Title: Ethanol exposure regulates \textit{Gabra1} expression via histone deacetylation at the promoter in cultured cortical neurons

Authors: John Peyton Bohnsack, Vraj K. Patel, and A. Leslie Morrow

Affiliations:

1. \textbf{JPB, ALM} = Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7178 USA.
2. \textbf{ALM} = Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7178 USA.
3. \textbf{JPB, VKP, and ALM} = Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599-7178, USA.
MC1568(2E)-3-[5-[(1E)-3-(3-fluorophenyl)-3-oxo-1-propen-1-yl]-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide
PKA, protein kinase A
PKC, protein kinase C
PTM, post-translational modifications
RGPF966 (2E)-N-(2-amino-4-fluorophenyl)-3-[1-(3-phenyl-2-propen-1-yl)-1H-pyrazol-4-yl]-2-propenamide
SAHA, Suberoylanilide Hydroxamic Acid
sgRNA, small-guide RNA
TSA, Trichostatin A
VTA, Ventral tegmental area

**Recommended section:** Neuropharmacology
Abstract (250 words): GABA<sub>A</sub>-Rs mediate the majority of inhibitory neurotransmission in the adult brain. α1-containing GABA<sub>A</sub>-Rs are the most prominent subtype in the adult brain, and are important in both homeostatic function and several disease pathologies including alcohol dependence, epilepsy, and stress. Ethanol exposure causes a decrease of α1 transcription and peptide expression both in vivo and in vitro, but the mechanism that controls the transcriptional regulation is unknown. Since ethanol is known to activate epigenetic regulation of gene expression, we tested the hypothesis that ethanol regulates α1 expression through histone modifications in cerebral cortical cultured neurons. We found that class I histone deacetylases (HDACs) regulate ethanol-induced changes in α1 gene and protein expression as pharmacological inhibition or knockdown of HDAC1-3 prevent effects of ethanol exposure. Targeted histone acetylation associated with the Gabra1 promoter using CRISPR dCas9-P300 (a nuclease-null Cas9 fused with a histone acetyltransferase) increases histone acetylation and prevents the decrease of Gabra1 expression. In contrast, there was no effect of a mutant histone acetyltransferase or generic transcriptional activator or targeting P300 to a distant exon. Conversely, using a dCas9-KRAB construct that increases repressive methylation (H3K9me3) does not interfere with ethanol-induced histone deacetylation. Overall our results indicate that ethanol deacetylates histones associated with the Gabra1 promoter through class I HDACs and that pharmacological, genetic, or epigenetic intervention prevents decreases in α1 expression in cultured cortical neurons.
Introduction (750 words max):

γ-Aminobutyric acid type A receptors (GABA<sub>A</sub>-Rs) are Cl⁻ ion channels that mediate the majority of inhibitory neurotransmission in the adult brain. GABA<sub>A</sub>-Rs are usually heteropentamers and with different subunits of the GABA<sub>A</sub>-R conveying heterogeneity of function, localization, and pharmacology (Uusi-Oukari and Korpi, 2010). GABA<sub>A</sub>-R expression is differentially regulated in different disease states, including alcohol withdrawal (Devaud et al., 1997), epilepsy (Lund et al., 2008), autism (Fatemi et al., 2008), depression (Poulter et al., 2008), and schizophrenia (Hoftman et al., 2013).

GABA<sub>A</sub>-Rs have long been known to be involved in acute alcohol intoxication, dependence, and withdrawal symptoms (Kumar et al., 2009), with acute alcohol potentiating GABAergic function and chronic alcohol exposure leading to GABA<sub>A</sub>-R hypofunction. Increasing GABA<sub>A</sub>-R function via pharmacological agents such as benzodiazepines is useful for treating the symptoms of acute alcohol withdrawal syndrome such as increased anxiety, seizure susceptibility, central and autonomic nervous system hyperexcitability, and tremor (also known as delirium tremens) (Amato et al., 2010). One issue with benzodiazepine treatment is that decreases in GABA<sub>A</sub>-R expression occur (Uusi-Oukari and Korpi, 2010), suggesting the need to find alternative strategies for increasing GABA<sub>A</sub>-R expression in acute alcohol withdrawal syndrome and other disease states where GABA<sub>A</sub>-R expression is dysregulated.

The α1-containing GABA<sub>A</sub>-Rs are the most abundant subtype in the adult brain, and are down-regulated in both alcohol withdrawal (Devaud et al., 1997) and epilepsy (Lund et al., 2008) in rodents. Chronic ethanol exposure decreases Gabra1 transcript
expression (Montpied et al., 1991; Devaud et al., 1995b), α1 protein expression (Devaud et al., 1997; Kumar et al., 2002; Cagetti et al., 2003) and GABA$_A$-R hypofunction (Morrow et al., 1988; Cagetti et al., 2004; Liang et al., 2004) that is associated with increased withdrawal symptoms such as increased seizure susceptibility (Devaud et al., 1995a), cross-tolerance to benzodiazepines (Cagetti et al., 2003; Liang et al., 2007) and tremor (Kralic et al., 2005) in rodents. Four hour ethanol exposure (50 mM) in cerebral cultured cortical neurons mimics changes in GABA$_A$-R α1 expression that have been shown in chronic ethanol exposure and withdrawal models \textit{in vivo} and this system has been used to determine molecular mechanisms that control their function and expression (Kumar et al., 2010; Carlson et al., 2013). Ethanol exposure also diminishes GABA$_A$-R function in cortical cultured neurons, including zolpidem potentiation (Kumar et al., 2010) and changes in mini-inhibitory postsynaptic current characteristics (Werner et al., 2011), but the molecular mechanisms that regulate the decreases in $\text{Gabra}1$ transcription after ethanol exposure are still poorly understood.

