Ethanol Induced Acetylation of Histone at G9a Exon1 and G9a-Mediated Histone H3 Dimethylation leads to Neurodegeneration in Neonatal Mice

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Abstract

The transient exposure of immature rodents to ethanol during postnatal day 7 (P7), comparable to a time point within the third trimester of human pregnancy, induces neurodegeneration. However, the molecular mechanisms underlying the deleterious effects of ethanol on the developing brain are poorly understood. In our previous study, we showed that a high dose administration of ethanol at P7 enhances G9a and leads to caspase-3-mediated degradation of dimethylated H3 on lysine 9 (H3K9me2). In this study, we investigated the potential role of epigenetic changes at G9a exon1, G9a-mediated H3 dimethylation on neurodegeneration and G9a-associated proteins in the P7 brain following exposure to a low dose of ethanol. We found that a low dose of ethanol induces mild neurodegeneration in P7 mice, enhances specific acetylation of H3 on lysine 14 (H3K14ace) at G9a exon1, G9a protein levels, augments the dimethylation of H3K9 and H3 lysine 27 (H3K27me2). However, neither dimethylated H3K9 nor K27 underwent degradation. Pharmacological inhibition of G9a activity prior to ethanol treatment prevented H3 dimethylation and neurodegeneration. Further, our immunoprecipitation data suggest that G9a directly associates with DNA methyltransferase (DNMT3A) and methyl-CpG-binding protein 2 (MeCP2). In addition, DNMT3A and MeCP2 protein levels were enhanced by a low dose of ethanol that was shown to induce mild neurodegeneration. Collectively, these epigenetic alterations lead to association of G9a, DNMT3A and MeCP2 to form a larger repressive complex and have a significant role in low dose ethanol-induced neurodegeneration in the developing brain.

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INTRODUCTION

Exposure to ethanol during pregnancy is one of the most common known causes of preventable birth defects (Ikonomidou et al., 2000). Prenatal ethanol exposure can lead to long-term deficits in physical and cognitive growth and development (Mattson and Riley, 1998, Mattson et al., 1998, Mattson et al., 2011). The developmental period in which the brain is particularly vulnerable and sensitive to ethanol is known as the brain growth spurt. This window occurs during postnatal days (P4–10) in rodents, which is equivalent to the third trimester of pregnancy in humans (Bayer et al., 1993). A single episode of ethanol intoxication on P7 triggers a massive wave of apoptotic neurodegeneration in the developing rodent brain (Ikonomidou et al., 2000), leading to neuronal dysfunction (Izumi et al., 2005, Wilson et al., 2011, Sadrian et al., 2012, Subbanna et al., 2013a). However, the mechanism(s) of ethanol-induced developmental neurotoxicity remains elusive.

Histone and epigenetic alterations have been shown to play a role in a diverse set of processes, both normal and disease-related, and have been implicated in several human developmental disorders (Campuzano et al., 1996, Petronis, 2003, Makedonski et al., 2005, Ryu et al., 2006, Warren, 2007, Gavin and Sharma, 2010). One well-studied mechanism of chromatin remodeling is mediated by histone methylation in the nervous system (Tsankova et al., 2006). Dimethylation of histone H3K9 correlates with transcriptional silencing, whereas the trimethylation of histone H3 at lysine 4 (H3K4me3) is linked to active transcription (Schneider et al., 2004). These events are dynamically controlled by euchromatic histone methyltransferases (HMTase), such as G9a (Tachibana et al., 2002) and G9a-related protein (GLP) (Ogawa et al., 2002), which can repress gene activity by inducing local H3K9 and H3K27 dimethylation at target promoters. Both G9a and GLP are ubiquitously expressed, and when G9a is knocked out throughout the limbic forebrain, cognitive deficits arise that are reminiscent of a mental retardation syndrome in humans (Balemans et al., 2010). Recently, G9a and GLP were shown to be essential for neuronal functions during early brain development (Schaefer et al., 2009). DNA methylation and histone tail modifications have been extensively studied as the two major components of epigenetic codes. DNMT3A (DNA methyltransferase) is required for maintaining DNA methylation in central nervous system (CNS) neurons from an early postnatal stage (Feng et al., 2010), and methyl-CpG binding proteins (MeCP2) have been shown to function as the molecular linker between these two codes. Although the molecular events related to DNMT3A and MeCP2 under ethanol-induced neurodegeneration are not known, rats exposed to a higher dose of ethanol show reduced DNMT mRNA levels in the sperm (Bielawski et al., 2002) and in the 21-day-old (Perkins et al., 2013) or adult brain (Wiebelhaus et al., 2012). Furthermore, many histone genes related to the epigenetic regulation of transcription are affected by ethanol (Zhou et al., 2011) at early neurulation and may alter chromatin organization, affecting transcription at a global level (Berger, 2002). Recently, we reported that high dose ethanol administration at P7, which induces robust apoptotic neurodegeneration in neonatal mice, enhances G9a activity followed by increased histone H3 lysine 9 (H3K9me2) and 27 (H3K27me2) dimethylation (Subbanna et al., 2013b). In addition, we found that the increased dimethylation of H3K9 makes it susceptible to proteolytic degradation by caspase-3 under conditions in which ethanol induces robust neurodegeneration. Further, pharmacological inhibition of G9a activity prior to ethanol treatment at P7 normalizes the dimethylation of the H3K9 and H3K27 proteins to basal levels and prevents neurodegeneration in neonatal mice (Subbanna et al., 2013b).
report in this study that administration of a low ethanol dose at P7 causes mild neurodegeneration with an increase in H3K14ace at G9a exon1, G9a-mediated histone methylation, DNMT3A and MeCP2. The pharmacological blockade of G9a provides protection against ethanol-induced neurodegeneration.

