# Acute Ethanol Alters Multiple Histone Modifications at Model Gene Promoters in the Cerebral Cortex

Andrey Finegersh and Gregg E. Homanics

**Background:** Ethanol (EtOH) exposure alters gene expression in the cerebral cortex (CCx); however, mechanisms of EtOH-induced gene regulation are not well understood. We hypothesized that EtOH regulates gene expression by differentially altering histone modifications at gene promoters that are up- and down-regulated by EtOH. Such epigenetic mechanisms may ultimately contribute to EtOH-induced neuro-adaptations that underlie tolerance, dependence, and EtOH-use disorders.

**Methods:** Eight-week-old, male C57BL/6J mice were treated with 3 g/kg EtOH (intraperitoneally) or saline and sacrificed 6 hours after injection; the CCx and hippocampus (HC) were immediately removed and flash frozen. Chromatin immunoprecipitation was used to study the association of model gene promoters with histone modifications. Western blot was used to detect global changes in the histone modifications studied. We also used a polymerase chain reaction (PCR) array to identify changes in expression of chromatin-modifying enzymes.

**Results:** In CCx, acute EtOH decreased expression of *Gad1*, *Hdac2*, and *Hdac11*, which was associated with decreased histone acetylation at the *Gad1* and *Hdac2* promoters; we also identified increased expression of *Mt1*, *Mt2*, *Egr1*, which was associated with increased H3K4me3 levels at the *Mt2* promoter and decreased H3K27me3 levels at the *Mt1* promoter. We identified an increase in global levels of H3K4me3 in CCx as well as a global increase in H3K9ac and H3K14ac in HC. The PCR array identified decreased expression of *Csrp2 bp*, *Hdac2*, and *Hdac11* as well as increased expression of *Kat2b* in CCx.

**Conclusions:** Acute EtOH induces chromatin remodeling at model up- and down-regulated genes in CCx. Different patterns of histone modifications at these gene promoters indicate that EtOH may be acting through multiple histone-modifying enzymes to alter gene expression; in particular, differential expression of *Kat2b*, *Hdac2*, *Hdac11*, and *Csrp2 bp* in CCx may mediate EtOH-induced chromatin remodeling. Additional studies are necessary to determine the relationship between EtOH-induced changes in histone-modifying enzymes, specific EtOH-induced histone modifications, and gene expression.

**Key Words:** Epigenetics, Ethanol, Alcohol, Chromatin Immunoprecipitation, Histones.

ETHANOL (ETOH) DEPENDENCE is associated with widespread changes in gene expression across multiple brain structures (Lewohl et al., 2000; Mayfield et al., 2002; Ponomarev et al., 2012; Zhou et al., 2011). These changes underlie neuronal and glial adaptations to the environmental stress of repeated EtOH exposure and may contribute to the reinforcing effects of EtOH that incentivize further consumption (Robinson and Berridge, 1993). In rodents, a single, binge-like exposure to EtOH is also associated with up- and down-regulation of genes in the cortex, nucleus accumbens, and ventral tegmental area (Kerns et al., 2005; Treadwell and Singh, 2004; Wolen et al., 2012). Despite the importance of differential gene expression for EtOH action, mechanisms of gene regulation by EtOH are poorly understood.

Gene expression is regulated by epigenetic mechanisms, which include covalent modifications to histones and DNA (Kouzarides, 2007). These modifications alter affinity of histones for DNA to inhibit or promote transcription factor binding. In particular, modifications to histone N-terminal tails, including acetylation and methylation of lysine residues, are catalyzed by a large group of histone-modifying enzymes and represent a rapid, reversible method of chromatin alteration (Smith and Shilatifard, 2010). These enzymes can be induced by drugs of abuse, like EtOH, to establish chromatin alterations that promote drug-seeking behavior and addiction (Robison and Nestler, 2011).

Recent evidence highlights the role of EtOH in inducing epigenetic disruptions and modulating histone-modifying enzyme expression in the brain. Studies of patients with EtOH dependence have identified altered distribution of

*From the Departments of Anesthesiology and Pharmacology & Chemical Biology (AF, GEH), University of Pittsburgh, Pittsburgh, Pennsylvania.*

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*Reprint requests: Gregg E. Homanics, PhD, Departments of Anesthesiology and Pharmacology & Chemical Biology, University of Pittsburgh, 6060 Biomedical Science Tower-3, 3501 Fifth Avenue, Pittsburgh, PA 15261; Tel.: 412-648-8172; Fax: 412-383-5267; E-mail: homanicsge@anes.upmc.edu*



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histone trimethylation in the hippocampus (HC) and cortex, which correspond to gene expression changes in these regions (Ponomarev et al., 2012; Zhou et al., 2011). In rodents, acute EtOH is associated with increased histone acetylation in the central nucleus of the amygdala (Pandey et al., 2008), altered histone modifications at the prodynorphin and pronociceptin promoters in the amygdala (D'Addario et al., 2013), and altered expression of histone-modifying enzymes in the striatum and prefrontal cortex (Botia et al., 2012); moreover, pretreatment with the histone deacetylase (HDAC) inhibitor sodium butyrate blocks EtOH-induced behavioral sensitization, indicating histone deacetylation plays a critical role in the neuroadaptive response to EtOH administration (Legastelois et al., 2013). A recent study pointed to HDAC2 expression in the amygdala as a critical regulator of EtOH preference and anxiolytic response to acute EtOH, identifying a specific histone-modifying enzyme in mediating EtOH action (Moonat et al., 2013).

