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G9a-Mediated Histone Methylation Regulates Ethanol-Induced Neurodegeneration in the Neonatal Mouse Brain

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Abstract

Rodent exposure to binge-like ethanol during postnatal day 7 (P7), which is comparable to the third trimester of human pregnancy, induces neuronal cell loss. However, the molecular mechanisms underlying these neuronal losses are still poorly understood. Here, we tested the possibility of histone methylation mediated by G9a (lysine dimethyltransferase) in regulating neuronal apoptosis in P7 mice exposed to ethanol. G9a protein expression, which is higher during embryogenesis and synaptogenic period compared to adult brain, is entirely confined to the cell nuclei in the developing brain. We found that ethanol treatment at P7, which induces apoptotic neurodegeneration in neonatal mice, enhanced G9a activity followed by increased histone H3 lysine 9 (H3K9me2) and 27 (H3K27me2) dimethylation. In addition, it appears that increased dimethylation of H3K9 makes it susceptible to proteolytic degradation by caspase-3 in conditions in which ethanol induces neurodegeneration. Further, pharmacological inhibition of G9a activity

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prior to ethanol treatment at P7 normalized H3K9me2, H3K27me2 and total H3 proteins to basal levels and prevented neurodegeneration in neonatal mice. Together, these data demonstrate that G9a mediated histone H3K9 and K27 dimethylation critically regulates ethanol-induced neurodegeneration in the developing brain. Furthermore, these findings reveal a novel link between G9a and neurodegeneration in the developing brain exposed to postnatal ethanol and may have a role in fetal alcohol spectrum disorders.

Keywords

Developing brain; Fetal alcohol syndrome; Methyltransferase; Neuronal loss; Bix

Introduction

Prenatal exposure to ethanol is one of the most common causes of birth defects (Jones and Smith, 1973) and can lead to fetal alcohol spectrum disorder (FASD) (Streissguth et al., 1990). FASD is one of the main causes of intellectual disability in Western nations (Mattson et al., 2011); it is characterized by neurobehavioral hallmarks, such as learning and memory deficits (Goodman et al., 1999; Mattson et al., 1999). As many as 1 in 100 children born in the United States and Canada are diagnosed with FASD (Chudley et al., 2005; May and Gossage, 2001). In murine models, the brain developmental period between postnatal days 4 and 10 (P4–10) is equivalent to the third trimester of pregnancy in humans (Bayer et al., 1993). The brain is particularly sensitive to ethanol during this period; therefore, binge models have been established to examine the effects of ethanol consumption on fetal brain development in humans (Gil-Mohapel et al., 2010). A single day of ethanol intoxication in P7 mice triggers a massive wave of neurodegeneration in several brain regions including hippocampus and cortex (Ikonomidou et al., 2000; Sadrian et al., 2012; Saito et al., 2010a; Wilson et al., 2011) and impairs spatial memory task performance into adulthood, and perturbs local and interregional brain circuit integrity in the olfacto-hippocampal pathway (Sadrian et al., 2012; Wilson et al., 2011; Wozniak et al., 2004).

Posttranslational modification of histone proteins play a role in normal and disease processes, and they have been implicated in several human developmental disorders (Campuzano et al., 1996; Gavin and Sharma, 2010; Makedonski et al., 2005; Petronis, 2003; Ryu et al., 2006; Warren, 2007). A well-studied histone modification mechanism in the nervous system is mediated by histone methylation (Tsankova et al., 2006). Dimethylation of histone H3K9 is correlated with transcriptional silencing, whereas 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 (HMTases), 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. In addition to histone acetylation and phosphorylation, histone methylation represents a basic molecular mechanism that regulates chromatin structure and influences changes in gene expression (Lagali et al., 2010). Recently, G9a and GLP were revealed to be essential for neuronal function during brain development (Schaefer et al., 2009) and many histone genes are affected by ethanol exposure (Zhou et al., 2011) during early development. Thus, histone modification may be another potential mechanism by which ethanol alters neuronal survival and may be an important candidate mechanism of postnatal ethanol-induced neurodegeneration. The present study was undertaken to evaluate G9a, histone methylation and their relationship with the apoptogenic effects of ethanol. We also examined the neuroprotective role of G9a inhibition against ethanol-induced neurodegeneration in neonatal mice.

Materials and methods

Animals and Treatment

C57BL/6J mice were housed in groups under standard laboratory conditions (12 hr light / 12 hr dark cycle) with food and water available ad libitum. Animal care and handling procedures followed Institutional (NKI IACUC) and National Institutes of Health guidelines. An ethanol treatment paradigm, which has been previously shown to induce robust apoptotic neurodegeneration in P7 mice (Olney et al., 2002) and causes no lethality, was used in the current study. Half of the pups (male and female) in each litter were treated subcutaneously (s. c.) with saline and the other half with ethanol at P7 (based on the day of birth) (1.0 or 2.5 g/kg s. c. at 0 h and again at 2 h) as described previously by our laboratory (Sadrian et al., 2012; Wilson et al., 2011). For blood ethanol levels (BEL), pups were euthanized by decapitation; truncal blood was collected at 1, 3, 5, 7 and 9 hr following the second ethanol injection. The concentrations of ethanol in pup serum were then determined using a standard alcohol dehydrogenase-based method (Lundquist, 1959). For the Bix experiments, Bix (Cayman, Michigan, USA) was dissolved in 10 µl of ethanol followed by a few drops of Tween 80 and then volume was made up with sterile saline solution. The Bix solution was administered by s. c. injection at a volume of 5 µl/g body weight 30 min before ethanol treatment. In some experiments, Bix was administered along with ethanol or after 1 h post ethanol treatment. The Bix vehicle solution was injected as a control. Mice were kept with the dams until the pups were sacrificed and their brains removed 4–24 h after the first saline/ethanol injection. Bix treatment did not alter P7 ethanol induced intoxication (sleeping time) at the time of brain harvest. Bix alone treated P7 mice looked normal like saline treated mice and did not cause any inflammation or bleeding in any of the organs. In some experiments, broad-spectrum caspase inhibitor, quinoline-Val-Asp(Ome)-CH₂-O-phenoxo (Q-VD-OPh) was administered by s. c. injection at a volume of 5 µl/g body weight 30 min before ethanol treatment. The brains were processed for several analyses, as described below. Three to 15 animals were used for each data point.