**EXPERIMENTAL PROCEDURES**

**Animals and treatment**

Animals were housed in groups under standard laboratory conditions (12 h light / 12 h dark cycle) with food and water available *ad libitum*. The animal care and handling procedures followed the Institutional (NKI IACUC) and National Institutes of Health guidelines. A low dose ethanol, non-lethal treatment paradigm, which has been previously shown to induce mild apoptotic neurodegeneration in P7 mice (Olney et al., 2002), was used in the current study. P7 mice (C57BL/6J) were treated subcutaneously (s.c.) with either normal saline or ethanol (1.0 g/kg s.c. at 0 h and again at 2 h) as described previously by our laboratory (Subbanna et al., 2013a, Subbanna et al., 2013b). The blood ethanol levels (BEL) of the pups were determined by an enzymatic method (Lundquist, 1959). The small molecule BIX, a diazepin-quinazolin-amine derivative, inhibits G9a enzymatic activity and reduces the H3K9me2 levels of several G9a target genes. For the BIX experiments, BIX (Cayman, Michigan, USA) was dissolved in 10 µl of ethanol (100%) followed by 2–3 drops of Tween 80 and then diluted in sterile saline solution as described previously (Subbanna et al., 2013b). The BIX solution was administered by s.c. injection at a volume of 10 ml/kg body weight 30 min prior to the ethanol administration. The BIX vehicle solution was injected as a control. BIX administration does not alter BELs or intoxication (sleeping time) at the time of brain harvest. P7 mice treated with BIX alone looked normal as saline-treated mice, and the treatment did not cause any inflammation or bleeding in any organ (Subbanna et al., 2013b). The mice were kept with the dams until the brains were removed 4–24 h after the first saline/ethanol injection. The brains were processed for several analyses as described below. Ten to 15 animals were used for each data point.

**Immunohistochemistry**

We perfused mice when maximum caspase-3 activation (in one or more brain regions) is present (8 h) (Ikonomidou et al., 2000, Wilson et al., 2011, Subbanna et al., 2013b) with a solution containing 4% paraformaldehyde and 4% sucrose in 0.05 M cacodylate buffer (pH 7.2), and the brains were further processed according to our previously described protocols (Subbanna et al., 2013a, Subbanna et al., 2013b). We have used several combinations of antibodies in our immunohistochemistry analysis. First, free-floating sections were obtained from ethanol- and saline-exposed brains (8 h of exposure) and immunostained using an antibody against cleaved caspase-3 (Asp175) (CC3) (Cell Signaling Technology, Danvers, MA, USA) with ABC reagents (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA) and a peroxidase substrate (DAB) kit (Vector Labs) to label neurodegenerating neurons. Second, we used a dual immunofluorescence method as described previously (Subbanna et al., 2013a, Subbanna et al., 2013b). We used two antibodies [anti-rabbit-cleaved caspase-3 (Asp175) or anti-mouse-caspase 3-cleaved-tau (cTau] to label neurodegenerating neurons. In anti-mouse-H3K9me2 (1:200) antibody (Abcam) staining, we used anti-rabbit-cleaved caspase-3 (Asp175, 1:1000). In anti-rabbit-H3K27me2 (1:200) (Cell Signaling Technology) staining, we used anti-mouse-caspase 3-cleaved-tau (cTau) (1:200). The secondary antibodies conjugated with Alexa Fluor 488 and 568 (Invitrogen, NY, USA) were used in these studies. The primary antibodies were omitted from the reactions as a control for secondary antibody specificity. In addition, pre-incubation with blocking peptides for the anti-CC3 and anti-cTau (GenScript, Piscataway, NJ, USA) completely blocked the immunostaining of these antibodies. All photomicrographs were
taken through a 2.5×, or 40× objective with a Nikon Eclipse TE2000 inverted microscope attached to a digital camera (DXM1200F, Morrell Instrument Company, Melville, NY, USA).