While epigenetic effectors of EtOH are now seen as potential targets for the treatment of alcohol use disorders, several important questions remain to be resolved. Of note, studies of acute EtOH have focused on epigenetic modifications at up-regulated genes; however, gene expression studies indicate both up- and down-regulation of genes in response to acute EtOH (Kerns et al., 2005; Treadwell and Singh, 2004; Wolen et al., 2012). Moreover, while acute EtOH-induced histone modifications have been characterized in the amygdala using chromatin immunoprecipitation (ChIP) (D'Addario et al., 2013; Moonat et al., 2013), no studies have examined broader effects of acute EtOH in the cerebral cortex (CCx). The CCx is a critical site of EtOH's effect on $GABA_A$ receptor potentiation (Devaud et al., 1995; Nestoros, 1980), motor control (Ziemann et al., 1995), and neuronal toxicity (Chandler et al., 1993; Harper and Matsumoto, 2005). Due to its sensitivity to EtOH and diversity of cell types, the CCx may be especially important for identifying general epigenetic effects of EtOH in the brain that will be useful for screening potential drug targets. Therefore, we identified a need to study the effects of acute EtOH on multiple histone modifications at promoters of both up- and down-regulated genes in the CCx.

In this study, we examine how EtOH alters histone acetylation and methylation at the promoters of 3 model up- and down-regulated genes, global levels of those histone modifications, and the expression of histone-modifying enzymes in CCx. To study these changes, C57BL/6J male mice, a high-drinking and well-characterized strain for studying EtOH mechanisms, were injected with a single binge-like dose of EtOH (3 g/kg, intraperitoneally (i.p.)) known to produce sustained alterations in gene expression in mouse CCx (Pignataro et al., 2007). Tissue was extracted at a time point (6 hours) previously shown to alter gene expression in this mouse strain (Treadwell and Singh, 2004). These results are expected to have implications for identifying epigenetic mechanisms of acute EtOH.

## MATERIALS AND METHODS

### *Animals and Treatments*

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All experiments were performed using 8-week-old, EtOH-naïve, specific pathogen free male C57BL/6J mice (20 to 25 g) from the Jackson Laboratory (Bar Harbor, ME). Mice were habituated to the University of Pittsburgh animal facility for 1 week prior to initiation of experiments. Mice were housed under 12-hour light/dark cycles and had ad libitum access to food and water.

All treatments were administered during the light cycle between 08:00 and 10:00. Mice were given i.p. injections containing 0.02 ml/g of either 15% EtOH solution in saline (3 g/kg EtOH) or saline alone. After injections, mice were individually housed for 6 hours with ad libitum access to food and water.

At 6 hours postinjection, mice were rapidly sacrificed by carbon dioxide asphyxiation and decapitated. The brain was immediately removed and placed on a petri dish on ice. The cerebellum was removed and cerebral hemispheres separated at midline. The olfactory bulbs were removed and the telencephalon (CCx) was carefully dissected from the diencephalon and midbrain. The HC was dissected and removed from the CCx. The remaining left and right CCx and HC were flash frozen separately in liquid nitrogen. All experiments were performed using either the left or right CCx or HC.

### *Reverse Transcription Quantitative Polymerase Chain Reaction*

Total RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), purified with DNase digestion (Qiagen, Valencia, CA), and 1 μg of RNA was synthesized into cDNA using reverse transcription (RT) (Bio-Rad, Hercules, CA). A no-RT reaction was used as a negative control. Reactions were carried out in duplicate for each gene. SYBR green fluorescent master mix (Bio-Rad) was added to each well and visualized using a Bio-Rad iCycler. All primers were optimized for 90 to 110% efficiency at the following conditions: 10 minutes at 95°C (initial denaturation) followed by 40 cycles of 30 seconds at 95°C (denaturation), 1 minute at 60°C (annealing), and 30 seconds at 72°C (extension). Primer sequences for *β-actin*, *Gad1*, *Mt1*, *Mt2*, *Egr1, Hdac2, Hdac11, Csrp2 bp,* and *Kat2b* are shown in Table S1. Threshold cycle (Ct) values were calculated for each well and duplicate values averaged. The difference between specific genes and *β-actin* (ΔCt) was calculated for each animal and normalized to the average of saline-treated animals (ΔΔCt). Fold change over saline controls was calculated for each animal using the following formula: $2^{-\Delta\Delta Ct}$.

### *ChIP and ChIP-Quantitative Polymerase Chain Reaction*

Chromatin was isolated from the CCx using a standard protocol with minor modifications (Millipore EZ-Magna ChIP; Billerica, MA). The CCx was minced on a petri dish over ice using a razor blade. DNA was cross-linked to histones by incubating minced tissue in 1% formaldehyde in phosphate-buffered saline (PBS) at 37°C for 10 minutes. The formaldehyde reaction was quenched using glycine and the tissue was washed 3 times in PBS with protease inhibitor cocktail (#04693116001; Roche, South San Francisco, CA). Cell lysis buffer (Millipore) with protease inhibitor was added and nuclei pelleted. The nuclear pellet was incubated on ice in 500 μl nuclear lysis buffer (Millipore) with protease inhibitor to generate chromatin. Chromatin was sheered in an ice water bath using 4 bursts of 15 seconds at 35% output and 80% duty cycle on a Branson Sonifier S-250A (Branson Ultrasonics Corporation, Danbury, CT). An

aliquot of sheered, cross-linked DNA was removed and run on a 1.5% agarose gel to ensure the majority of DNA was between 200 and 600 bp. Chromatin was aliquotted and stored in dilution buffer (Millipore) with protease inhibitor at −80°C until immunoprecipitation experiments.