Immunohistochemistry

Previous studies indicate that the maximum caspase-3 activation (in one or more brain regions) was induced between 8 and 24 h after the first ethanol injection (Ikonomidou et al., 2000; Wilson et al., 2011). Therefore, 8 or 18 hours after the first ethanol/saline injection, the pups were anesthetized with isoflurane and perfused 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 (Wilson et al., 2011). Briefly, the free-floating sections obtained from ethanol- and saline-exposed brains (8 h of exposure) were immunostained using an antibody anti-rabbit cleaved caspase-3 (Asp175) (CC3) (polyclonal, #9961, 1:1000, Cell Signaling, Danvers, MA, USA) either by the ABC reagents (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA) with a peroxidase substrate (DAB) kit (Vector Labs) or by a dual immunofluorescence method, as described previously (Saito et al., 2007; Wilson et al., 2011). For dual labeling, anti-rabbit-G9a (polyclonal, #09-071, 1:100, Millipore, Billerica, MA, USA) and anti-mouse-caspase 3-cleaved-tau (cTau) (monoclonal, # 27027, 1:200, Millipore, Billerica, MA, USA) antibodies or anti-mouse NeuN (monoclonal #MAB377, 1:200, Millipore, Billerica, MA, USA) or anti-mouse-H3K9me2 (monoclonal #ab1220, 1:200, Abcam, Cambridge, MA, USA) or anti-rabbit-H3K27me2 (monoclonal #9728, 1:200, Cell Signaling, Danvers, MA, USA), or anti-mouse GFAP (monoclonal #3670, 1:200, Cell signaling) antibodies were applied, and the secondary antibodies conjugated with anti-rabbit Alexa Fluor 488 (# A21206, 1:200, Invitrogen, Grand Island, NY, USA) and anti-mouse Alexa Fluor 568 (#A11031, 1:200, Invitrogen, Grand Island, NY, USA) were used. In some experiments, anti-rabbit-G9a was counterstained with nuclear 4',6-Diamidino-2-phenylindole (DAPI). The primary antibodies

were omitted from the reactions as a control for secondary antibody specificity. Also, pre-incubation with blocking peptides for the anti-rabbit CC3, anti-mouse-cTau (GenScript, Piscataway, NJ, USA), anti-mouse H3K9me2 (Abcam, Cambridge, MA, USA), anti-rabbit G9a (Millipore, Billerica, MA, USA), anti-rabbit-H3K27me2 antibodies (Cell Signaling Technology, Danvers, MA, USA) completely blocked the immunostaining of these antibodies. The free-floating sections obtained from ethanol- and saline-exposed brains (18 h of exposure) were processed for F-J (Millipore, Billerica, MA, USA) staining (Saito et al., 2007; Wilson et al., 2011). The extent of the neurodegeneration detected by F-J staining was quantified as described previously (Wilson et al., 2011). All photomicrographs were taken through a 2.5X, 5X, 20X, or 40X objective with a Nikon Eclipse TE2000 inverted microscope attached to a digital camera (DXM1200F, Morrell Instrument Company, Melville, NY, USA).

Electron microscopy

For conventional electron microscopy (EM), 24 h after the first saline or ethanol injection, the pups were anesthetized with isoflurane and the pups brains (n = 4 pups/group) were fixed by cardiac perfusion using 2% glutaraldehyde-4% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, postfixed in 1% osmium tetroxide and processed as described previously (Yang et al., 2008). Images were captured on a digital camera (Hamamatsu; model C4742-95) using the Advantage charge-coupled device camera system software (Advanced Microscopy Techniques).

Electrophoresis and immunoblot

For Western blot analysis, 4–24 h after the first saline or ethanol injection, pups were sacrificed by decapitation, hippocampus and neocortex were dissected, flash frozen and stored at -80°C . Homogenates from the hippocampus and neocortex of the pups were processed as described previously (Lubin and Sweatt, 2007). Tissue homogenates containing freshly added 1% protease inhibitor mixture (Roche, Indianapolis, IN, USA) and phosphatase inhibitors were centrifuged at 7700 g for 1 min, and the supernatant (cytosolic fraction) was aspirated and stored at -80°C until use. The pellet (nuclear fraction) was then resuspended in a nuclear extraction buffer (Grabowski, 2005) and nuclear fraction was prepared according to the manufacturer instruction (Thermo Fisher Scientific, Suwanee, GA). The nuclear extracts were stored at -80°C until use. In some experiments, the cytosolic and nuclear fractions were mixed and referred to as total extracts. The samples were prepared in a sample buffer as previously described (Basavarajappa et al., 2008). Developmental brain samples were prepared by scarifying the P0 to P12 and P90 mice according to their date of birth respectively. The blots were incubated in primary antibody anti-rabbit CC3 (Asp175) (polyclonal, #9661, 1:1000, Cell Signaling), anti-rabbit-G9a (monoclonal, #3306, 1:1000, Cell Signaling), anti-rabbit-H3K9me2 (monoclonal #4658, 1:1000, Cell Signaling) anti-rabbit-H3K27me2 (monoclonal #9728, 1:1000, Cell Signaling, Danvers, MA, USA), or anti-rabbit-Histone H3 (polyclonal #9715, 1:1000, Cell Signaling, Danvers, MA, USA), anti-mouse-cTau (monoclonal #27027, 1:5000, Millipore, Billerica, MA, USA), anti-mouse- β -actin (monoclonal, #ab24701, 1:40,000, Abcam, Cambridge, MA, USA) for 3 h at room temperature or overnight at 4°C and processed as previously described by our laboratory (Basavarajappa et al., 2008). Incubation of blots with a secondary antibody (goat anti-mouse peroxidase conjugate, #AP 124P, 1:5000, Millipore; goat anti-rabbit, #AP132P, 1:5000, Millipore) alone did not produce any bands.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

