been precise. In this study, we examined the involvement of HDAC1–3 through CB1 receptors in PEE-induced neurodegeneration and neurobehavioral abnormalities. We found that CB1 receptor antagonism and HDAC inhibition rescue PEE-induced neurodegeneration, synaptic plasticity-related gene expression, synaptic plasticity, learning, and memory. Our results provide novel underlying brain mechanisms and are relevant to the development of pharmacological treatments for FASD.

Introduction

Exposure to ethanol in utero can cause detrimental effects to the developing fetus brain, leading to a wide range of neurobehavioral abnormalities collectively referred to as fetal alcohol spectrum disorders (FASDs). FASDs are estimated to occur in 2% to 5% of live births in the United States (Riley et al., 2011) and continue to be major health problems in Western nations. The Centers for Disease Control (CDC) suggest that more than 3 million childbearing women may be at risk of exposing their developing babies to the potentially harmful effects of alcohol (CDC, 2016). Despite public health warnings, approximately 1 in 8 pregnant women (500,000/year) continue to drink alcohol throughout pregnancy, and approximately 80,000 binge drink (Floyd et al., 2009). Among several potential adverse consequences of ethanol exposure during fetal development, damage to the developing brain and significant persistent neurobehavioral anomalies are the most prevalent (Coles et al., 2011; Riley et al., 2011). Neurobehavioral problems involving aspects such as impulsivity, response inhibition, attention, learning, and memory (Coles et al., 2011; Graham et al., 2013; Basavarajappa and Subbanna, 2015) are common in children diagnosed with FASDs. Several FASD animal models established with varying ethanol doses have successfully recapitulated these behavioral deficits (Berman and Hannigan, 2000; Ikonomidou et al., 2000; Brown et al., 2007; Leraci and Herrera, 2007; Joshi et al., 2019). However, the underlying molecular mechanisms responsible for these neurobehavioral deficits are somewhat unclear. Substantial evidence (Basavarajappa and Subbanna, 2016; Lussier et al., 2017) suggests that ethanol exposure during early brain development induces chromatin remodeling in many brain regions (Bekdash et al., 2013; Nagre et al., 2015; Zhang et al., 2015); such changes could play important roles in the development of ethanol-induced brain disorders (Coles et al., 2011; Riley et al., 2011; Lunde et al., 2016). In our recent studies using a third trimester-equivalent human pregnancy animal model, we have provided evidence to suggest that epigenetic changes are responsible for some of the abnormalities found in postnatal day 7 and adult mice (for a review, see Basavarajappa and Subbanna, 2016).

Posttranslational acetylation of DNA-associated histone proteins is orchestrated through the vigorous activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Maze et al., 2013; Basavarajappa and Subbanna, 2016). Eighteen different HDACs have been characterized to date and are classified into 3 major classes (I, II, and III) (de Ruijter et al., 2003). Class I HDACs are expressed efficiently in developing and adult brains, emphasizing their central roles in brain development and function (Brodie et al., 2007). Specifically, HDAC1/2 has been shown to be involved in neurodegeneration (Rardai et al., 2012), and limited evidence suggests that histone acetylation-regulated gene expression might be associated with neurodegenerative processes (Konsoula and Barile, 2012). Furthermore, histone acetylation has also been shown to regulate the expression of genes important for synaptic plasticity and memory formation [e.g., early growth response 1 (Egr1), activity-regulated cytoskeleton-associated protein (Arc), brain-derived neurotrophic factor (Bdnf), and member of the fos family proto-oncogene (cFos)] (Subbanna et al., 2015; Epstein and Finkbeiner, 2018). Thus, based on these previous observations, histone acetylation remodeling may be a potential mechanism by which ethanol affects neuronal survival and by which postnatal ethanol (PE) induces neurobehavioral anomalies. In the present study, we explored HDAC1–HDAC3, acetylated histone, and Egr1 and Arc levels and their association with the neurodegenerative effects of ethanol. Because CB1R regulates ethanol-induced abnormalities in this model, we examined the involvement of CB1R in ethanol-induced activation of HDAC1–3. We also examined the critical functions of HDACs in mediating PE-induced persistent epigenetic and neurobehavioral abnormalities in adult mice.

Materials and Methods

Animals

Cannabinoid receptor type 1 (CB1R) wild-type (WT) and global CB1R knockout (KO) mice lacking a functional CB1R gene in all tissues [generated by Dr Andreas Zimmer from the National Institute of Mental Health (NIMH) (Steiner et al., 1999)] were produced on a C57BL/6J background from a CB1R heterozygous breeding colony at the Nathan Kline Institute for Psychiatric Research. The C57BL/6J, CB1RWT, and CB1RKO mice were housed in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines (McGrath et al., 2010). Our animal care and handling procedures followed the Nathan Kline Institute for Psychiatric Research Institutional Care and Use Committee and National Institutes of Health guidelines. The CB1R WT and KO mouse genotypes were determined as described previously (Basavarajappa et al., 2003). Male mice were used for all the studies except for studies involving pan-HDAC inhibitor trichostatin A (TSA) due to a lack of sex differences in the previous P7 ethanol studies (Subbanna et al., 2018b; Joshi et al., 2019). Five to 10 mice were used for each group.