For immunoprecipitation, chromatin in dilution buffer was thawed on ice and 2% of the volume was removed and saved as the input. The remaining chromatin was incubated at 4°C overnight with antibody and Protein A/G magnetic beads (Millipore) with end-over-end rotation. The following antibodies were used for immunoprecipitation reactions: histone subunit H3-acetylated at lysines 9 and 14 (H3K9,14ac) (#06-599; Millipore), histone subunit H3 trimethylated at lysine 4 (H3K4me3) (#A-4033; Epigentek, Farmingdale, NY), and histone subunit H3 trimethylated at lysine 27 (H3K27me3) (#17-622; Millipore). IgG (#PP64B; Millipore) was used as a negative control. Antibodies were screened by assessing enrichment of the constitutively active gene, *β-actin*, over the neuronally repressed gene, *ε-globin*; there was no difference in enrichment between EtOH and saline-treated animals (Fig. S1) (Kurita et al., 2013). Antibodies were also validated by assessing binding to peptide arrays containing 46 histone modifications to the H3 N-terminal tail (#16-667; Millipore); H3K27me3 and H3K4me3 antibodies bound to their stated histone modifications while the H3K9,14ac antibody bound H3K9ac but not H3K14ac (data not shown). After incubation, magnetic beads containing antibody-chromatin complexes were immobilized on a magnetic rack and washed once with low salt, high salt, and LiCl immune complex wash buffers and TE. Elution buffer (Millipore) with proteinase K was added and the complexes were incubated at 65°C for 2 hours to elute enriched DNA. Immunoprecipitated and input DNA was purified using a ChIP DNA kit (Zymo Research, Irvine, CA) and eluted in 100 µl elution buffer.

For quantitative polymerase chain reaction (qPCR), 5 µl of immunoprecipitated or input DNA was used in each well and carried out in duplicate or triplicate for each primer pair. qPCR conditions were the same as reported in the RT-qPCR section and all ChIP-qPCR primers were optimized to perform at 90 to 110% efficiency. Primer sequences for promoter regions of *β-actin*, *ε-globin*, *Gad1*, *Mt1*, *Mt2*, *Egr-1*, *Hdac2*, and *Hdac11* are listed in Table S1. Ct values were normalized to input DNA and a negative control region not enriched for the histone modification (Meyer et al., 2008; Yu et al., 2012; Zhao et al., 2007). For H3K9,14ac and H3K4me3, *ε-globin* served as the negative control region; for H3K27me3, *β-actin* served as the negative control region (Fig. S1). Data are presented as fold enrichment over saline controls.

### *RT-qPCR Array*

An RT-qPCR array containing primers for 84 chromatin-modifying enzymes was used to screen EtOH-induced gene expression changes according to the manufacturer's protocol (#PAMM-085; SA Biosciences, Frederick, MD; full gene list shown in Table S2). RNA was extracted and purified according to the RT-qPCR section, converted to cDNA, and 8.5 ng of cDNA used per well according to the manufacturer's protocol. A total of 6 PCR arrays were used (3 saline-treated and 3 EtOH-treated animals). qPCR conditions were 10 minutes at 95°C (initial denaturation) followed by 40 cycles of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing and extension). Ct values from each PCR array were normalized to the median Ct value of that array (median normalization). Median normalized Ct values were further normalized to the average of saline controls (ΔΔCt) and fold change values calculated using the following formula: $2^{-\Delta\Delta Ct}$.

Genes whose expression was changed >100% after EtOH treatment or had a $p$-value <0.1 and change in expression >25% were chosen for validation by RT-qPCR using an additional 6 mice per group.

### *Western Blot*

Histone lysates were extracted using the Qiagen Qproteome kit (Valencia, CA) according to the manufacturer's protocol and quantified using a Bradford assay. Twenty micrograms of histone lysate was loaded onto 4 to 20% Novex Tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were blocked in Odyssey buffer (LiCor Biosciences, Lincoln, NE) and incubated overnight with primary antibodies. The following antibodies were used for Western blots according to the manufacturer's protocol: histone subunit H3 (#sc-8654; Santa Cruz Biotechnologies, Dallas, TX), H3K9ac (#9671s; Cell Signal Technologies, Danvers, MA), H3K14ac (#07-353; Millipore), H3K4me3 (#A-4033; Epigentek), and H3K27me3 (#17-622; Millipore). After overnight incubation, membranes were incubated with secondary fluorescent antibodies according to the manufacturer's protocol (LiCor Biosciences) and visualized using the Odyssey Infrared Imaging System (Licor Biosciences). The total intensity of each band was divided by the total intensity of histone subunit H3 and presented as a percent change relative to the average of saline controls. For CCx, a total of 6 animals per group were assessed on 2 separate membranes. For HC, a total of 3 animals per group were assessed on 2 separate membranes. Membranes were stripped 4 times using stripping buffer (LiCor Biosciences) between incubations with primary antibody.