For the qPCR studies, 4–24 h after the first saline or ethanol injection, pups were sacrificed by decapitation, 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). 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 (Umapathy et al., 2010). The primers for the qPCR analysis were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA): forward, 5'-AGGAGCCAACATCAATGCCGTAGA-3'; reverse, 5'-TCAGTAGCAGGCTGACCATTTCCTCA-3'; and probe, 5'-AAGCAACAACGCACGCCACTAATGGA-3'. Hypoxanthinephosphoribosyltransferase (HPRT) was used as an endogenous mRNA control. The detailed procedure for qPCR was similar to a previously published procedure (Umapathy et al., 2010). qPCR for histone H3 was performed with the integrated thermocycler and fluorescence detector ABI PRISM 7900HT Sequence Detector (Applied Biosystems) using the Pre-developed TaqMan® Gene Expression Assays, Mm00787223_s1 (H3f3b) 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. Data obtained was analyzed with the use of SDS2.4 software (Applied Biosystems). The amount of target (G9a, H3f3b), normalized to endogenous reference (Hprt, Gapdh) and relative to a calibrator was given by $-2\Delta\Delta Ct$.

G9a enzyme activity

G9a enzyme assay was done according to the manufacturer's instructions for the colorimetric assay kit (Epigentek, Brooklyn, NY, USA). Equal amounts of hippocampus and neocortex extracts from either saline- or ethanol treated mice (after 8h) were subjected to G9a enzyme activity in the presence and absence of G9a inhibitor, Bix (4 μ M). G9a enzymes transfer a methyl group to histone H3 substrate from Adomet to methylate the substrate at lysine 9. The methylated histone H3K9 was captured with a high-affinity antibody. The ratio or amount of methylated H3K9, which is directly proportional to enzyme activity, was quantified through a HRP conjugated secondary antibody-color development system. The HMT activity was then calculated based on the amount of methylated H3K9 converted by the G9a.

Statistical analysis

Unless indicated otherwise, the experiments evaluated with equal number pups per treatment and were performed in triplicate. All of the data are presented as the mean $\pm$ SEM. A statistical comparison of the data was performed by either a student's t test or one-way analysis of variance (ANOVA) 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 the Prism software (GraphPad, San Diego, CA).

Results

Activation of G9a during ethanol-induced neurodegeneration in the neonatal brain

We first injected ethanol (2.5 g/kg body weight $\times$ 2) and measured blood ethanol levels (BELs) at several time points. This paradigm resulted in persistently elevated BELs (Fig. 1A) that remained at or above the toxic threshold, which has been previously demonstrated to produce apoptotic neurodegeneration in neonatal mice (Ikonomidou et al., 2000).

We then examined ethanol-induced neurodegeneration in the brains of neonatal mice. We found widespread neurodegeneration throughout the forebrain [hippocampus, ($F_{1, 11} = 80$, $p < 0.001$) and cortex ($F_{1, 11} = 800$, $p < 0.001$) regions], as indicated by caspase-3 activation (generation of CC3) in ethanol-exposed brains (one-way ANOVA) (Fig. 1B and C). We subsequently evaluated neurodegeneration in hippocampal and neocortical protein extracts by Western blot analysis. Comparisons using one-way ANOVA with Bonferroni's post hoc tests indicated that the 8 and 24 h ethanol groups had significantly greater proportions of neuronal death in both the hippocampus ($F_{3, 28} = 90$, $p < 0.001$) and neocortex ($F_{3, 28} = 95$, $p < 0.001$) (Fig. 1D). It is interesting to note the long-lasting activation of caspase-3 after blood normalization (12 h) of ethanol levels. It seems that ethanol-induced cascades are still active even after blood normalization of ethanol levels. It also suggests that ethanol-induced activation of caspase-3 may not involve direct interaction with caspase-3.

Next, a conventional electron microscopy (EM) analysis was performed on neonatal mouse brains to examine ultrastructural changes caused by apoptosis. These analyses revealed that ethanol-exposed neurons exhibited developmentally regulated apoptotic features (Fig. 1E). The most striking ethanol-induced apoptotic changes involved the formation of one or more chromatin balls (CBs) of various sizes and densities in the nuclei (white arrows) and the migration of chromatin to the periphery or one pole of the nucleus (black arrows). Taken together, our experimental conditions recapitulated the apoptotic patterns and severity described for this ethanol treatment paradigm in the developing brain (Ikonomidou et al., 2000; Olney et al., 2002).

Because ethanol-induced neuropathology involves characteristic biochemical and morphological features that take place both outside and inside the nucleus, we tested the possible role of G9a in the nuclear changes experienced by apoptotic neuron in ethanol treated P7 mice. We first confirmed G9a nuclear localization in brain (Supplementary Fig. 1A and B). To identify patterns of G9a protein expression during mouse brain development, we used a well characterized G9a specific antibody (Ling et al., 2012; Ueda et al., 2006) which detects the G9a-L (165 kDa) and G9a-S (140 kDa) isoforms and these bands were close to each other in our 10% SDS PAGE. Protein extracts from mouse brain neocortex tissues at several developmental stages were determined by Western blot analysis. G9a protein levels were substantially higher during embryogenesis and synaptogenesis compared to the levels in the adult brain (P90) ($F_{8, 45} = 160$, $p < 0.001$) (Fig. 2A). Consistent with previous studies (Guo et al., 2011; Jacob et al., 2011), the levels of the housekeeping protein actin did not change significantly during various stages of brain development.