Ethanol Administration

In the current study, we used an ethanol treatment paradigm that has previously been shown to induce widespread neurodegeneration in many brain regions, including the...
hippocampus (HP) and neocortex (NC), without causing any lethality in postnatal day 7 (P7) mice (Olney et al., 2002). Ethanol was administered as described before (Nagre et al., 2015; Subbanna et al., 2015; Joshi et al., 2019). More details are provided in the supplementary Methods section.

Drug Treatment

In the current study, we used the pan-HDAC inhibitor TSA (Cayman, MI) to inhibit HDACs activity in vivo. Different doses of TSA were injected s.c. at a volume of 5 μL/g body weight 30 minutes before the first high-dose ethanol treatment. We used an optimum dose of SR141716A (SR) to inhibit CB1R activity based on our previous extensive studies (Subbanna et al., 2013a, 2015, 2018b; Joshi et al., 2019) with this drug. Individual drugs (TSA or SR) were dissolved in ethanol (10 μL) followed by Tween 80 (10 μL) and brought to the correct volume with a sterile saline solution (vehicle). The vehicle was administered as a control. The TSA-, SR- and vehicle-treated P7 mice were kept with the dams until the time of sacrifice. The brains were removed 4-24 hours or 90 days (at P90) after the first saline/ethanol administration. The brains were processed for several analyses as described below. Neither TSA nor SR treatment altered the sleeping time (intoxication) of P7 ethanol-exposed mice at the time of brain harvest (8 hours). In addition, neither TSA nor SR alone promoted bleeding or inflammation in any of the organs in P7 mice. In some experiments, TSA (3 mg/kg, i.p.) was administered to P90 mice (yielding saline+TSA and ethanol+TSA mice) for 3 consecutive days (from P90 to P93; each injection was 24 hours apart). The dose was selected based on previous studies (Pandey et al., 2015). Y-maze tests to assess spatial memory and social recognition memory tests were performed 24 hours after the final TSA injection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

HP and NC tissue samples at different time points were analyzed by qRT-PCR in a Stratagene Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA). The mRNA expression of Hdac1, Hdac2, Hdac3, Bdnf, Cfos, Egr1, and Arc was measured using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) as we described previously (Subbanna et al., 2015; Joshi et al., 2019). The relative gene expression levels of Hdac1, Hdac2 and Hdac3, Egr1, and Arc were calculated by the 2^(-ΔΔCt) method, and the results are presented as fold increases (Subbanna et al., 2015; Joshi et al., 2019).

Immunohistochemistry (IHC)

We used coronal brain sections collected 8 hours after the first injection of ethanol/saline for the IHC study. At the 8-hour time point, ethanol has been shown to induce optimum activation of caspase-3 in P7 mice as previously described (Subbanna et al., 2015; Joshi et al., 2019). Detailed methods and antibody source are provided in the supplementary Methods section.

Protein Extraction, Electrophoresis, and Immunoblotting

For the immunoblot procedure, HP and NC cytosolic or nuclear fractions were prepared using tissue homogenates of P7 pups and adult animals (Subbanna et al., 2015; Joshi et al., 2019). The immunoblot procedure was similar to a previously described method (Subbanna et al., 2015; Joshi et al., 2019). Antibody sources and detailed methods are provided in the supplementary Methods.

Chromatin Immunoprecipitation (ChIP)-qPCR Assay

A ChIP-qPCR assay was conducted as described previously (Subbanna et al., 2015; Joshi et al., 2019). Antibody sources and detailed methods are provided in the supplementary Methods.

Spatial Memory (SM) Task Using a Y-Maze

An SM task was performed using a symmetrical Y-maze exactly as described previously (Subbanna et al., 2015; Joshi et al., 2019). Separate cohorts of adult male and female (diestras phase) mice exposed to saline, ethanol, saline + TSA, or ethanol + TSA at P7 were used in these studies (for details, see supplementary Methods). The discrimination ratios for arm entries and dwell time were determined using the following formula for the preference for the novel arm over the familiar other arm: novel/novel+other.

Novel Object Recognition (ORT) Task

Separate cohorts of adult male mice were tested in an plexiglass square open-field box with a mean floor light intensity of approximately 20 lux, as described previously by our laboratory (Subbanna et al., 2015; Joshi et al., 2019) (for details, see supplementary Methods).

Social Recognition Memory (SRM)

Separate cohorts of adult male mice treated with saline, ethanol, saline+TSA, or ethanol+TSA at P7 were used in the SRM task, which was performed as described previously (Subbanna and Basavarajappa, 2014; Subbanna et al., 2015; Joshi et al., 2019) (for details, see supplementary Methods).

Long-Term Potentiation (LTP)

HP slices (400 μm) were prepared from male adult mice exposed to saline, ethanol, saline + TSA, or ethanol + TSA at P7, and LTP experiments were performed as described previously (Subbanna et al., 2015; Joshi et al., 2019). More details are provided in the supplementary Methods section.