**Electrophoresis and immunoblot**

For quantitative Western blot analysis, homogenates from the hippocampus and cortex of the pups were processed 4–24 h after saline or ethanol injection as described previously (Lubin and Sweatt, 2007, Subbanna et al., 2013a, Subbanna et al., 2013b). Cytosolic and nuclear fractions from tissue homogenates were prepared as described in our recent publication (Basavarajappa and Subbanna, 2013). The samples were prepared in a sample buffer as previously described by our laboratory (Basavarajappa et al., 2008). The blots were incubated with the following primary antibodies: anti-rabbit-CC3 (Asp175) (polyclonal, #9661, 1:1000), anti-rabbit-G9a (monoclonal, #3306, 1:1000), anti-rabbit-H3K9me2 (monoclonal, #4658, 1:1000) anti-rabbit-H3K27me2 (monoclonal, #9728, 1:1000), anti-rabbit-Histone H3 (monoclonal, #9715, 1:1000), anti-rabbit-DNMT3A (monoclonal, #3598, 1:1000), anti-rabbit-MeCP2 (monoclonal, #3456, 1:1000) (Cell Signaling Technology), anti-mouse-cTau (monoclonal, #27027, 1:5000, Millipore, Billerica, MA, USA), and anti-mouse-β-actin (monoclonal, #ab24701, 1:5,000, Abcam, Cambridge, MA, USA). The blots were incubated with the primary antibodies for 3 h at room temperature or overnight at 4 °C and processed as previously described by our laboratory (Basavarajappa et al., 2008). Incubation with a secondary antibody alone did not produce any bands (data not shown).

**Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

For the qPCR studies, pups were sacrificed by decapitation 4–24 h after the first saline or ethanol injection, and the neocortex and hippocampus were dissected, flash frozen and stored at −80 °C. The samples were subjected to a total RNA preparation using the RNeasy mini kit (Qiagen, Valencia, CA). The mRNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using the Maxima First Strand cDNA Synthesis Kit from Fermentas on a programmable thermal cycler (PCR-Sprint, Thermo Fisher, Milford, MA, USA). A 193-bp fragment of Mus musculus G9a (Accession No. NM_145830.1) was amplified from 50 ng of cDNA in each of the real-time polymerase chain reactions using the Fermentas SYBR Green qPCR reagents (Thermo Fisher Scientific, Suwanee, GA, USA) in an Applied Biosystems StepOne PCR machine (Life Technologies, Carlsbad, California, USA). The detailed method for qPCR was similar to a previously published procedure (Subbanna et al., 2013a, Subbanna et al., 2013b). The following primers for the qPCR analysis were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA): forward, 5′-AGGAGCCAACATCAATGCCGTAGA-3′; reverse, 5′-TCAGTAGCAGTAGCATTCCA-3′; and probe, 5′-AAGCAACAACGACGCCACTAATGGA-3′. Hypoxanthinephosphoribosyltransferase (hprt) was used as an endogenous mRNA control. For DNMT3A and MeCP2, qPCR was performed with an integrated thermocycler and fluorescence detector ABI PRISM 7900HT Sequence Detector (Applied Biosystems) using TaqMan® Gene Expression Assays Mm00432881_m1 (dnmt3a), Mm00521967_m1 (mecp2) and 4352932 (gapdh) (Applied Biosystems). Gapdh was used as an endogenous mRNA control. Three independent runs were carried out for each set of samples. For each run, triplicate reactions were carried out for each sample. The obtained data were analyzed using SDS 2.4 software (Applied Biosystems). The amount of the target (G9a, dnmt3a and mecp2), normalized to an endogenous reference (hprt, gapdh) and relative to a calibrator, was determined using 2-ΔΔCt.
**Chromatin immunoprecipitation assay**

Chromatin Immunoprecipitation (ChIP) assay was performed as described elsewhere (Lubin et al., 2008, Martinez-Finley et al., 2011). For ChIP assay, 8 h after the first saline or ethanol injection, pups were sacrificed by decapitation and hippocampus and neocortex were dissected. Tissue (25 mg) was fixed by 1% formaldehyde, homogenized, and subjected to DNA shearing and the amount of sample normalized to contain equivalent protein amounts. Chromatin was immunoprecipitated with anti-acetyl histone H3K14 (# 07-353), anti-acetyl histone H4K8 (# 07-328) (Millipore, Billerica, MA, USA) antibodies and anti-dimethyl histone H3K9Me2 antibody. As a control, samples were immunoprecipitated with rabbit IgG (Millipore). Immune-complexes were collected with Protein A-agarose beads, cross-links were reversed, followed by protein digestion and DNA extraction. Immunoprecipitated DNA was subjected to quantitative real-time PCR with the RT² Sybr Green Master Mix (Thermo Fisher Scientific, Suwanee, GA, USA) using primers for mouse G9a exon I (mouse G9a 118 F 5'-CGAAGCCTGCTCTCGCT--3', mouse G9a 245 R 5'-GGGCTCCTTCTCCAGCA-3'). Relative quantification for acetylated and methylated histone associated gene in saline and ethanol group was calculated by the ΔΔCt method (Schmittgen and Livak, 2008).

**Immunoprecipitation assay**

Equal amounts of nuclear extract proteins from the cortices of either saline- or ethanol- (1.0 g/kg x2) treated mice were mixed with the antibody against G9a (#09-071, Millipore), and the resulting immunoprecipitation was processed as previously described (Rao et al., 2011). Immunoprecipitates (IPs) were washed and fractionated on polyacrylamide gels along with 5–10% of the supernatant from the IPs and the input. The gels were transferred to nitrocellulose membranes and immunoblotted with antibodies against G9a (#3309; 1:1000), DNMT3A (#3598; 1:1000) and MeCP2 (#3456; 1:1000) (Cell Signaling Technology).