### *Statistical Analysis*

For RT-qPCR, ChIP-qPCR, and Western blot quantification, a 2-tailed, unpaired $t$-test was used to compare the EtOH and saline-treated groups. For validation of antibodies used for ChIP, a 2-way ANOVA was used to compare *β-actin* and *ε-globin* expression in the EtOH and saline-treated groups. Statistical significance was defined by a $p$-value <0.05. All data are presented as mean ± SEM.

## RESULTS

### *EtOH-Induced Epigenetic Regulation of Model Down-Regulated Genes*

We first identified candidate genes down-regulated by acute EtOH exposure using previously published microarray data (Kerns et al., 2005; Treadwell and Singh, 2004; Wolen et al., 2012) and the RT-qPCR array used in this study. Six hours after injection of 3 g/kg EtOH or saline, there was significantly decreased expression of glutamic acid decarboxylase 1 (*Gad1*) ($p < 0.05$), histone deacetylase 2 (*Hdac2*) ($p < 0.05$), and *Hdac11* ($p < 0.05$) in the CCx of EtOH-treated compared to saline-treated mice (Fig. 1*A*).

ChIP assays revealed changes to histone modifications at the *Gad1* and *Hdac2* promoters 6 hours after injection of 3 g/kg EtOH (Fig. 1*B–D*). There was a significant decrease in the association of the *Gad1* and *Hdac2* promoters with H3K9,14ac in the CCx of EtOH-treated compared to saline-treated mice ($p < 0.05$). There was also a paradoxical decrease in the association of the *Gad1* promoter with H3K27me3 in the CCx of EtOH-treated compared to saline-treated mice ($p < 0.05$). We did not identify EtOH-induced changes in the association of either promoter with H3K4me3 or the association of the *Hdac11* promoter with the studied histone modifications.

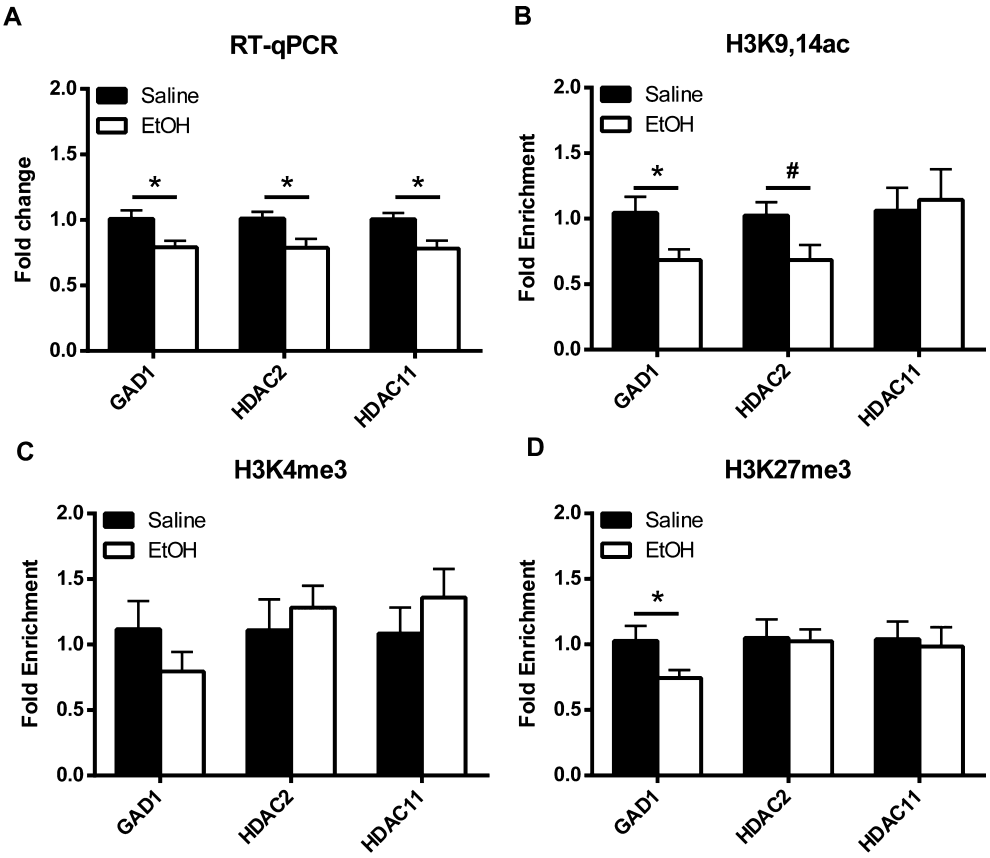


**Fig. 1.** Acute ethanol (EtOH) induces epigenetic modifications at the *Gad1* and *Hdac2* promoters. Reverse transcription quantitative PCR (RT-qPCR) and chromatin immunoprecipitation (ChIP) were used to study how acute EtOH alters the association of the model down-regulated genes, *Gad1*, *Hdac2*, and *Hdac11* with histone modifications at its promoter. (**A**) Using RT-qPCR, we found that acute EtOH exposure significantly decreased expression of *Gad1*, *Hdac2*, and *Hdac11*; data normalized to *β-actin* expression. ChIP-qPCR studies (**B**–**D**) were normalized to input and a negative control region and revealed that the *Gad1* and *Hdac2* promoters (**B**) had a significantly decreased association with H3K9,14ac, (**C**) no change in association of either gene with H3K4me3, and (**D**) that the *Gad1* promoter has a significantly decreased association with H3K27me3. $n = 6$/group, $*p < 0.05$, $\#p = 0.052$.