Statistical Analysis

Where possible, we sought to randomize and blind the sample data. Due to the presence of white marks on the backs of the ethanol-treated adult animals, the adult behavioral and LTP experiments were not blinded and randomized, but they were controlled studies. The unblinded experimental data were analyzed in an identical manner for all conditions to eliminate possible experimenter bias. All experiments were evaluated using equal numbers of P7 and adult mice per treatment and were performed in triplicate. All the data are presented as the mean ± SEM. We performed 1-way or 2-way ANOVA with Bonferroni’s post hoc test to statistically compare the data. In all the comparisons, P < .05 was considered to indicate statistical significance. Prism software (GraphPad, San Diego, CA) was used for all statistical analysis.

Results

Developmental Changes in HDAC1-3 Proteins Occur in Mouse Brain

Figure 1A provides the experimental design used in the current study. Before conducting studies to address the role of HDACs in
the PE effects, we first examined the expression levels of HDAC1-3, which is not well described in mouse brain during various stages of development. NC nuclear protein extracts at developmental stages P2 to P90 were used in western-blot analysis. HDAC1 and HDAC2 protein levels were substantially higher during synaptic development (synaptogenesis) than they were in the adult brain (P90). The expression of HDAC3 gradually increased from P4 to P30 compared with that in the P2 developmental stage and gradually stabilized to P4 levels at developmental stages P45 to P90 (P < .05) (Figure 1B).

**PEE Activates Caspase-3 and Enhances HDAC1-3 Expression**

We injected P7 mice with a low (1.0 g/kg, s.c.) or high (2.5 g/kg, s.c.) dose of ethanol at 0 hours and again at 2 hours. Blood ethanol levels (BELs) were monitored at 3 and 9 hours posttreatment (supplementary Table 1). We also monitored cleaved caspase-3 (CC3) (generation of CC3 as a marker for neurodegeneration) in the brains of P7 mice 8 hours after the first low or high dose of ethanol or saline (postnatal saline [PS]) treatment. Low-dose PEE induced mild caspase-3 activation, whereas high-dose PEE caused robust, widespread caspase-3 activation (P < .05) (Figure 1C). In our previous studies, we demonstrated dose- and time-dependent (4–24 hours) activation of caspase-3 by PEE (Subbanna et al., 2014; Joshi et al., 2019).

Low-dose PE enhanced HDAC1-3 protein levels in the HP and NC at the 8- to 24-hour (Figure 1D) (after the first ethanol exposure) time points compared with the corresponding levels in the PS group (0 hours). Low-dose PE decreased Hdac1 mRNA levels in HP and NC tissues (4–24 hours), reduced Hdac2 mRNA levels (4–24 hours) in the HP but enhanced these levels in the NC at 4 and 8 hours, and decreased them at 24 hours. Hdac3 mRNA levels were increased in both the HP and NC at 8 and 24 hours compared with the levels at 0 hours (P < .05) (Figure 1D). Results suggested that high-dose PE increased HDAC1-3 protein expression in the HP and NC at the 4- to 24-hour (after the first ethanol exposure) time points compared with corresponding expression in the PS group (0 hours) (P < .05) (Figure 1F). High-dose PE reduced the Hdag1 mRNA levels in both HP and NC tissues. Furthermore, Hdag2 mRNA levels were also significantly reduced in HP tissue and enhanced in NC tissue. Hdag3 mRNA levels were increased at 8 and 24 hours in HP and at 4–24 hours in NC tissues (P < .05) (Figure 1G). Overall, PE enhanced HDAC1-3 protein expression and reduced or increased Hdag mRNA in HP or NC tissues.

In dual IHC studies, Manders’ overlap coefficient analysis revealed that compared with PS treatment, PEE (8 hours) significantly enhanced HDAC1-3 positive neuronal nuclear antigen (NeuN) neurons in the HP CA1 and retrosplenial cortex (RSC) brain regions (P < .05) (Figure 2).

**PEE Inhibits Histone H3K14 and H4K8 Acetylation**

Low-dose PEE significantly reduced H3K14ac and H4K8ac levels (P < .05) without affecting the total histone proteins (Figure 3A–B). High-dose PEE not only reduced H3K14ac and H4K8ac levels but also reduced the total H3 and H4 proteins. However, high-dose PEE enhanced H4K8ac and total H4 levels at 24 hours (P < .05) (Figure 3C–D). It has been shown that high-dose PEE results in the caspase-3-mediated degradation of total H3 proteins (Subbanna et al., 2013b) and, possibly, H4 proteins as well.

**CB1R Gene Ablation Before PEE Prevents Loss of H3K14 and H4K8ac in P7 Mice**

It was shown previously that genetic ablation or blockade of CB1R prevents PE-induced neurodegeneration without affecting ethanol metabolism in P7 mice (Subbanna et al., 2015; Joshi et al., 2019). High-dose PEE in CB1R KO mice failed to induce the loss of H3K14ac and H4K8ac in the HP and NC brain regions (Figure 4A,B).

**PEE Impairs Synaptic Plasticity-Related Gene Expression in P7 Mice**

RT-qPCR analysis indicated that a high dose of PEE (8 hours) significantly reduced Bdnf, C-fos, Egr1, and Arc mRNA (P < .05) (Figure 4C) levels in P7 HP and NC tissues. PEE also significantly reduced Egr1 protein expression in P7 HP and NC tissues (P < .05) (Figure 4D). In our previous study (Subbanna et al., 2015), we showed that PEE significantly reduced Arc protein expression in P7 HP and NC tissues.