We used multiple approaches to examine the influence of ethanol exposure on G9a function. First, G9a protein levels were determined. Compared with control, ethanol increased G9a protein levels in the hippocampus ($F_{3, 28} = 21$, $p < 0.001$) and neocortex ($F_{3, 28} = 22$, $p < 0.001$) (Fig. 2B) at all time points measured. Second, G9a mRNA levels were determined. The results demonstrated that ethanol significantly enhanced G9a mRNA levels at all the time points measured in the hippocampus ($F_{3, 28} = 58$, $p < 0.001$) and neocortex ($F_{3, 28} = 109$, $p < 0.001$) (Fig. 2C). 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). Additional experiments were carried out to examine whether observed ethanol effects are dose-dependent. Results suggest that P7 ethanol treatment significantly enhanced G9a protein levels in a dose-dependent manner and lower dose (1gx2) which induces mild neurodegeneration ($p < 0.001$) was less effective than higher dose (2.5gx2) in both hippocampus ($F_{1, 20} = 90$, $p < 0.001$) and neocortex ($F_{1, 20} = 110$, $p < 0.001$) (one-way ANOVA) (Supplementary Fig. 2A & B). Third, we measured G9a enzyme activity. Consistent with the increased protein levels, G9a enzyme activity was also enhanced after ethanol exposure (8 h) compared with its activity in the saline-treated hippocampus ($F_{1, 20} =$

168, $p < 0.001$) and neocortex ($F_{1, 20} = 121$, $p < 0.001$) (one-way ANOVA) (Fig. 2D). The addition of a G9a-specific inhibitor (BIX 0129)4, 4 μ M (Bix) to the enzyme assay significantly reduced (55%) G9a activity in both saline and ethanol treated tissues. Two-way ANOVA revealed the significant effects of ethanol (vs. saline) ($F_{1, 28} = 365.6$, $p < 0.001$) and inhibitor Bix (vs. saline) ($F_{1, 28} = 117$, $p < 0.001$) and a significant interaction between ethanol and Bix ($F_{1, 28} = 24$, $p < 0.001$). Although P7 ethanol enhanced G9a protein expression in both the brain regions, the dramatic increase in G9a gene expression is not reflected in protein levels suggesting that ethanol-induced mRNA may be unstable or ethanol may be inhibiting additional enzymes which may be required for successful translation of G9a mRNA in to G9a proteins.

To examine the link between G9a expression and ethanol-induced neurodegeneration, we assessed the association of G9a and cTau (marker for neurodegeneration) in saline- and ethanol-exposed brain sections. Recent studies have revealed that ethanol induces Tau cleavage at Asp421/422 through caspase-3 activation in ethanol-exposed mouse brains (Saito et al., 2010b). In both control and ethanol-treated samples, G9a was widely expressed in all brain regions, and the cingulate cortex region is shown as an example. Confocal images revealed G9a nuclear expression in cTau-positive neurons in the ethanol-treated brain. cTau staining was observed in the cell soma and neurites, whereas G9a association was observed only in the soma of degenerating neurons (Fig. 2E).

H3 modification after ethanol exposure in neonatal mice

As we found enhanced G9a activity in ethanol exposed neonatal mice, we examined H3K9me2 and H3K27me2 levels following ethanol treatment by Western blot analysis (Fig. 3A). Surprisingly, the results demonstrated that ethanol significantly reduced H3K9me2 levels in the hippocampus ($F_{3, 28} = 42$, $p < 0.001$) and neocortex ($F_{3, 28} = 13$, $p < 0.001$) when blots were normalized with β -actin as a loading control [Fig 3A (i)] at 8 and 24h. P7 ethanol did not reduce H3K27me2/ β -actin ratio in the hippocampus ($F_{3, 28} = 1.6$, $p > 0.05$) and neocortex ($F_{3, 28} = 1.5$, $p > 0.05$) [Fig. 3A (ii)].

This specific reduction in dimethylated H3K9 protein/ β -actin ratio prompted us to further examine total histone H3 protein levels using an H3 C-terminal-specific antibody. We found that ethanol significantly reduced the amount of total histone H3 at 8 and 24 h after treatment in the hippocampus ($F_{3, 28} = 24$, $p < 0.001$) and neocortex ($F_{3, 28} = 55$, $p < 0.001$) [Fig. 3A (iii)]. This effect of ethanol on total H3 proteins appears to be specific because ethanol in the same condition failed to change total H4 proteins in the hippocampus ($F_{3, 28} = 1$, $p > 0.05$) and neocortex ($F_{3, 28} = 2$, $p > 0.05$) at any time points measured (Supplementary Fig. 3). To address this issue further, we calculated H3K9me2 or H3K27me2/total H3 ratio. The results demonstrated that ethanol significantly enhanced H3K9me2/H3 ratio in the hippocampus ($F_{3, 28} = 32$, $p < 0.001$) and neocortex ($F_{3, 28} = 118$, $p < 0.001$) [Fig. 3A (iv)]. Similar increase was also observed in H3K27me2/H3 ratios in the hippocampus ($F_{3, 28} = 213$, $p < 0.001$) and neocortex ($F_{3, 28} = 259$, $p < 0.001$) [Fig. 3A (v)]. We also measured the mRNA transcripts for H3 in the ethanol (treated for 8h) samples. Ethanol slightly enhanced the amount of mRNA in the hippocampus ($p < 0.05$), with no significant changes in the neocortex ($p > 0.05$) (Fig. 3B) suggesting the loss of total H3 is not due to inhibition of its transcription. Altogether, these findings suggest that ethanol enhances H3K9 and K27 dimethylation but dimethylated H3K9 were undergoing degradation without affecting H3K27.