**Statistical analysis**

Unless otherwise indicated, each experiment was performed at least in triplicate, and 10–15 pups were evaluated per treatment. All of the data are presented as the mean ± SEM. A statistical comparison of the data was performed by either a two-tailed Student’s t-test or a two-way ANOVA with Bonferroni’s post hoc test. In all of the comparisons, p < 0.05 was considered to indicate statistical significance. The statistical analyses were performed using Prism (GraphPad, San Diego, CA, USA).

**RESULTS**

**A low dose of ethanol induces mild neurodegeneration in the neonatal brain**

We first examined the effects of a low dose of ethanol exposure on neurodegeneration, G9a expression, H3 modification and H3K9 and H3K27 dimethylation in the hippocampus and cortex, two of the many brain regions affected by ethanol treatment in P7 mouse pups. We injected low concentrations of ethanol (1.0 g/kg body weight x 2) and measured the blood ethanol levels (BELs) at several intervals. This paradigm resulted in elevated BELs (Fig. 1A) and remained for 3–5 hours after the second dose of ethanol at the toxic threshold, which has been previously noted to produce mild apoptotic neurodegeneration in neonatal mice (Wozniak et al., 2004, Subbanna et al., 2013b).

We also examined the neurodegeneration induced by ethanol exposure in the brains of neonatal mice. We found a mild pattern of neurodegeneration throughout the forebrain, as indicated by caspase-3 activation [formation of cleaved caspase-3 (CC3)] in ethanol-exposed brains (Fig. 1B and C) (p < 0.001). These results confirmed previous studies.
describing neurodegeneration in various brain regions, including the hippocampus (Ikonomidou et al., 2000, Wilson et al., 2011, Subbanna et al., 2013a, Subbanna et al., 2013b). Subsequently, we also evaluated neurodegeneration in the hippocampus and cortex protein extracts by quantitative Western blot analysis. The results demonstrated that the caspase-3 activation was observed in the hippocampus (8 h) and neocortex (Fig. 1D) at 4 (p < 0.05) and 8 h (p ≤0.001).

**Neonatal exposure to a low dose of ethanol enhances G9a protein levels through transcriptional activation of the G9a gene**

In our previous studies, a high dose of ethanol enhanced G9a protein levels through transcriptional activation of the G9a gene (Subbanna et al., 2013b). To confirm that a low dose of ethanol also enhances G9a protein levels through transcriptional activation of the G9a gene, we first measured the G9a protein levels. Compared with the control, a low dose of ethanol increased the G9a protein levels in the hippocampus (F3, 28 = 21, p<0.001) and neocortex (F3, 28 = 22, p<0.001) (Fig. 2A) at all of the time points measured. Next, the G9a mRNA levels were determined, and we found that ethanol significantly enhanced the G9a mRNA levels at all of the time points measured in the hippocampus (F3, 28 = 58, p<0.001) and neocortex (F3, 28 = 109, p<0.001) (Fig. 2B). The levels of the endogenous mRNA control hprt were not significantly changed at any time point or in any of the brain regions examined (data not shown).

**Neonatal exposure to a low dose of ethanol enhances H3K14 acetylation at G9a exon1**

We used ChIP assay to determine whether G9a transcriptional activation involves specific epigenetic modification of histone proteins at the exon I of G9a gene. The results indicated that low dose of ethanol treatment increased acetylated H3K14 levels (Fig. 3A) at G9a exon I in the hippocampus and neocortex (p < 0.001). However, ethanol failed to alter acetylated H4K8 (Fig. 3B) or H3K9me2 (Fig. 3C) levels (p > 0.05) at the exon 1 of G9a gene. This suggests that transcriptional activation of G9a by ethanol treatment could be related to specific increase in histone H3K14 acetylation of G9a exon I gene.

**A low dose of ethanol enhances the G9a substrates H3K9me2 and H3K27me2**

Because we found enhanced G9a protein levels in ethanol-exposed neonatal mice, we used Western blot analysis to examine H3K9me2 and H3K27me2 levels following ethanol treatment. The results demonstrated that ethanol significantly enhanced H3K9me2 (Fig. 4A) and H3K27me2 (Fig. 4B) levels in the hippocampus (F3, 28 = 42, p<0.001) and neocortex (F3, 28 = 13, p<0.05) at all of the time points measured.

In our previous study, we found that all the H3K9me2-positive neurons were also stained for H3K27me2, suggesting that both H3K9me2 and H3K27me2 are confined to neuronal nuclei in the developing brain but not in GFAP-positive cells (Subbanna et al., 2013b). Here we assessed the association of H3K9me2 and CC3 or H3K27me2 and cTau in saline- and ethanol-exposed brain sections. H3K9me2 staining was found to be interspersed with CC3-positive neurons (Fig. 4C, white arrows; ethanol merged panel). Consistent with the Western blot data, H3K9me2 staining was increased in ethanol- compared to saline-treated brain sections (Fig. 4D) (p > 0.01). H3K27me2 staining was not found to be interspersed with cTau-positive neurons (Fig. 4E, ethanol merged panel). Consistent with the Western blot data, H3K27me2 staining was increased in ethanol- (Fig. 4F) compared to saline-treated brain sections (p > 0.01). Collectively, these findings suggest that enhanced G9a protein levels lead to an increase in H3K9me2 and H3K27me2 levels, and only H3K9Me2 is interspersed with neurons undergoing neurodegeneration in the developing brain.
Pharmacological in vivo inhibition of G9a activity prevents the dimethylation of H3K9 and H3K27 proteins and rescues neurodegeneration in neonatal mice