**PEE Impairs Persistent Expression of Egr1 and Arc Through Gene Locus–Specific Epigenetic Changes in Adult Mice**

As Egr1 and Arc expression are severely affected by PEE in P7 mice, we extended these 2 target genes for adult studies. We have used adult HP tissues for mechanism studies as these analyses are related to synaptic plasticity, learning, and memory behavior and HP region is most important for learning and memory. PEE significantly reduced Egr1 protein and mRNA levels as well as Arc protein and mRNA (P < .05) (Figure 4E–H) levels in adult mouse HP tissues. Furthermore, HP ChIP analysis results suggested that PEE significantly enriched HDAC2 and HDAC3 in the Egr1 and Arc gene promoter region. Reduced CBP was found at both Egr1 and Arc promoter regions. Increased HDAC1 and H3K14ac were found at the Egr1 gene promoter region. However, no change in HDAC1 (P > .05) and reduced H3K14ac were found at the Arc gene promoter region (P < .05) (Figure 4I,J). No alteration in H4K8ac was found at the Egr1 and Arc promoter regions (P > .05).

**Pan HDAC Inhibitor (TSA) Rescues Neurodegeneration by Normalizing H3K14 and H4K8ac in PEE P7 Mice**

In these studies, we used TSA, which potently inhibits both class I (isoforms 1–3, 8, and 11) and II HDACs (isoforms 4–7, 9, and 10) but not class III HDACs (Sirt2). TSA has also been applied to enhance H3/H4ac at specific gene promoters (You et al., 2014; Sharma et al., 2015). Preadministration of TSA (30 minutes before the first ethanol dose) effectively and dose dependently prevented the generation of CC3 fragments in P7 mice (P < .05) (Figure 5A) without any detectable toxicity. Thus, we used TSA at 0.25 mg/kg in all our subsequent studies. Administration of the optimum dose of TSA (0.25 mg/kg, 30 minutes) before the first ethanol treatment did not change the BELs (supplementary Table 1), indicating that TSA does not modulate ethanol metabolism. We also determined the CC3-positive cells at 8 hours in all treatment groups (saline + vehicle, ethanol + vehicle, saline + TSA, and ethanol + TSA) using the IHC method. These results strongly demonstrated that TSA significantly inhibited caspase-3 activation in the HP and RSC brain regions. The saline and saline + TSA groups were not significantly different (P > .05), whereas the PE-treated mice had significantly larger proportions of CC3-positive cells in both the RSC and the HP (P < .05) (Figure 5B). Further, preadministration of TSA significantly rescued the PE-induced loss of H3K14ac and H4K8ac in NeuN-positive cells in both RSC and CA1 brain regions (supplementary Figure 1).
Figure 1. PEE increases histone deacetylase (HDAC1–HDAC3) levels in P7 mouse brain hippocampus (HP) and neocortex (NC) tissues. (a) Experimental design indicates developmental age, timing of ethanol, trichostatin A (TSA), or SR141716A (SR) administration and various analysis at early (P7) as well as at adult stage. (b) Changes in HDAC1–HDAC3 protein levels in the mouse NC during postnatal development. Western-blot analysis of HDAC1–HDAC3 expression in neocortical nuclear extracts obtained from 11 postnatal developmental stages of mouse brains. Error bars, SEM (*P < .05 vs the P2 [HDAC1] group, #P < .05 vs the P2 [HDAC2] group, and $P < .05 vs the P2 [HDAC3] group, n = 10 pups/group). (c) Mice were exposed to 1.0 g/kg (low-dose) or 2.5 g/kg (high-dose) ethanol s.c. at 0 hours and again at 2 hours (8 hours), and CC3 levels were evaluated in HP and NC brain region samples obtained 8 hours after the first saline (S) or ethanol (E) exposure by western-blot analysis. (d–g) Increased HDAC1–HDAC3 protein expression with differential mRNA levels in P7 mouse HP and NC tissues in response to low- and high-dose ethanol exposure. HP and NC nuclear extracts procured 4–24 hours after the first saline or low-dose (d and e)/high-dose (f and g) ethanol exposure were used in these experiments. HDAC1–HDAC3 protein levels were determined using western-blot analysis. The protein samples were equally loaded, confirmed with Ponceau S staining and normalized to β-actin. Hdac1–Hdac3 mRNA levels were determined by qRT-PCR analysis. Hypoxanthine-guanine phosphoribosyltransferase (Hprt) and glyceraldehyde 3-phosphate dehydrogenase (Gadph) mRNA were used as the internal controls for normalization of Hdac mRNA levels. For the 0 hours ethanol group, saline was administered. Error bars, SEM (*P < .05 vs the saline [0 h] group, n = 10 pups/group).
Figure 2. Increased colocalization of HDAC1–HDAC3 protein with neuronal nuclear antigen (NeuN)-positive neurons was observed in P7 mouse brain regions after high-dose ethanol exposure. Free-floating coronal brain sections (CA1 and retrosplenial cortex [RSC]) exposed to saline and ethanol (8 hours) were subjected to immunohistochemistry (IHC) with anti-mouse HDAC2 or HDAC3 and anti-rabbit NeuN antibodies or with anti-rabbit HDAC1 and anti-mouse NeuN antibodies to label HDAC1-positive, HDAC2-positive, and HDAC3-positive NeuN in neurons. HDAC1-positive, HDAC2-positive, and HDAC3-positive NeuN neurons in the CA1 and RSC brain regions were quantified using Manders’ coefficient analysis. Error bars, SEM (*P < .05 vs the saline [0 hours] group, n = 6 pups/group). Scale bars = 10 μm.
Preadministration of TSA Prevents Loss of Egr1 and Arc Expression via Chromatin Remodeling at Their Gene Promoter Regions in PEE Adult Mice