### *EtOH-Induced Epigenetic Regulation of Model Up-Regulated Genes*

Metallothioneins are components of the cellular response to oxidative stress whose expression is robustly increased by EtOH exposure (Kerns et al., 2005; Treadwell and Singh, 2004). Early growth response 1 (*Egr1*) is an immediate early gene whose expression also increases following EtOH exposure (Bachtell and Ryabinin, 2001). Six hours after injection of 3 g/kg EtOH or saline, there was significantly increased expression of metallothioneins 1 (*Mt1*) ($p < 0.05$), 2 (*Mt2*) ($p < 0.01$), and *Egr1* ($p < 0.05$) in the CCx of EtOH-treated compared to saline-treated mice (Fig. 2*A*).

ChIP assays revealed changes to histone modifications at the *Mt1* and *Mt2* but not *Egr1* promoters 6 hours after injection of 3 g/kg EtOH (Fig. 2*B–D*). There was a significant decrease in the association of the *Mt1* promoter with H3K27me3 but not H3K9,14ac or H3K4me3 in the CCx of EtOH-treated compared to saline-treated mice ($p < 0.05$). There was a significant increase in the association of the *Mt2* promoter with H3K4me3 but not H3K9,14ac or H3K27me3 in the CCx of EtOH-treated compared to saline-treated mice ($p < 0.05$). Surprisingly, there were no EtOH-induced changes in the association of model up-regulated gene promoters with H3K9,14ac.

### *EtOH Alters Global Levels of H3K4me3*

To study whether EtOH treatment alters global levels of the histone modifications assessed using ChIP, we performed Western blot on histone lysates generated from the CCx. There was a significant increase in global levels of H3K4me3 but no significant change in H3K9ac, H3K14ac, or H3K27me3 levels in the CCx 6 hours after injection of 3 g/kg EtOH compared to saline (Fig. 3).

### *EtOH Alters Expression of Chromatin-Modifying Enzymes*

To identify mechanisms of EtOH-induced changes to histone modifications, we used an RT-qPCR array containing primers for all known HDAC as well as histone acetyltransferases (HAT), methyltransferases, and other

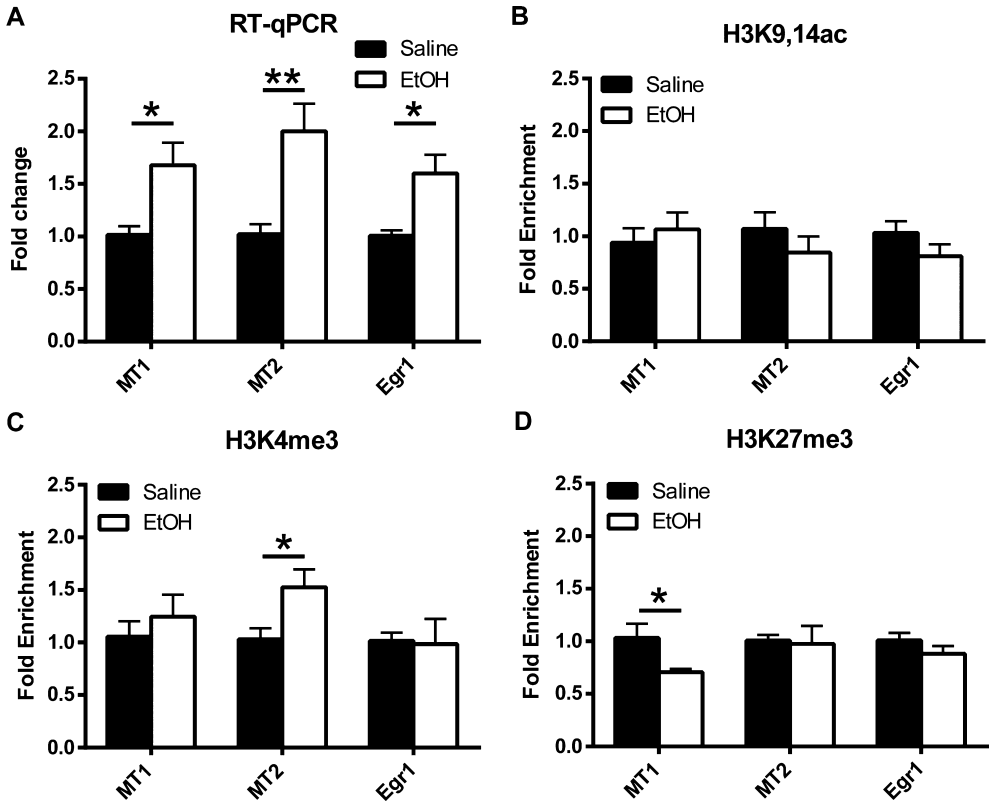


**Fig. 2.** Acute ethanol (EtOH) induces epigenetic modifications at the *Mt1* and *Mt2* promoters. Reverse transcription quantitative PCR (RT-qPCR) and chromatin immunoprecipitation (ChIP) were used to study how EtOH alters the association of the model up-regulated genes, *MT1*, *MT2*, and *Egr-1*, with histone modifications at their promoters. (**A**) Using RT-qPCR, we found that EtOH exposure significantly increased expression of *Mt1, Mt2*, and *Egr1*; data normalized to *β-actin* expression. ChIP-qPCR studies (**B**–**D**) were normalized to input and a negative control region and revealed (**B**) no changes in association of up-regulated genes with H3K9,14ac, (**C**) increased association of the *Mt2* promoter with H3K4me3, and (**D**) decreased association of the *Mt1* promoter with H3K27me3. $n = 6$/group, $*p < 0.05$, $**p < 0.01$.