Because increased H3K9me2 is found within degenerating neurons, we next sought to identify whether an increase in G9a activity is responsible for ethanol-induced neurodegeneration. We used a potent and selective noncompetitive inhibitor of G9a activity (BIX 01294) (Kubicek et al., 2007, Subbanna et al., 2013b) due to the lack of viable G9a knockout mice, which die between embryonic days 9.5 and 12.5 (Tachibana et al., 2002). In our previous dose-dependent studies, the maximum inhibition of G9a-mediated ethanol-induced activation of caspase 3 (CC3 levels) was observed at 1 mg/kg (Bix) (Subbanna et al., 2013b). Administration of Bix 30 min before ethanol treatment was more effective in inhibiting G9a-mediated ethanol-induced activation of caspase-3 than co-treatment of ethanol and Bix together or administration of Bix 1 h after ethanol treatment (Subbanna et al., 2013b). Thus, we used Bix at 1 mg/kg in all of our present studies. Consistent with our previous findings with a high dose of ethanol (Subbanna et al., 2013b), the administration of Bix (1 mg/kg) before a low dose ethanol treatment did not alter the BELs, indicating that Bix does not modulate ethanol metabolism.

First, we tested whether Bix could prevent the enhanced dimethylation of H3K9 and H3K27 proteins observed after low dose ethanol treatment. We found that Bix prevented G9a-mediated ethanol-induced dimethylation of H3K9 and H3K27 (Fig. 5) in the hippocampus and neocortex (p < 0.001). A two-way ANOVA for the H3K9me2 or H3K27me2 total H3 ratio suggested a significant effect of ethanol (vs. saline) (hippocampus; H3K9me2; F_{1, 28} = 7, p < 0.01; H3K27me2; F_{1, 28} = 6, p < 0.05) (neocortex; H3K9me2; F_{1, 28} = 5, p < 0.05; H3K27me2 F_{1, 28} = 114, p < 0.001) and a significant interaction between ethanol and Bix (hippocampus; H3K9me2; F_{1, 28} = 18, p < 0.001; H3K27me2; F_{1, 28} = 7, p < 0.05) (neocortex; H3K9me2; F_{1, 28} = 13, p < 0.001; H3K27me2; F_{1, 28} = 218, p < 0.001) (**p < 0.01, ***p < 0.001). Bix significantly rescued the ethanol-induced increase in the H3K9me2/total H3 and H3K27me2/total H3 ratios. In the same experimental conditions, both ethanol and Bix alone failed to alter H3 levels (p > 0.05). Taken together, our results suggest that the inhibition of G9a activity by Bix prevents the ethanol-induced increase in H3K9me2 and H3K27me2 levels in the hippocampus and neocortex of the neonatal mouse brain.

The neurodegeneration markers CC3 and cTau were evaluated in cytosolic extracts by the Western blot method. The results strongly demonstrated that inhibition of G9a by Bix completely blocked cTau and CC3 generation (Fig. 6) in the hippocampus and neocortex (p < 0.001). A two-way ANOVA followed by Bonferroni’s post hoc test demonstrated significant effects of ethanol (vs. saline) (hippocampus; CC3; F_{1, 20} = 13, p < 0.001; cTau; F_{1, 20} = 7, p < 0.001) (neocortex; CC3; F_{1, 20} = 14, p < 0.001; cTau; F_{1, 20} = 29, p < 0.001) and a significant interaction between ethanol and Bix (hippocampus; CC3; F_{1, 20} = 13, p < 0.001; cTau; F_{1, 20} = 6, p < 0.001) (neocortex; CC3; F_{1, 20} = 16, p < 0.001; cTau; F_{1, 20} = 27, p < 0.001). Bix or vehicle alone had no significant effects on cTau or CC3 levels in the absence of subsequent ethanol treatment (p > 0.05).

G9a associates with the proteins DNMT3A and MeCP2, and a low dose of ethanol enhances DNMT3A and MeCP2 protein levels in the neonatal mouse brain

We examined the possible association of G9a with other gene-repressive proteins such as DNMT3A and MeCP2 (Kouzarides, 2002, Epstean-Litman et al., 2008). Immunoprecipitation experiments revealed that both DNMT3A and MeCP2 co-immunoprecipitated with the G9a antibody (Fig. 7A). Together, these results demonstrate that G9a associates with (and/or recruits) DNMT3A and MeCP2 and forms a repressive complex. Because we found an association of G9a with DNMT3A and MeCP2, we examined whether a low dose of ethanol could affect these proteins. Our results
demonstrated that ethanol significantly enhanced the DNMT3A protein (Fig. 7B) levels in the hippocampus ($F_{3, 28} = 42, p<0.001$), at all of the time points measured, and in the neocortex ($F_{3, 28} = 13, p<0.001$) at 4 and 8 h. In addition, ethanol also enhanced DNMT3A mRNA levels ($p < 0.05$) consistent with the protein levels (Fig. 7C). Ethanol significantly enhanced the MeCP2 protein levels in the hippocampus ($F_{3, 28} = 42, p<0.001$) and neocortex ($F_{3, 28} = 13, p<0.001$) at all of the time points measured (Fig. 7B). However, the MeCP2 mRNA levels were increased in the neocortex at 4 h and were reduced in the hippocampus at 4 and 8 h (Fig. 7D) by ethanol treatment.