To examine whether the inhibition of HDACs in P7 mice averts the persistent PE-induced loss of Egr1 and Arc levels in HP tissue at adulthood, we determined Arc and Egr1 mRNA and protein levels. Pretreatment of TSA significantly prevented the PE-induced loss of Egr1 and Arc mRNA and protein levels in the adult HP brain region (P < .05) (Figure 6A,B). Further, preadministration of TSA significantly rescued the PE-induced enrichment of H3K14ac at the Egr1 and Arc promoter regions (P < .05) (Figure 6C).

Administration of SR Prior to PEE Prevents Loss of Egr1 and Arc Expression via Chromatin Remodeling at Their Gene Promoter Regions in Adult Mice

Because inhibition of CB1R prior to PEE rescued H3/H4 acetylation, we examined the influence of CB1R on chromatin modification at the Egr1 and Arc promoter regions. Preadministration of SR significantly rescued the PE-induced deficits of Egr1 and Arc mRNA (P < .05) (Figure 6D,E) and enrichment of HDAC1, HDAC2, HDAC3, H3K14ac, and CBP in the Egr1 promoter region (P < .05) (Figure 6F). Additionally, SR rescued the PE-enhanced enrichment of HDAC2, HDAC3, and reduced H3K14ac as well as CBP at the Arc (Figure 6G) promoter region.

Preadministration of TSA Prevents Learning and Memory Behavior Deficits in Both Male and Female PEE Adult Mice

Although our previous studies indicated that ethanol has no sex effects and we therefore used males for all the studies, we included females in TSA behavioral studies to examine how TSA influences behavior in female mice compared with male mice. The results demonstrated that PS-exposed male and female mice with or without TSA entered the arms more regularly and spent more time in the novel, formerly unvisited arm of the maze.
(Figure 7A). However, PEE male and female mice displayed a reduced preference for the novel arm ($P < .05$) and spent less time (dwell time) ($P < .05$) in the novel arm than did the PS-exposed mice after 24 hours of retention. TSA-treated PEE mice exhibited a greater preference toward exploration of the novel arm ($P < .05$) and increased time spent ($P < .05$) in the novel arm. These
results suggested that PE-activated HDACs significantly contributed to the onset of spatial recognition memory abnormalities in adult male and female mice. Furthermore, the saline- or saline+TSA-treated mice (male and female) preferred the novel arm as the first choice, whereas the PE-treated adult mice (male and female) exhibited an impaired preference for the novel arm. TSA preadministration rescued the PE-impaired preference for the novel arm. The other experiments were conducted with only male mice due to the lack of a significant influence of sex in the Y-maze behavioral studies.

We also evaluated the ORT task, and the results indicated that there was no significant effect of saline, ethanol, S + TSA, or E + TSA treatment on total exploration times at e1 (T1) or e2 (T2) in the ORT task (P > .05) (Figure 7B). PEE mice exhibited a significantly reduced ORT performance compared with that of PS-treated mice. Interestingly, TSA preadministration significantly prevented PE-induced ORT defects compared with those in PEE mice. TSA alone had no significant influence (P > .05) on ORT performance, and these mice displayed normal ORT performance.

**P7 Preadministration of TSA Before PEE Prevents PE-Induced SRM Abnormalities in Adult Mice**

The social investigation behavior of PEE adult mice was significantly impaired compared with that of PS-exposed mice. Pretreatment with TSA significantly prevented the PE-caused SRM abnormalities compared with those of PS mice administered with or without TSA (Figure 7C). Importantly, PS mice treated with TSA displayed no significant effects (P > .05) with respect to SRM, and these mice displayed normal social investigation compared with that of the PS-treated mice (P > .05). These findings demonstrate that PE-enhanced HDACs during postnatal development contribute to SRM loss in adult mice.

**Administration of TSA Before PEE Prevents LTP Impairments in Adult Mice**

Robust I/O responses were detected in all treatment groups and were not changed by ethanol or TSA administration (P > .05) (data not shown). The baseline fEPSP was recorded for 10 minutes at 60-second intervals with a stimulation intensity equivalent to approximately 35% of the maximum evoked response. Application of theta-burst stimulation (TBS) in PS-exposed animals elicited robust LTP and was constant over 120 minutes. However, the application of TBS in HP slices from PEE mice caused markedly declined LTP (P < .05). TSA treatment before PEE significantly rescued the TBS-evoked LTP abnormalities in adult mice (Figure 7D). TSA treatment before PS exposure failed to affect LTP in adult mice HP (P > .05).