We also examined the link between H3K9 and H3K27 dimethylation and ethanol-induced neurodegeneration. We initially sought to identify the staining pattern of H3K9me2 and H3K27me2 in P7 mice brain sections. We found that all the H3K9me2 positive neurons were also stained for H3K27me2 suggesting that both H3K9me2 and H3K27me2

(Supplementary Fig. 4A) are associated with same population of cells in the developing brain. In addition, all the H3K27me2 positive cells were associated with NeuN positive cells (Supplementary Fig. 4B) but not GFAP positive cells (Supplementary Fig. 4C). These results together suggest that H3K9me2 and H3K27me2 are confined to neuronal nuclei in the developing brain. Further, we assessed the association of H3K9me2 and CC3 or H3K27me2 and cTau in saline- and ethanol-exposed brain sections. H3K27me2 staining is increased in ethanol compared to saline treated brain sections (Fig. 3C) (Supplementary Fig. 4D) and majority of H3K27me2 staining was found to be associated with cTau positive neurons [white arrows; Fig. 3C ethanol merged panel]. Consistent with Western blot data, H3K9me2 staining is reduced in ethanol (Fig. 3D) (Supplementary Fig. 4D) compared to saline treated brain sections and found no association of H3K9me2 staining with CC3 positive neurons (Fig. 3D ethanol merged panel) possibly due to H3K9me2 degradation. These findings collectively raise a question that enhanced G9a expression may regulate caspase-3 activation and caspase-3 inhibitors could reverse the ethanol-induced degradation of H3 proteins.

To test this possibility, we used a third generation dipeptidyl broad-spectrum caspase inhibitor, Q-VD-OPh, in our experiments. Q-VD-OPh (Renolleau et al., 2007) inhibits caspase-3 cleavage into its 17 kDa active fragment in vivo in P7 rats. This compound was significantly more effective and non toxic than the widely used caspase-3 inhibitors in preventing apoptosis [for references see (Renolleau et al., 2007)]. We found that Q-VD-OPh rescued ethanol-induced formation of CC3 (hippocampus; $F_{1,28} = 80$, $p < 0.001$; neocortex; $F_{1,28} = 95$, $p < 0.001$) and prevented H3K9me2 (hippocampus; $F_{1,28} = 15$, $p < 0.01$; neocortex; $F_{1,28} = 25$, $p < 0.001$) and total H3 protein (hippocampus; $F_{1,28} = 12$, $p < 0.01$; neocortex; $F_{1,28} = 29$, $p < 0.001$) degradation in the hippocampus and neocortex (Figs. 4A and B). Saline and Q-VD-OPh-saline groups were not significantly different ($p > 0.05$). In addition, we also found enhanced H3K9me2 levels ($p < 0.01$) in the hippocampus and neocortex of ethanol treated P7 mice due to inhibition of ethanol-induced activation of caspase-3 by Q-VD-OPh. Taken together, our results suggest that ethanol-induced degradation of H3 proteins by caspase-3 occurs through G9a mediated dimethylation of H3K9.

Pharmacological in vivo inhibition of G9a activity prevents ethanol-induced H3 modification and apoptotic neurodegeneration in neonatal mice

We then sought to identify whether increased G9a mediated H3K9me2 and H3K27me2 are responsible for ethanol-induced neurodegeneration. We used a potent and selective noncompetitive inhibitor of G9a activity (BIX 01294) (Kubicek et al., 2007) due to the lack of viable G9a knockout mice, which die between embryonic days 9.5 and 12.5 (Tachibana et al., 2002). We evaluated the effects of ethanol (8 h) on neurodegeneration and total histone H3, H3K9me2 and H3K27me2 levels in the presence and absence of Bix treatment. We determined the effects of various doses of Bix (0.25, 0.5, and 1 mg/kg) on ethanol-induced activation of caspase 3 in neocortex. Our results suggest that Bix dose-dependently inhibited G9a mediated ethanol-induced activation of caspase 3 (CC3 levels) ($F_{3,28} = 14$, $p < 0.001$) (Fig. 5A). Thus, we used Bix at 1mg/kg in all our subsequent studies. Administration of Bix 30 min before ethanol treatment was more effective in inhibiting G9a mediated ethanol-induced activation of caspase-3 than co-treatment (ethanol and Bix together) or administration of Bix 1h after ethanol treatment (supplementary Fig. 5 A). Administration of Bix (1mg/kg) before ethanol treatment did not alter the BELs (Supplementary Fig. 5 B), indicating that Bix does not modulate ethanol metabolism.

We used several approaches to investigate the neuroprotective effects of Bix on G9a mediated ethanol-induced neurodegeneration. First, immunohistochemistry with CC3 demonstrated the complete inhibition of CC3 generation in the Bix-ethanol groups

(compared with ethanol group) in the brain regions studied (hippocampus, ($F_{3, 33} = 95$, $p < 0.001$; cortex, $F_{3, 33} = 766$, $p < 0.001$, two-way ANOVA) (Fig. 5B). Two-way ANOVA demonstrated the significant effects of ethanol (vs. saline) and a significant interaction between ethanol and Bix treatment. Saline and Bix-saline groups were not significantly different ($p > 0.05$), whereas ethanol mice had significantly greater proportions of neuronal death in both the retrosplenial cortex and the hippocampus (treatment $\times$ CC3-positive cells/ mm^2) ($p < 0.001$) (Fig. 5C). Second, the neurodegeneration markers CC3 and cTau were evaluated in cytosolic extracts by Western blot method. The results strongly demonstrated that inhibition of G9a by Bix completely blocked cTau and CC3 generation (Fig. 5D) in the hippocampus and neocortex ($p < 0.001$). Two-way ANOVA with Bonferroni's post hoc demonstrated the 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 ($p > 0.05$). Third, the extent of neurodegeneration was evaluated by Fluoro-Jade (F-J) staining (Wilson et al., 2011). The results demonstrated a lack of F-J-positive neurons in the Bix-ethanol group (compared with the ethanol group) in the studied brain regions (hippocampus, ($F_{3, 12} = 400$, $p < 0.001$; neocortex, $F_{3, 12} = 695$, $p < 0.001$, two-way ANOVA) (Fig. 5E). Two-way ANOVA demonstrated the significant effects of ethanol (vs. saline) and a significant interaction between ethanol and Bix treatment. Saline and Bix-saline groups were not significantly different ($p > 0.05$), whereas ethanol mice had significantly greater proportions of neuronal death in both the retrosplenial cortex and the hippocampus (treatment $\times$ FJ-positive cells/ mm^2) ($p < 0.001$). Bix pre-treatment before ethanol administration completely prevented neurodegeneration in both brain regions compared to the ethanol-treated mice (Fig. 5E). Representative images used for F-J counting are provided in Supplementary Fig. 5C.