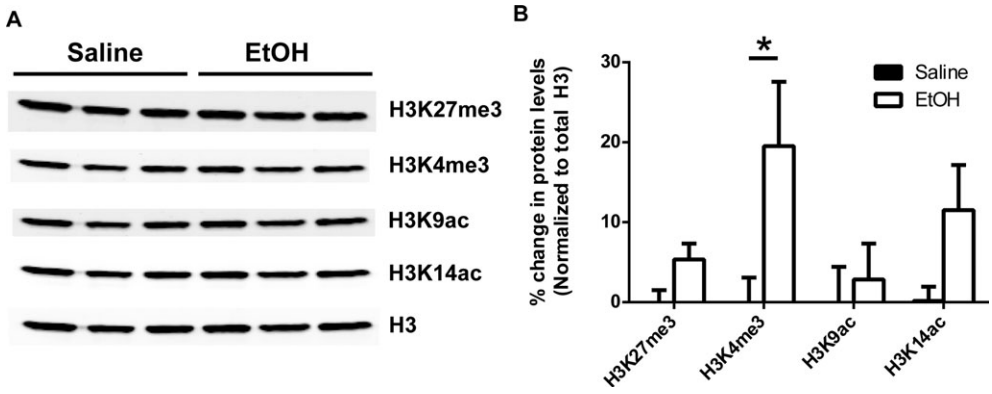


**Fig. 3.** Global levels of histone modifications after acute ethanol (EtOH) treatment. Western blot was used to assess global levels of histone modifications; data were quantified from 6 independent samples per EtOH and saline groups run across 2 separate gels. (**A**) Representative Western blot of EtOH and saline-treated animal histone lysates. (**B**) Quantification of protein levels normalized to total H3 presented as percent change over saline controls. $n = 6$/group, $*p < 0.05$.

enzymes involved in covalent modification of chromatin (full results in Table S2). The RT-qPCR array demonstrated low variability between EtOH- and saline-treated animals (Fig. S2) with few changes in the expression of chromatin-modifying enzymes associated with EtOH treatment in the CCx (Fig. 4*A*). This low power, screening assay revealed that *Csrp2 bp*, *Hdac2*, *Hdac11*, and *Kat2b* met criteria for validation with an additional 6 animals per group.

PCR validation revealed 6 hours after injection of 3 g/kg EtOH or saline there was significantly decreased expression of *Hdac2*, *Hdac11*, and *Csrp2 bp* and a near-significant trend for increased expression of *Kat2b* in the CCx of EtOH-treated compared to saline-treated mice (Fig. 4*B*).

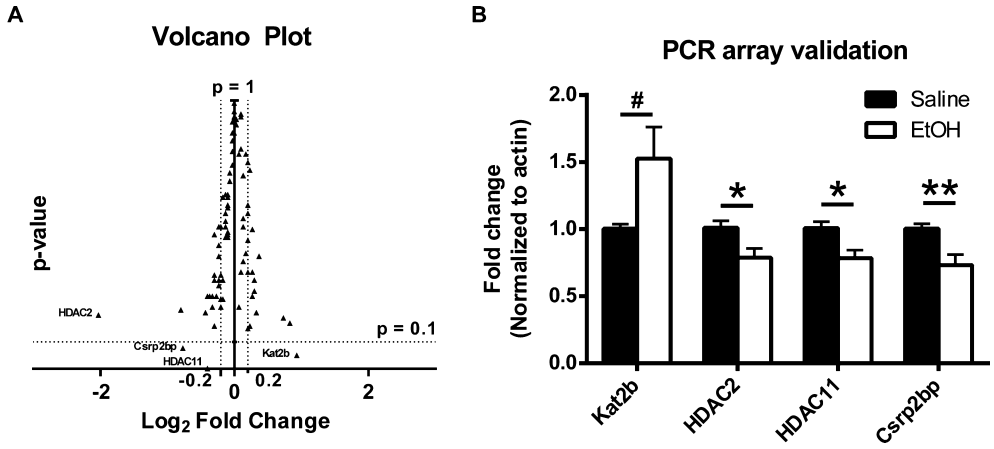


**Fig. 4.** Reverse transcription quantitative polymerase chain reaction (PCR) array of ethanol (EtOH)-induced changes in expression of chromatin-modifying enzymes. (**A**) Volcano plot showing the *p*-value (*y*-axis) and fold regulation [$\log_2$(fold change)] (*x*-axis) of all 84 chromatin-modifying enzymes with positions of *Csrp2 bp, Hdac2, Hdac11*, and *Kat2b* indicated; vertical dashed lines indicate 25% change in expression and the horizontal dashed line indicates a *p*-value of 0.1 for EtOH-treated animals compared to saline controls. (**B**) PCR validation of *Csrp2 bp, Hdac2, Hdac11*, and *Kat2b*. $n = 9$/group, $^*p < 0.05$, $^{**}p < 0.01$, $\#p = 0.056$. Note: *Hdac2* and *Hdac11* expression data is identical to that presented in Fig. 1.