**Administration of TSA at Adulthood Prevents Learning and Memory Behavior Deficits in PEE Adult Mice**

To further examine whether TSA treatment at the adulthood would prevent PE-induced behavioral deficits, PEE and postnatal saline exposed (PSE) adult mice were treated with TSA (3 mg/kg) for 3 consecutive days and behavioral studies were performed 24 hours after the last TSA injection. The Y-maze spatial memory results demonstrated that PSE male and female (combined due to lack of gender effects) (P < .05) mice with or without TSA entered the arms more regularly and spent more time (P < .05) in the novel, formerly unvisited arm of the maze. TSA treatment at the adulthood (P90–P93) rescued the PE-impaired preference...
Figure 6. Pretreatment of P7 mice with TSA or SR rescues Arc and Egr1 impairment through chromatin remodeling in adult mice. (a and b) P7 mice were exposed to saline (S) or a high dose of ethanol (E; 8 hours) after administration of 0.25 mg/kg TSA for 30 minutes (S + TSA or E + TSA, respectively), and Arc and Egr1 protein and mRNA levels were determined in adult HP brain samples by western blot and qRT-PCR analysis, respectively. The protein samples were equally loaded, confirmed with Ponceau S staining, and normalized to β-actin. (c) Epigenetic analysis of the promoter regions of the Egr1 and Arc genes. Chromatin immunoprecipitation (ChIP) analysis of the Egr1 and Arc gene promoters in adult HP tissues from the S + V, E + V, S + TSA and E + TSA-treated groups with anti-H3K14ac antibodies. Error bars, SEM (*P < .05 vs the saline group; #P < .05 vs the ethanol group, n = 10 mice/group). (d and e) P7 mice were exposed to saline (S) or a high dose of ethanol (E; 8 hours) after administration of 1 mg/kg SR for 30 minutes (E + SR or S + SR, respectively), and Arc and Egr1 mRNA levels were determined in adult HP brain samples by qRT-PCR analysis. For all qRT-PCR analyses, Hprt mRNA was used as the internal control for normalization of Egr1 and Arc mRNA. (f and g) Epigenetic analysis of the promoter regions of the Egr1 and Arc genes. ChIP analysis of the Egr1 and Arc gene promoters in adult HP tissues from the saline-, ethanol-, and SR- or vehicle-treated groups with anti-H3K14ac, anti-CBP, and anti-HDAC1–HDAC3 antibodies. The levels of Egr1 and Arc gene promoter chromatin enrichment in the immunoprecipitates were measured by qRT-PCR and are expressed as the percentage input. Error bars, SEM (*P < .05 vs the saline group; #P < .05 vs the ethanol group, n = 10 mice/group).
for the novel arm ($P < .05$), and the mice spent more time ($P < .05$) in the novel arm of the maze. In addition, the saline- or saline + TSA-treated mice preferred the novel arm as the first choice, whereas the PEE adult mice displayed lessened preference for the novel arm. TSA administration at adulthood rescued the PE-impaired preference for the novel arm. We also

Figure 7. TSA exposure prior to ethanol treatment in P7 mice or post ethanol treatment in adult mice ameliorates ethanol-induced behavioral abnormalities and LTP deficits in adult mice. (a and e) Spatial working memory was evaluated with a Y-maze in adult male and female mice exposed to saline (S) or ethanol (E) with or without TSA at P7 (a) or P90 (e). The discrimination ratios (preference for the novel arm over the familiar other arm [novel/novel + other]) for arm entries and dwell time (time spent in each arm) were determined for S + vehicle (V), E + V, S + TSA, and E + TSA-exposed mice 24 hours after the first encounter with the partially opened maze. The percentages of mice choosing the novel arm as the first choice are shown for S + V, E + V, S + TSA, and E + TSA-exposed mice 24 hours after the first encounter with the partially opened maze. Error bars, SEM (*$P < .05$ vs the saline group; # $P < .05$ vs the ethanol group, n = 8 mice/group). (b) Object recognition memory was evaluated in an open field in adult male mice exposed to S + V, E + V, S + TSA, or E + TSA at P7. The total level of exploration was measured at t1 and t2 (24 hours), and the time spent by all P7-treated adult male mice exploring the 2 objects in T1 and T2 (24 hours) was determined. Discrimination indices (d1) were calculated for the S + V, E + V, S + TSA, and E + TSA-treated mice at 24 hours retention intervals. Error bars, SEM (*$P < .05$ vs the saline group; # $P < .05$ vs the ethanol group, n = 8 mice/group). (c and f) The percentages of social investigation are shown for all the treated groups (S + V, E + V, S + TSA, and E + TSA) 24 hours after the first encounter with the same juvenile mice. TSA was administered either at P7 (c) or at P90 (f). Error bars, SEM (*$P < .05$ vs the saline group; # $P < .05$ vs the ethanol group, n = 8 mice/group). (d) The average field excitatory postsynaptic potential (fEPSP) slope at various time points obtained from P7 S + V, E + V, S + TSA, or E + TSA-treated adult male mice. For each slice, the fEPSP slopes were normalized against the average slope over the 10-minute recording period before long-term potentiation (LTP) stimulation. The arrows show the time of theta-burst stimulation (TBS) (4 pulses at 100 Hz; the bursts repeated at 5 Hz, and each tetanus included 3 different 10-burst trains separated by 15 seconds). The bar graph demonstrates the average fEPSP slopes at multiple time points after theta-burst stimulation (TBS) for all the treated groups. Error bars, SEM (*$P < .05$ vs the saline group, # $P < .05$ vs the ethanol group, n = 5 mice/group, 10 slices/group).
performed the SRM behavior of PEE mice treated with TSA at P90 to P93. The results suggested that treatment with TSA significantly prevented the PE-impaired SRM abnormalities (Figure 7I). Like the previous observations, P5 mice treated with TSA displayed no significant effects (P > 0.05), and these mice displayed normal SRM compared with that of the P5-treated mice (P > 0.05).