**DISCUSSION**

In this study, we demonstrate that administration of a low dose of ethanol to P7 mice enhances the dimethylation of H3K9 and H3K27 through transcriptional activation of G9a by specific H3K14 acetylation of G9a gene, leading to mild neurodegeneration in the developing brain. Previously, we reported that G9a protein expression, which is significantly higher during early development compared to the mature brain, is entirely confined to the neuronal nuclei in the developing brain (Subbanna et al., 2013b). Pharmacological inhibition of G9a activity prior to ethanol treatment inhibited H3K9 and H3K27 dimethylation and rescued mild neurodegeneration in neonatal mice. Our findings are consistent with another study in which hair cell damage induced by aminoglycosides was rapidly followed by an increase in H3K9me2, preceding the apoptotic death of the hair cells. Inhibition of G9a/GLP protects the auditory hair cells from apoptotic death in the neonatal organ of Corti explants in vitro. Pre-conditioning with Bix also prevents hair cell apoptosis induced by neomycin in vivo and improves the hearing threshold (Yu et al., 2013). The regulation of gene expression during development is not only under the control of transcriptional machinery, but it is also affected by histone tail modifications (Hsieh and Gage, 2005, Wu and Sun, 2006) mediated by several histone modifying enzymes (Kosztolanyi, 2011), including G9a (Ding et al., 2008, Rao et al., 2010). Chromatin modification, such as histone acetylation, has been implicated as a critical mechanism involved in the regulation of gene expression that may underlie long-lasting changes in behavior (Barrett and Wood, 2008, McClung and Nestler, 2008, McQuown and Wood, 2010). Currently, very little is known about the specific histone acetyl transferase that regulate histone acetylation (Bekdash et al., 2013) implicated in ethanol effects within the developing hippocampus or neocortex. This is the first study to demonstrate that low dose of ethanol epigenetically enhances G9a gene activation through specific H3K14 acetylation at G9a exon 1. Further, this study reveals that the dimethylation of H3 proteins at K9 and 27, catalyzed by epigenetically enhanced G9a by a low dose of ethanol, is responsible for mild neonatal neurodegeneration.

G9a protein and mRNA levels are positively correlated with H3K9me2 and H3K27me2 levels. These findings add gravity to our previous demonstration of increased H3K9me2 levels followed by caspase-3-mediated degradation in high ethanol-exposed neonatal mouse hippocampus and neocortex (Subbanna et al., 2013b). However, such degradation of H3K9me2 was absent under a low dose of ethanol, which activates low levels of caspase-3 in P7 mice. Although additional studies are required, our observation seems to suggest that widespread activation of caspase-3 (Subbanna et al., 2013b) with nuclear morphological changes that occur in degenerating neurons (Kamada et al., 2005, Subbanna et al., 2013a) may be associated with degradation of H3K9me2. Previous studies have also indicated that maternal cocaine exposure during the second and third trimesters of gestation might produce profound modifications of the epigenetic programs of neonatal mice, leading to altered gene expression (Novikova et al., 2008). The ethanol-induced delay in neural development reported by many investigators [For references see, (Subbanna et al., 2013a, Subbanna et al., 2013b)] may be the result of an effect on the transcription of specific genes (Naus and Bechberger, 1991). Thus, it remains to be established that ethanol-induced activation of G9a...
during early brain development may alter H3 dimethylation at promoters of the genes encoding survival factor (s) (e.g., BDNF, pCREB etc.) (Kokubo et al., 2009). These survival factors may suppress the transcription of genes and promote a delay in neuronal development. It should be mentioned that G9a expression inhibits skeletal muscle differentiation in a methyltransferase activity-dependent manner (Ling et al., 2012).

Increased histone methyl transferase, including G9a expression, was found to be associated with worsening of specific symptoms, longer durations of illness and a family history of schizophrenia, a developmental synaptic disorder (Chase et al., 2013). Future chromatin immunoprecipitation studies coupled to genome-wide analysis will further unveil the impact of increased G9a-mediated H3 modification on specific gene expression and ethanol teratogenesis.