### *EtOH Alters Global Histone Acetylation in HC*

We measured global levels of histone modifications and expression of chromatin-modifying enzymes that were found to be altered in CCx in HC tissue from the same mice to study whether EtOH-induced changes in CCx generalized to another brain structure. Western blot results revealed a significant increase in global levels of H3K9ac and H3K14ac as well as a nonsignificant trend for an increase in H3K4me3 with no change in levels of H3K27me3 6 hours after injection of 3 g/kg EtOH compared to saline (Fig. 5*A,B*). There were no significant differences in expression of *Hdac2, Hdac11, Csrp2 bp, or Kat2b* in EtOH-treated compared to saline-treated mice in the HC (Fig. 5*C*).

## DISCUSSION

Our data demonstrate novel epigenetic mechanisms of EtOH-regulated gene promoters, which indicate a diverse pattern of histone modifications induced by acute EtOH. Of note, this is the first study to identify histone deacetylation at gene promoters in the brain following acute EtOH exposure. We also identified 4 histone-modifying enzymes, *Hdac2*, *Hdac11*, *Kat2b*, and *Csrp2 bp*, whose expression is altered by acute EtOH in the CCx as well as an increase in H3K4me3 throughout the CCx and at the *Mt2* gene promoter. Finally, we identified increased histone acetylation in the HC with no change in expression of chromatin-modifying enzymes that were altered in CCx, suggesting differences in EtOH-induced epigenetic changes between these structures.

Histone deacetylation at our model down-regulated gene promoters indicates that acute EtOH leads to recruitment of HDACs to a subset of gene promoters. This finding is surprising considering that several studies show acute EtOH exposure is associated with decreased HDAC activity and expression (Botia et al., 2012; Pandey et al., 2008; Pascual et al., 2012; Sakharkar et al., 2012). However, there is also compelling evidence for histone deacetylation after acute EtOH exposure. In particular, down-regulation of genes by

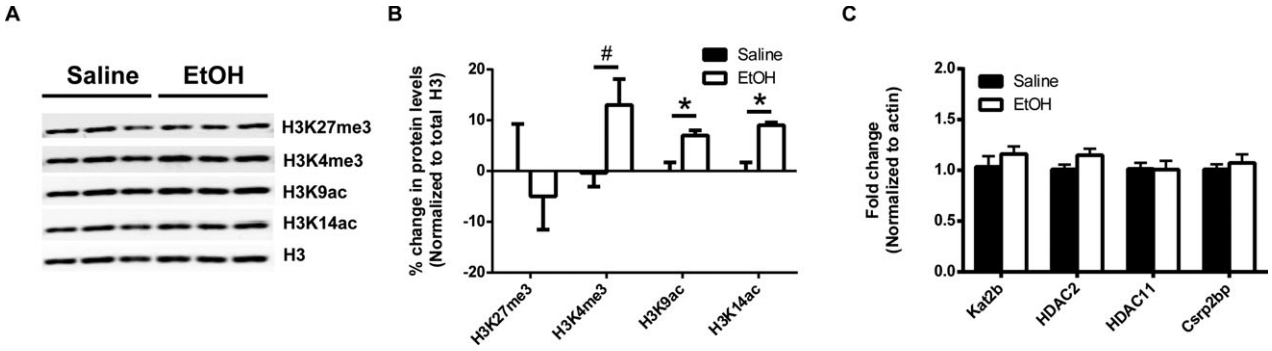


**Fig. 5.** Ethanol (EtOH)-induced epigenetic changes in cerebral cortex do not generalize to the hippocampus (HC). Western blot was used to assess global levels of histone modifications; data were quantified from 3 independent samples per EtOH and saline groups run across 2 separate gels. Reverse transcription quantitative polymerase chain reaction was used to study expression of chromatin-modifying enzymes ($n = 9$/group). (**A**) Representative Western blot of EtOH- and saline-treated animal histone lysates. (**B**) Quantification of protein levels normalized to total H3 presented as percent change over saline controls. (**C**) *Csrp2 bp, Hdac2, Hdac11*, and *Kat2b* expression was not altered in the HC in EtOH-treated compared to saline-treated mice. $^*p < 0.05$, $\#p = 0.0873$.

acute EtOH in the brain has been reported by several microarray studies (Kerns et al., 2005; Treadwell and Singh, 2004; Wolen et al., 2012) and implies a role for HDACs in mediating the acute effects of EtOH. A recent study also revealed decreased acetylation of histone subunit H4 in the nucleus accumbens 4 hours after the onset of binge drinking (Warnault et al., 2013). These studies highlight the diversity of epigenetic mechanisms induced by acute EtOH and importance of characterizing EtOH-induced histone modifications at specific gene promoters. This is especially important as several studies have proposed a role for HDAC inhibitors for modulating EtOH consumption (Moonat et al., 2013; Pandey et al., 2008; Sakharkar et al., 2012; Warnault et al., 2013). We expect future studies to expand on current findings by studying both temporal and gene-specific effects of EtOH on histone acetylation.