All the statistical details for each result are provided in supplementary Table 2.

Discussion

In the present paper, we report the roles of upregulated HDAC-mediated histone deacetylation on PE-induced neonatal neurodegeneration and persistent impairment of synaptic plasticity genes, LTP, and memory. The primary observation that inspired this study was the finding that enhancement of cAMP Response Element-Binding Protein (CREB) phosphorylation during memory formation is suppressed in a CB1R-dependent manner in mice exposed to PE (Subbanna et al., 2015, 2018a). Particularly, CREB can modify histone acetylation and expression of its target genes by recruiting the transcriptional coactivator and acetyl transferase such as CBP (Lonze and Ginty, 2002). On the other hand, HDACs that regulate histone acetylation are expressed in the central nervous system, often in a developmental stage-dependent manner (Broide et al., 2007; Murko et al., 2010). Studies using gene KO animals have shown that HDAC1/2 contribute to the regulation of genes that have vital functions in nervous system development and maintenance (Montgomery et al., 2009; Morris and Monteggia, 2013; Schroeder et al., 2017). However, the functions of HDACs in PE-induced developmental defects are not largely understood.

The first objective of this study was to demonstrate the developmental patterns of HDAC1–HDAC3 and to elucidate their neurodegenerative roles in PE-exposed neonatal mice. The results revealed that HDAC1 and HDAC2 were most highly expressed during synaptogenesis (P2–P15) (Yuste and Bonhoeffer, 2004) and that their levels dropped significantly by adulthood. However, HDAC3 was more highly expressed at the end of the synaptogenesis period (P15–P30) than in adulthood or the P2 developmental stage.

On the basis of the results presented, we propose that histone deacetylation initiated by PE in P7 mice causes neurodegeneration and that this effect can be ameliorated by enhancement of histone acetylation. The suggestion of this molecular mechanism is based on several crucial observations. First, PEE, which induces neurodegeneration in P7 mice, enhanced HDAC1–HDAC3 protein levels and reduced global H3K14ac and H4K8ac levels. The PE-enhanced HDACs were associated with mature neuronal markers, such as NeuN. Furthermore, treatment with TSA before PEE in P7 mice significantly attenuated the loss of H3K14ac and H4K8ac from NeuN-positive HP and RSC region cells and ameliorated neurodegeneration. Many studies on animal models of FASD have demonstrated that epigenetic changes, including DNA-associated histone modifications, are associated with the condition (Guo et al., 2011; Bekdach et al., 2013; Subbanna et al., 2013b, 2015; Subbanna and Basavarajappa, 2014; Nagre et al., 2015). Consistent with our findings, PEE has been found to enhance HDAC2 expression in a human neuronal cell line (Agudelo et al., 2011) and to increase HDAC activity in neurons (Agudelo et al., 2012). Another study found reduced expression of CBP (a HAT) and H3/H4 protein in the cerebella of rats exposed to PE during a period equivalent to the third trimester of human pregnancy (Guo et al., 2011). Additionally, reduced HDAC2 levels, Pomc gene expression, and β-endorphin protein production in the arcuate nucleus of the hypothalamus have been demonstrated in a PE model (Govorko et al., 2012). Similarly, in our previous studies, high-dose PEE was found to increase the specific enrichment of H4K8ac at the Cnr1 gene promoter, causing enhanced CB1R expression in P7 mouse brains (Subbanna et al., 2013).

Furthermore, low-dose PEE in P7 mice (eliciting mild neurodegeneration) enhanced enrichment of H3K14ac at the G9a gene promoter region (Subbanna and Basavarajappa, 2014). A recent study using neural crest cells as well as mouse embryos exposed to ethanol in vivo revealed that ethanol decreased H3K9ac at the Bcl-2 gene promoter region, while pharmacological augmentation of H3K9ac at the Bcl-2 gene promoter rescued Bcl-2 expression and apoptosis (Yuana et al., 2018). In our recent studies, PEE persistently reduced enrichment of H3K14ac at the Arc gene promoter and expression of the Arc gene (Subbanna et al., 2018a). These findings together demonstrate that global and gene promoter-specific decreases in H3ac/H4ac induced by PE cause neurodegeneration through regulation of HDAC expression during a highly sensitive period of neurodevelopment.