Our previous studies demonstrated that ethanol-induced apoptotic neurodegeneration results from the concomitant upregulation of G9a, and not all G9a/H3K9me2-positive neurons are undergoing neurodegeneration, implying that only enhanced G9a expression/H3K9me2 leads to neurodegeneration. In addition, it appears that not all neurons are sensitive to ethanol-induced apoptosis simultaneously (Ikonomidou et al., 2000). Consistent with our previous studies, increased G9a activity was associated with increased dimethylation of H3K9 and H3K27 proteins, suggesting that the G9a-mediated effects on neurodegeneration may be dependent upon its catalytic activity on histones. In vivo pharmacological inhibition of G9a activity with a specific inhibitor (Bix) strongly demonstrates the G9a-mediated mechanisms through which ethanol could induce neonatal neurodegeneration. G9a-deficient mice die between embryonic days 9.5 and 12.5 and display severe developmental growth retardation (Tachibana et al., 2002, Tachibana et al., 2005) due to aberrantly high levels of programmed cell death during embryogenesis (Tachibana et al., 2002). In another study, the demethylation of H3K9me3 by BIX-01294 resulted in significantly increased levels of Bad expression and consequent Leydig cell apoptosis (Choi et al., 2013). These observations imply that the tight control of G9a expression and its substrates may be required for the homeostatic regulation of crucial genes necessary for specific stages of brain development. In future experiments, it remains to be established that the activation of G9a and subsequent mild neuronal loss leads to any developmental defects during the early stages of development, and whether that activation could lead to the long lasting synaptic defects found in adult animals exposed to a high dose of ethanol at P7 (Izumi et al., 2005, Wilson et al., 2011, Sadrian et al., 2012, Subbanna et al., 2013a).

Although the identity of the H3K9 histone methyltransferase that binds to MeCP2 is still unclear, our data demonstrate that the G9a enzyme binds to MeCP2 and DNMT3A in the neonatal brain. Our observation seems to suggest that MeCP2 mRNA and translation into protein occur differentially with deregulated time kinetics in hippocampus and cortex brain regions in P7 mice treated with ethanol. The reason for such a dysregulation needs to be investigated in the future. Our observations reveal that G9a, potentially through built-in methyl-CpG-binding domains (Kouzarides, 2002), might be directly recruited to the sites of CpG methylation. Overall, enhanced DNMT3A and MeCP2 by ethanol, which also enhances G9a, may lead to a bigger repressive complex, and this repressive environment provides a mechanism for coordinated H3K9 and H3K27 (and possibly DNA) methylation by ethanol treatment; this could in turn suppress specific developmental regulators during early brain development. In another study, prenatal low dose ethanol treatment for several days also increased repressive marks (H3K9me2, G9a, Setdb1), DNMT1, and MeCP2, and a gestational choline treatment normalized the ethanol-induced increase in repressive marks (H3K9me2, G9a, Setdb1, DNMT1, and MeCP2) (Bekdash et al., 2013). Further studies are needed to determine whether G9a, DNMT3A and MeCP2 associate at a specific gene promoter and whether this association regulates specific gene expression.
In conclusion, the present study demonstrates that in vivo Bix treatment at P7 exerts neuroprotection against low dose ethanol-induced mild neurodegeneration in neonatal mice. Bix appears to be highly specific for G9a in animals in vivo (Maze et al., 2010, Gupta-Agarwal et al., 2012, Subbanna et al., 2013b), but the consequences of Bix treatment on human fetal development remain to be investigated. Furthermore, the results presented in this study indicate that chromatin structure is altered during ethanol-exposure in neonatal mice and may significantly contribute to ethanol teratogenesis. If pharmacological interventions can reduce this altered chromatin state in FASD, inducing a type of “genome softening,” then neuronal gene expression can be enhanced, thus allowing for an improved therapeutic response.

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**HIGHLIGHTS**

- Low dose ethanol treatment at P7 induces mild neurodegeneration.
- Results in enhanced acetylation of H3 on lysine 14 at G9a exon1 and G9a expression.
- Leads to enhanced dimethylation of H3K9 and H3 lysine 27.
- Blockade of G9a rescue H3K9 and H3K27me2 levels and neurodegeneration.
- Enhanced G9a, DNMT3A and MeCP2 directly associate to form a suppressive complex.
Fig. 1.
A low dose of ethanol induces apoptotic neurodegeneration in the P7 mouse brain. (A) Blood ethanol curve associated with low ethanol concentration regimens (after two doses of 1.0 g/kg ethanol). Each point on the line graph represents the mean ± SEM (n = 6 pups/group). (B) Coronal brain sections (hippocampus and retrosplenial cortex) from saline- and ethanol-treated animals were immunostained with an anti-rabbit CC3 antibody. The black arrows indicate CC3-positive neurons in the hippocampus and retrosplenial cortex. Scale bars = 200 µm. The respective images were enlarged to show CC3-positive cells (*). The scale bars represent 50 µm. (C) CC3-positive cells were counted in the hippocampus and retrosplenial cortex. The number of CC3-positive neurons was significantly higher in the ethanol-treated group compared to the saline group. (D) Western blot analysis of CC3 and β-actin expression in the hippocampus (HP) and retrosplenial cortex (NC) of saline- and ethanol-treated animals. The CC3/β-actin ratio was significantly higher in the ethanol-treated group compared to the saline group. The results are presented as mean ± SEM (n = 6 pups/group).
retrosplenial cortex (n = 10 pups/group) (Student’s t test). ***p < 0.001. Error bars, SEM.