We identified EtOH-induced epigenetic changes at 2 model up-regulated gene promoters. Notably, H3K4me3 was increased at the *Mt2* promoter and H3K27me3 was decreased at the *Mt1* promoter. EtOH-induced changes to these histone modifications have been reported previously (D'Addario et al., 2013). However, we did not observe an increase in H3K9,14ac at any of our model up-regulated gene promoters. This finding was surprising, as several studies have found that acute EtOH increases histone acetylation in the brain (Moonat et al., 2013; Pandey et al., 2008; Sakharkar et al., 2012). One explanation for no change in histone acetylation at these gene promoters is that they have returned to a baseline state. Importantly, *Mt1*, *Mt2*, and *Egr1* expression has been shown to be elevated 4 hours following EtOH exposure (Kerns et al., 2005), so that it is possible that the promoters are no longer active at the 6-hour time point studied in this paper. This finding highlights the importance of temporal dynamics in studying epigenetic gene regulation induced by EtOH, and we anticipate future studies will identify gene promoter states at multiple time points.

We identified a significant increase in global H3K4me3 in mice treated with acute EtOH. Increased levels of H3K4me3 have been reported in the cortex of patients with alcoholism (Ponomarev et al., 2012) and our results indicate that this occurs after a single EtOH exposure. Importantly, H3K4me3 exclusively marks active and poised promoters near transcriptional start sites (Black et al., 2012; Schneider et al., 2004), so that a global increase in this histone modification likely reflects EtOH-induced chromatin remodeling to promote gene expression. While our RT-qPCR array did not detect a change in expression of lysine methyltransferases or demethylases, these enzymes are regulated by posttranslational modifications that could account for increased histone trimethylation after EtOH exposure (Black et al., 2012). Further studies are needed to identify how EtOH induces trimethylation of lysine 4 on histone subunit H3 without altering expression of histone methyltransferases.

Our analysis of chromatin-modifying enzyme expression following acute EtOH exposure identified 2 HDACs and 2 HATs whose expression is altered in the CCx. Notably, decreased expression of *Hdac2* and *Hdac11* after acute EtOH exposure has been reported in the striatum (Botia et al., 2012), indicating down-regulation of these genes is a fundamental neurobiological mechanism of EtOH. *Hdac2* expression has been implicated in conferring preference for EtOH (Moonat et al., 2013) and down-regulation promotes memory formation (Guan et al., 2009), so that decreased expression may be important for promoting further EtOH consumption. Differential expression of *Hdac11* was found to regulate EtOH drinking behavior (Wolstenholme et al., 2011), though specific mechanisms of *Hdac11* in the brain have not been identified. Based on our findings and similar changes in expression in the striatum (Botia et al., 2012), we feel that studying EtOH-induced regulation of *Hdac2* and *Hdac11* will likely help elucidate mechanisms of EtOH-induced gene expression.

Changes in expression of 2 HATs in the CCx after EtOH exposure likely reflect competition between HAT complexes. *Csrp2 bp* is a component of the Ada2a-containing (ATAC) complex, which acts as a HAT that regulates cell cycle progression (Orpinell et al., 2010). *Kat2b* is a component of a HAT complex with cAMP response element binding protein (CREB), which is critical for memory formation (Maurice et al., 2007). Interestingly, the ATAC complex was found to be mutually exclusive with a HAT complex containing *Kat2b* (Nagy et al., 2010), so that inverse expression of *Kat2b* and *Csrp2 bp* may reflect EtOH-induced mechanisms that activate one HAT complex and repress the other. This idea is supported by studies indicating the importance of CREB for mediating EtOH action (Moonat et al., 2010) and also suggest repression of the ATAC complex as a mechanism of EtOH. Studying how modulating the ATAC and CREB complexes affects EtOH consumption would further support the idea that EtOH induces reciprocal activation of these HAT complexes.

Our study raises important points about studying epigenetic mechanisms of EtOH. We identified general mechanisms of acute EtOH on histone modifications and chromatin-modifying enzyme expression as well as specific mechanisms at gene promoters using ChIP in the CCx. Our findings indicate that changes seen on a global level do not generalize to all or even most gene promoters across the CCx. This is likely due to temporal dynamics and differences among neuronal subpopulations that influence EtOH-induced epigenetic changes and our ability to identify specific effects at gene promoters. It is also interesting that chromatin-modifying enzymes whose expression was altered in CCx were not altered in HC, suggesting changes in CCx expression may be driven by a specific region or population of cells. Recent work indicates that EtOH-induced epigenetic changes are different even between different subregions of the same brain structure (Botia et al., 2012; Moonat et al., 2013; Pandey et al., 2008; Sakharkar et al., 2012), so that it is not unexpected EtOH-induced gene expression changes differ between

CCx and HC. We anticipate these issues precluded identification of a discrete pattern of EtOH-induced histone modifications shared by any of our 6 studied promoters. We expect future studies to examine additional time points of EtOH exposure as well as study neuronal subtypes by utilizing laser-capture microdissection or fluorescence-activated cell sorting. Last, as the current study utilized C57BL/6J male mice, it is of interest to determine whether the results observed generalize to females and to mice of other genetic backgrounds.

In conclusion, this study introduces new epigenetic mechanisms of EtOH, including histone deacetylation at down-regulated gene promoters, increased global H3K4me3, and altered expression of histone-modifying enzymes in the CCx following acute EtOH exposure.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Validation of ChIP antibodies using *β-actin* and *ε-globin*.

**Fig. S2.** Scatter plot of EtOH-induced changes to chromatin-modifying enzymes.

**Table S1.** Primer Sequences.

**Table S2.** Full RT-qPCR Array Results.