Although PEE of P7 mice also affected Hdac mRNA, the results were not always consistent with the protein levels. Increased protein expression due to PE may activate a feedback mechanism to regulate mRNA levels and therefore control protein levels. This type of discrepancy with HDACs has also been observed in other tissues (Lee et al., 2015). Currently, it is not clear whether an autoregulatory loop, as demonstrated for HDAC1 (Canettieri et al., 2010), exists for other HDACs and thus may be responsible for the discrepancy between the mRNA and protein levels of specific HDACs.

PEE has also been shown to impact several aspects of neurobehavioral function at the adult stage (Bonthius and West, 1991; Girard et al., 2000; Noel et al., 2011; Subbanna and Basavarajappa, 2014; Basavarajappa, 2015; Subbanna et al., 2015), but the mechanisms are not clear. Based on the findings presented using TSA, we propose that histone deacetylation initiated by PEE at the P7 stage causes long-lasting synaptic and behavioral deficits in adult mice through epigenetic remodeling of chromatin. As numerous studies have demonstrated, pCREB and histone acetylation promote the transcription of memory genes such as Bdnf, c-Fos, Egr1, and Arc, which are essential in the hippocampal function and process of memory formation (Perissi et al., 1999; Subbanna et al., 2015, 2018a). We now corroborate these data by showing that reductions of histone acetylation in the HP and NC tissues of PE-exposed P7 mice are associated with reduced expression of the synaptic plasticity (Bdnf, C-Fos, Egr1, and Arc) genes. We selected the Egr1 and Arc genes and HP region, important for learning and memory, for further detailed studies in adult animals, and the findings demonstrate that their expression is persistently impaired in HP tissues of PEE-exposed adults. Persistent loss of Egr1 and Arc expression may involve epigenetic remodeling of their respective gene promoters via enhanced recruitment of HDAC1–HDAC3, which results in greater chromatin condensation at the Egr1 and Arc promoter regions. Despite the increases in HDAC1–HDAC3 and the decreases in CBP recruitment, as indicated by the ChiP experiment, we found increased H3K14ac on the Egr1 gene promoter region. These data may indicate the functions of other histone acetylation markers (Hendrickz et al., 2014) in the regulation of Egr1 expression. The reduced levels of H3K14ac on the Arc gene promoter region might be due to reduced recruitment of CBP.

Furthermore, pretreatment with drugs (TSA and SR) that are shown to prevent PE-induced neurodegeneration before PEE...
Egr1 and Arc gene expression are associated with persistent neurobehavioral defects caused by developmental ethanol exposure; thus, epigenetic pharmacotherapy may be a promising area of potential to reverse the transcriptional dysregulation and behavioral abnormalities. Our conclusion is supported by numerous additional critical observations.

Preadministration of TSA before the first high-dose PEE ameliorated persistent abnormalities associated with learning, memory, and social interaction behaviors in both male and female adult mice. Furthermore, TSA prevented PE-induced LTP deficits. In addition, TSA treatment in adulthood was also effective in preventing PE-induced SM and SRM deficits. Altogether, it is clear that ethanol exposure at a distinct developmental period may promote a repressive chromatin state at the global histone level, although the specific signaling pathway that elicits such changes in epigenetic states and gene expression that causes enduring behavioral impairment is unclear (Subbanna et al., 2013a, 2013b, 2014, 2015). Further, most of our current experiments were carried out with a pan-HDAC inhibitor, which offers no insight into the roles of individual HDACs role and warrants further studies to establish a direct mechanistic link between PE-induced specific HDAC-mediated epigenetic changes to behavioral abnormalities.

Despite the encouraging results highlighting the potential of TSA for the treatment of FASD in animal models, additional research is required before its application to humans. Indeed, reasons of concern include the multi-targeted and multicellular effects exerted by pan-inhibitors such as TSA. Nonetheless, the current findings strongly demonstrate that PE-induced activation of HDACs can impact developmental programs and contribute to persistent synaptic plasticity gene expression and neurobehavioral defects. These findings add a novel mechanism of PEE that leads to FASDs.

Conclusions

In summary, ethanol exposure during the active brain growth period impairs H3K14ac and H4K8ac via upregulation of HDAC1–HDAC3. Pharmacological inhibition of HDACs or CB1R prior to PEE prevents loss of H3K14ac and H4K8ac and activation of caspase-3 in P7 mice. The PEE-induced impairments, which occur within cells of the developing brain, may cause defects in synaptic circuit maturation, leading to persistent deficits in synaptic plasticity and learning and memory behavior (Figure 8) similar to those found in human FASDs (Coles et al., 2011; Riley et al., 2011) or in animal models of FASD (Bonthius and West, 1991; Girard et al., 2000; Noel et al., 2011; Subbanna and Basavarajappa, 2014; Basavarajappa, 2015; Subbanna et al., 2015); however, these effects could be alleviated by TSA. These findings suggest that well-characterized pharmacological manipulation of epigenetic enzymes has the potential to reverse the transcriptional dysregulation and behavioral deficits caused by developmental ethanol exposure; thus, epigenetic pharmacotherapy may be a promising area of drug discovery for FASD.

Supplementary Materials

Supplementary data are available at International Journal of Neuropsychopharmacology (IJNPPY) online.

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**Statement of Interest**

None.

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