(D) Western blot analysis of CC3 using cytosolic extracts (20 µg) of hippocampal and cortical samples from the saline and ethanol groups (n = 15 pups/group). The graphs represent the ratio of the proteins normalized to the expression of β-actin (ratio multiplied by an arbitrary factor to set the control to 100). *p < 0.05, ***p < 0.001. The error bars represent the SEM. HP, hippocampus; NC, neocortex.
A low dose of ethanol increases G9a protein expression through transcriptional activation of the G9a gene in the P7 mouse brain. (A) Western blot analysis of G9a and β-actin (loading control) in hippocampal and neocortical extracts from the saline and ethanol groups (n = 15 pups/group) [***p < 0.001; compared with respective saline (0 h) group]. (B) qPCR analysis of G9a mRNA expression in hippocampal and cortical extracts from the saline and ethanol groups (n = 6 pups/group) (***p < 0.001). All statistics were calculated using One-way ANOVA with Bonferroni’s post hoc tests. The error bars represent the SEM.
Fig. 3.
A low dose of ethanol enhances H3K14 acetylation at the exon1 of G9a gene. ChIP analysis of G9a exon1 gene in hippocampal and neocortical tissues from the saline and ethanol groups (n = 8 pups/group) with anti-acetylated H3K14 (A) or anti-acetylated H4K8 (B) or anti-H3K9me2 antibodies and levels of G9a exon 1 in the IPs were measured by quantitative PCR. [***p < 0.001; compared with respective saline group]. The error bars represent the SEM (Student’s t test).
Fig. 4.
A low dose of ethanol increases the dimethylation of H3K9 and H3K27 and their colocalization with degenerating neurons. (A–B) Western blot analysis of H3K9me2 and H3K27me2 levels in hippocampal and neocortical nuclear extracts from the saline or ethanol groups. β-actin and total H3 were used as a loading control. All statistics were calculated using One-way ANOVA with Bonferroni’s post hoc tests. The error bars represent the SEM. [***p < 0.001; compared with respective saline group]. (C) Retrosplenial cortex sections were dual-labeled with anti-mouse H3K9me2 and anti-rabbit CC3. Scale bars = 50 µm. The white arrows denote the association of H3K9me2 (merged ethanol panel) with CC3-positive neurons. (D) H3K9me2-positive cells were counted in the retrosplenial cortex (n =10 pups/group). (E) Retrosplenial cortex sections were dual-labeled with anti-rabbit H3K27me2 and anti-mouse cTau antibodies (E). Scale bars = 50 µm. (F) H3K27me2-positive cells were counted in the retrosplenial cortex (n =10 pups/group).
counted in the retrosplenial cortex (n =10 pups/group). The error bars represent the SEM (Student’s t test). **p < 0.01.
Fig. 5. G9a inhibition rescues the ethanol-induced increase in the dimethylation of H3K9 and H3K27 proteins. Mice pre-treated for 30 min with Bix (1 mg/kg) or vehicle were exposed to ethanol for 8 h. Western blot analysis of H3K9me2 and H3K27me2 in hippocampal and neocortex nuclear extracts from the four (S+V, E+V, S+Bix and E+Bix) groups (n = 15 pups/group). The total H3 and β-actin were used as a loading control. All statistics were calculated using Two-way ANOVA with Bonferroni’s post hoc tests. ***p < 0.001. The error bars represent the SEM. a, compared with respective saline control; b, compared with respective ethanol group in all of the bar graphs.
Fig. 6.
Pharmacological inhibition of G9a rescues low dose ethanol-induced neurodegeneration in the neonatal mouse brain. Mice pre-treated for 30 min with Bix (1 mg/kg) or vehicle were exposed to ethanol for 8 h. Brain samples from the four groups S+V, E+V, S+Bix and E+Bix (n = 15 pups/group) were collected 8 h after treatment and processed to analyze cTau and CC3 levels by Western blotting. β-actin was used as a loading control. Representative blots are shown for the hippocampal and neocortical cytosolic extracts. All statistics were calculated using Two-way ANOVA with Bonferroni’s post hoc tests. (***p < 0.001). The error bars represent the SEM. a, compared with respective saline control; b, compared with respective ethanol group in all of the bar graphs.
Fig. 7.
Association of G9a with DNMT3A and MeCP2 proteins in the P7 mouse brain and a low dose of ethanol enhances the DNMT3A and MeCP2 levels in P7 mice. (A) Cortical nuclear extracts were prepared from the brains of P7 mice and subjected to immunoprecipitation using a G9a antibody. Equal amounts of input, non-immune and immunoprecipitant were analyzed by Western blotting using G9a, DNMT3A and MeCP2 antibodies. NI, non-immune serum; IP, immunoprecipitant. (B) Western blot analysis of DNMT3A and MeCP2 protein levels in the hippocampus and neocortex nuclear extracts from the saline or ethanol groups. β-actin was used as a loading control. One-way ANOVA with Bonferroni’s post hoc tests. (C–D) qPCR analysis of DNMT3A and MeCP2 mRNA expression in the hippocampal and cortical extracts from the saline and ethanol groups (n = 6 pups/group) (Student’s t test) (*p < 0.05; **p < 0.01; ***p < 0.001). The error bars represent the SEM.