Next, we tested whether Bix rescue reduced H3K9me2 and total H3 proteins observed after ethanol treatment. We found that Bix rescued G9a mediated ethanol-induced decrease in H3K9me2 levels and prevented total H3 proteins degradation in the hippocampus and neocortex (Figs. 6 i and ii) ($p < 0.001$). In the same experimental conditions, either ethanol or Bix altered H3K27me2 levels (Fig. 6 i and ii) ($p > 0.05$). Two-way ANOVA for the total H3/ β -actin ratio suggest the significant effects of ethanol (vs. saline) (hippocampus; $F_{1, 28} = 13$, $p < 0.001$; neocortex; H3; $F_{1, 28} = 11$, $p < 0.001$) and a significant interaction between ethanol and Bix (hippocampus; H3; $F_{1, 28} = 10$, $p < 0.001$; neocortex; $F_{1, 28} = 18$, $p < 0.001$). Saline and Bix-saline groups were not significantly different ($p > 0.05$). Further, two-way ANOVA for the H3K9me2 or H3K27me2/ total H3 ratio suggest the significant effects 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 ethanol increased H3K9me2/total H3 and H3K27me2/total H3 ratio without affecting the basal levels of H3K9me2 and H3K27me2 ($p > 0.05$) (Fig. 6iii and iv). Taken together, our results suggest that inhibition of G9a activity by Bix rescued ethanol-induced changes in H3K9me2 and H3K27me2 levels and prevented total H3 protein degradation in the hippocampus and neocortex of neonatal mice brain.

Discussion

In this study, we demonstrate that single-day binge like ethanol intoxication on P7 induces enhanced H3K9 and H3K27 dimethylation through transcriptional activation of G9a leading

to enhanced neurodegeneration in the developing brain. This conclusion is supported by the demonstration that G9a protein expression, which is significantly enhanced during early brain development compared to that in the mature brain, is entirely confined to the neuronal nuclei in the developing brain, as previously demonstrated in cell lines (Esteve et al., 2005). Pharmacological inhibition of G9a activity prior to ethanol treatment prevented neurodegeneration in neonatal mice. It should be noted that changes in gene expression during development are not only controlled by the transcriptional machinery; but also affected by modifications of histone tails (Hsieh and Gage, 2005; Wu and Sun, 2006) that are mediated by several histone modifying enzymes (Kosztolanyi, 2011), including G9a (Ding et al., 2008; Rao et al., 2010). This study reveals a major role for the G9a in the developing mouse brain exposed to ethanol and their influence on neonatal neurodegeneration.

To the best of our knowledge, this is the first study on the effect of postnatal ethanol exposure on G9a and histone H3 modification in the developing brain. However, previous studies have indicated that maternal cocaine exposure during the second and third trimesters of gestation, might produce profound structural and functional modifications of the epigenetic programs of neonatal mice (Novikova et al., 2008). The ethanol-induced delay in neural development reported by many investigators [For review see, ((Medina, 2011))] may be the result of an effect on the transcription of specific genes (Naus and Bechberger, 1991). Thus, it is possible that during early brain development, ethanol-induced activation of G9a may suppress transcription of genes encoding for survival factor (s) (e.g., BDNF, pCREB etc.) (Kokubo et al., 2009) that induces a delay in neuronal development. Future chromatin immunoprecipitation studies coupled to genome wide analysis will further unveil the impact of increased G9a expression on specific gene expression and function in FASD models.

Furthermore, our data demonstrate that ethanol-induced G9a expression is associated with neurodegeneration marker, cTau, implying that ethanol-induced apoptotic neurodegeneration results from the concomitant upregulation of G9a. It should be noted that 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 at the same time (Ikonomidou et al., 2000). Increased G9a activity was associated with increased dimethylation of H3K9 and H3K27 proteins suggesting that G9a mediated effects on neurodegeneration may be dependent of 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. Functionally, G9a is crucial for more differentiated somatic cells and may be dispensable for the survival of undifferentiated cells (Tachibana et al., 2002). It should be noted that 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). It was demonstrated that this developmental growth arrest is at least in part due to aberrantly high levels of programmed cell death during embryogenesis (Tachibana et al., 2002). Taken together, the evidence suggests that tightly controlled G9a expression is required for the homeostatic regulation of crucial genes necessary for specific stages of brain development. Thus, activation of G9a and subsequent neuronal loss could cause serious developmental defects during early stages of development, which may lead to long-lasting synaptic dysfunction in the adult animals (Izumi et al., 2005; Sadrian et al., 2012; Wilson et al., 2011) due to ethanol administration during early brain development.

This study also suggests that these G9a-induced histone modifications may be responsible for ethanol-induced neurodegeneration because Bix inhibited ethanol-induced G9a activity as well as increased formation of H3K9me2 and H3K27me2 proteins. In addition, our

findings suggest that ethanol activated caspase-3 is in fact responsible for H3 protein degradation. Although H3K9me2 and H3K27me2 are associated with same population of neurons but not with glial cells, the ethanol-induced loss of H3 proteins may be specific to dimethylation of H3K9 proteins. Similar ethanol treatment conditions did not alter total H4 proteins. Many studies have revealed that histone levels are controlled by several distinct mechanisms, including transcriptional control (Osley, 1991) and protein stability through phosphorylation and ubiquitination (Singh et al., 2009), as well as acetylation and methylation (Strahl and Allis, 2000). Clearly, posttranslational modification of histone proteins have a profound effect on chromosome stability, transcription, cell growth and survival (Libuda and Winston, 2010). Many substrates for caspase-3 have been identified in the nucleus (Fischer et al., 2003) and caspase-3 is believed to play an important role in the nuclear morphological changes that occur in apoptotic cells (Kamada et al., 2005). Thus, our study, for the first time, suggests that methylated H3 proteins may act as substrates for activated caspase-3 due to ethanol-induced neurotoxicity in the developing brain.

The present study demonstrates that in vivo Bix treatment at P7 exerts neuroprotection against ethanol-induced neuroapoptosis in neonatal mice. We used an acute dose of ethanol that achieved high BELs that were maintained for more than 12 h, and nonetheless, Bix treatment reversed the ethanol-induced neurodegeneration without effecting ethanol metabolism. Another important aspect of our study is that a biologically active G9a inhibitor produced no toxic effects by itself in P7 mice but rescued ethanol-induced neonatal neurodegeneration (Fig. 7) when administered before or with ethanol but not after ethanol-induced onset of neurodegeneration. As discussed above, tightly controlled G9a expression is required for proper brain development, it is possible that the disruption caused by inhibiting (acute) G9a in the saline treated P7 mice is small compared with the extensive damage that occurs with P7 ethanol exposure and/or may have effects on other aspects of brain development that are not detected with the measures used in the current study. It should be mentioned that G9a expression inhibits skeletal muscle differentiation in a methyltransferase activity-dependent manner (Ling et al., 2012). In another study, pharmacological modulation of another histone H3 (K9) methyltransferase that trimethylates the H3 protein and follows different gene silencing mechanisms was confirmed to be an important target for neuronal survival and function in Huntington's disease (HD) (Ryu et al., 2006). In conclusion, Bix appears to be highly specific for G9a in vivo in animals (Gupta-Agarwal et al., 2012; Maze et al., 2010), although the consequences of Bix treatment on human fetal development are currently unknown, this study provides incentive to explore the clinical potential of well characterized G9a inhibitor against developmental synaptic disorders, including FASD and many drugs that have similar mechanisms of action as ethanol such as anticonvulsant, anesthetics and analgesics (Ikonomidou et al., 2001; McCann and Soriano, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Ethanol treatment at P7 induces apoptotic pattern described for binge model of FASD.
- Results in enhanced G9a expression and increased H3K9me2 and H3K27me2/H3 ratios.
- Leads to H3K9me2 specific degradation of H3 proteins by activated caspase-3.
- Blockade of G9a before ethanol exposure rescue H3K9me2, H3K27me2 and H3 levels.
- Bix 01294 prevents neurodegeneration and may have a clinical potential against FASD.

Fig. 1.

Blood ethanol levels as a function of post-treatment time and ethanol - induced apoptotic neurodegeneration in the P7 mouse brain. (A) Blood ethanol curves associated with the ethanol regimen. Each point on the line graph represents the mean $\pm$ 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. Black and white arrows indicate CC3-positive neurons in the hippocampus and retrosplenial cortex respectively. Scale bars = 200 μ m. The respective images were enlarged to show CC3 positive cells (*). The scale bars represent 50 μ m. (C) CC3-positive cells were counted in the hippocampus and the retrosplenial cortex ($n = 10$ pups/group). Statistical analysis was calculated using Student's t test; *** $p < 0.001$. Error bars, SEM. (D) Western blot analysis of CC3 using cytosolic extracts (20 μ g) 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). One-way ANOVA with Bonferroni's post hoc tests; * $p < 0.05$, *** $p < 0.001$. Error bars, SEM. HP, hippocampus; NC, neocortex. (E) Coronal brain sections processed for conventional electron microscopic analysis ($n = 3-4$ pups/group). Representative areas of the CA1 region of the hippocampus and retrosplenial cortex are shown. N, nucleus. Scale bars = 1 μ m. A neuron in an early apoptotic stage with small condensed CBs in the nucleus (white arrow) and a nucleus undergoing separation of its elements (black arrows).

Fig. 2.

Expression of G9a during mouse brain development and the influence of ethanol on G9a expression and activity in the P7 mouse brain. (A) Western blot analysis of G9a expression during mouse brain development using total neocortical extracts. Representative blot shows developmental changes in G9a expression (all data were compared with the P90 group) (** $p < 0.01$; *** $p < 0.001$ $n = 10$ pups/group). (B) 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) [*** $p < 0.01$; *** $p < 0.001$; compared with respective saline (0 h) group]. (C) qPCR analysis of G9a mRNA expression in hippocampal and cortical extracts from the saline and ethanol groups ($n = 6$ pups/group) (** $p < 0.01$; *** $p < 0.001$). (D) G9a enzyme activity was determined in hippocampal and cortical extracts ($n = 8$ pups/group) by measuring the transfer of methyl groups to histone lysine side chains in the presence (+) or absence (-) of Bix (** $p < 0.01$; *** $p < 0.001$). All statistical analysis was done using One or Two-way ANOVA with Bonferroni's post hoc tests. Error bars, SEM. HP, hippocampus; NC, neocortex. (E) Retrosplenial cortex sections were dual-labeled with anti-rabbit G9a and anti-mouse cTau antibodies. i, saline; ii, ethanol; iii, higher magnification image of ethanol-treated sections; iv, confocal image showing a degenerating neuron. Scale bar = 20 μ m. White arrows denote the association of G9a with degenerating neurons.

Fig. 3.

Effect of P7 ethanol treatment on H3 modification. (A) Western blot analysis of H3K9me2 (i, $**p < 0.01$, $***p < 0.001$), H3K27me2 (ii, $p > 0.05$) and total H3 (iii, $***p < 0.001$) in hippocampus and neocortex nuclear extracts from saline or ethanol groups. β -actin was used as a loading control. Because, P7 ethanol treatment reduced total H3 proteins, in some experiments, we have calculated H3K9me2 (iv), H3K27me2 (v) and total H3 ratios [$*p < 0.05$, $**p < 0.01$, $***p < 0.001$; compared with respective saline (0 h) group]. (B) qPCR analysis of H3 mRNA in hippocampal ($*p < 0.05$) and neocortex ($p > 0.05$) extracts from saline and ethanol (treated for 8h) groups ($n = 6$ pups/group) (compared with the saline group). All statistics were calculated using One or Two-way ANOVA with Bonferroni's post hoc tests. Error bars, SEM. HP, hippocampus; NC, neocortex. (C, D) Retrosplenial cortex sections were dual-labeled with anti-mouse H3K9me2 and anti-mouse-CC3 or anti-rabbit H3K27me2 and anti-mouse cTau antibodies. Scale bars = 50 μ m. The white arrows denote the association of H3K27me2 (C, merged ethanol panel) or H3K9me2 (D, merged ethanol panel) with degenerating neurons.

Fig. 4.

Pharmacological inhibition of caspase-3 rescues P7 ethanol-induced cleavage of caspase-3 and prevents H3K9me2 and H3 protein degradation in the neonatal mouse brain. (A) Mice pre-treated for 30 min with Q-VD-Oph (1 mg/kg) or vehicle were exposed to ethanol and CC3 levels were determined by a Western blot analysis. β -actin was used as a loading control. Data were compared between S+V vs. E+V and E+V vs. E+Q-VD-Oph ($n=10$ pups/group; *** $p < 0.001$) (i). (B) Western blot analysis of total H3 and H3K9me2 (i and ii, # $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$) in hippocampus (i) and neocortex (ii) nuclear extracts from saline or ethanol groups with and without Q-VD-Oph. β -actin was used as a loading control. Because, P7 ethanol treatment reduced total H3 proteins, in some experiments, we have calculated H3K9me2 and total H3 ratios (iii) (* $p < 0.05$, # $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$). Statistical analysis was calculated using Two- and one-way ANOVA with Bonferroni's post hoc tests (in one-way ANOVA, the comparison was performed between S+V vs. E+V and E+V vs. E+Q-VD-Oph). Error bars, SEM. a, compared with respective saline control; b, compared with respective ethanol group. #, compared with E+V. HP, hippocampus; NC, neocortex.

Fig. 5.

Pharmacological inhibition of G9a rescues P7 ethanol-induced neurodegeneration in the neonatal mouse brain. (A) Mice pre-treated for 30 min with various doses of Bix (0, 0.25, 0.5 and 1 mg/kg) or vehicle were exposed to ethanol and CC3 levels were determined by a Western blot analysis and data were compared with 0 concentration of Bix in both P7 mice treated with saline or ethanol ($n = 6$ pups/group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (B) Mice pre-treated (30 min) with Bix (1 mg/kg) or vehicle were exposed to ethanol and were perfusion-fixed 8 h after treatment, and the brain sections from four groups [saline + vehicle (S+V), ethanol + vehicle (E+V), saline + Bix (S+Bix) and ethanol + Bix (E+Bix)] were stained with the anti-rabbit CC3 antibody. The images show the retrosplenial cortex and hippocampus. White arrows denote the CC3-positive neurons in the hippocampus and neocortex. Scale bar = 200 μ m. The respective images were enlarged to show CC3 positive cells (*). The scale bars represent 50 μ m. (C) CC3-positive cells were counted in the hippocampus and the retrosplenial cortex ($n = 15$ pups/group). *** $p < 0.001$. (D) Brain samples from four groups S+V, E+V, S+Bix and E+Bix ($n = 15$ pups/group) were collected 8 h after treatment and processed for Western blotting to analyze cTau and CC3 levels. β -actin was used as a loading control. Representative blots are shown for the hippocampal and neocortical cytosolic extracts (*** $p < 0.001$). HP, hippocampus; NC, neocortex. (E) Mice were perfused at 18 h after treatment, and brain sections from four groups (S+V, E+V, S+Bix and E+Bix) were stained with F-J. F-J-positive cells were counted in the hippocampus and the retrosplenial cortex ($n = 15$ pups/group; *** $p < 0.001$). Representative images used for counting are shown in supplementary Fig. 5C. Statistical analysis was calculated using Two- and one-way ANOVA with Bonferroni's post hoc tests (in one-way ANOVA, the comparison was performed between S+V vs. E+V and E+V vs. E+Bix). Error bars, SEM. a, compared with respective saline control; b, compared with respective ethanol group in all the bar graphs.

Fig. 6.

G9a inhibition rescue ethanol-induced increase in H3K9me2, H3K27me2 and reduction in total histone H3 proteins. Western blot analysis of total H3 (i and ii, $**p < 0.01$), H3K9me2 (i and ii, $**p < 0.01$), H3K27me2 (i and ii, $p > 0.05$) and β -actin (loading control) in hippocampal and neocortex nuclear extracts from the four (S+V, E+V, S+Bix and E+Bix) groups ($n = 15$ pups/group). P7 mice were treated with ethanol for 8h and Bix was pretreated for 30 min before P7 ethanol treatment. Western blot data of H3K9me2 (iii and iv, $**p < 0.01$; $***p < 0.001$) and H3K27me2 (iii and iv, $**p < 0.01$; $***p < 0.001$) were also calculated using total H3 as a loading control. Statistical analysis was done using One-way ANOVA with Bonferroni's post hoc tests. Error bars, SEM. a, compared with respective saline control; b, compared with respective ethanol group in all the bar graphs. HP, hippocampus; NC, neocortex.

Fig. 7.

A schematic representation of proposed mechanism of action by which G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in neonatal mice. Ethanol treatment at P7 enhances G9a expression and activity ($\rightarrow$) leading to caspase-3 activation. Enhanced G9a in turn increases net H3K9 dimethylation and H3K27me2/H3 ratio. These events lead to specific degradation of dimethylated H3K9me2, caspase-3 and tau proteins leading to neurodegeneration in neonatal mice. Inhibition of G9a activity ($\dashv$) prevents ethanol-induced increase in net H3K9 dimethylation and H3K27me2/H3 ratio, degradation of dimethylated H3K9 proteins and rescue neurodegeneration (Tau and caspase-3 cleavage) in neonatal animals. In addition, pharmacological inhibition of ethanol-induced caspase-3 activation also prevented generation of CC3 and degradation of H3K9me2 proteins. The

putative G9a mediated events may have a potential role in improving neuronal deficits observed in FASD.