Recent studies suggest that ethanol regulates gene expression through epigenetic pathways involving post-translational modifications on histone tails (Pandey et al., 2008; Wang et al., 2008; Warnault et al., 2013). Changes in post-translational modifications of histones allow for either permissive or prohibitive access for transcription factors and other key components of transcription machinery to bind to DNA to initiate transcription. Acetylation of histone tails is associated with increased or permissive transcription (Wang et al., 2008), and is facilitated by a class of enzymes called histone acetyltransferases (HAT) and removed by histone deacetylases (HDACs).
(Wang et al., 2009). The HDAC family consists of four different classes, that have different regulatory roles, subcellular localization, and pharmacology (Haberland et al., 2009). The Zn\textsuperscript{+} dependent HDACs (Class I, II and IV) are localized in the nucleus and cytoplasm and control a number of different cellular functions including gene transcription (Broide et al., 2007; Haberland et al., 2009; Wang et al., 2009). HDAC inhibitors have recently been suggested for the treatment of alcohol use disorders (Pandey et al., 2008; Warnault et al., 2013; Simon-O'Brien et al., 2015). Treatment with histone deacetylase inhibitors prevents GABA\textsubscript{A}-R hypofunction in the ventral tegmental area after chronic ethanol exposure possibly through restoring \(\alpha\)1 expression through either a trafficking or gene activating mechanism (Arora et al., 2013). Another study found that the HDAC inhibitors, enhanced folding, trafficking, and function of \(\alpha\)1(A322D), an \(\alpha\)1 mutation that causes epilepsy (Di et al., 2013). However, changes in histone acetylation associated with \textit{Gabra1} gene elements (i.e. promoter) after ethanol have not yet been identified, nor have the HDACs or HATs that may regulate changes in \textit{Gabra1} gene expression been elucidated.

Changes in histone acetylation have long been associated with changes in gene transcription, however, experiments using HDAC inhibitors found that HDAC inhibitors only regulate a small set of genes (~2%) via histone acetylation (Van Lint et al., 1996) and therefore until recently determining if changes in histone acetylation associated with a specific gene or corresponding regulatory elements was not possible. CRISPR Cas9 has recently become the premier tool for gene targeting and editing technology (Doudna and Charpentier, 2014) as the tool is highly selective for certain loci in the genome. A modified Cas9 (dCas9-P300) has been utilized to make epigenetic changes at specific
loci by utilizing gene targeting ability of Cas9 in combination with histone acetyltransferase capabilities of the HAT, P300 (Hilton et al., 2015).

We utilized a cerebral cultured cortical neuron system to determine the molecular mechanism that drives decreases in Gabra1 expression after ethanol exposure. We report that ethanol decreases Gabra1 expression through histone deacetylation at the Gabra1 promoter that is facilitated by class I HDACs. Furthermore, we demonstrate that increasing histone acetylation at the Gabra1 promoter using dCas9-P300 prevents ethanol-induced decreases in Gabra1 expression. Identification of the epigenetic regulators that modulate Gabra1 expression could be potentially useful for development of therapeutics for the treatment of various disorders where Gabra1 expression is dysregulated.

Material and Methods

Primary Cultured Cortical Neurons

Cerebral cortices from mixed sex (M/F ~50%) Sprague Dawley rat pups (pn = 0) were isolated then seeded at 1,000,000 neurons per well or 100,000 neurons per well and grown for 18 days in vitro (DIV18, DMEM + B27 + penicillin and streptomycin, 37°C, 5% CO₂) as described previously (Bohnsack et al., 2016). On DIV14 antibiotics were removed. All procedures were carried out in compliance with guidelines specified by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Drug Exposure
On DIV18, Trichostatin A, (TSA, 500 nM, Tocris), Suberoylanilide Hydroxamic Acid, (SAHA, 3 μM, Tocris), (2E)-N-(2-amino-4-fluorophenyl)-3-[1-(3-phenyl-2-propen-1-yl)-1H-pyrazol-4-yl]-2-propenamide (RGPF966, 24 nM, 80 nM, Cayman Chemical), (2E)-3-[5-[(1E)-3-(3-fluorophenyl)-3-oxo-1-propen-1-yl]-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide (MC1568, 1 μM, Cayman Chemical), or sodium butyrate (1 mM, Tocris) or equivalent volume vehicle (100% v/v DMSO) was added directly to cell culture media for 4h. The TSA concentration (500 nM) was chosen based on preliminary experiments showing increases in H3KAc in cell culture (data not shown). SAHA and sodium butyrate doses were chosen based on manufacturers recommendations. RGFP966 and MC1568 doses were chosen based on specificity for HDAC3 and class II HDACs respectively. Ethanol (50 mM) was co-exposed with drugs (or vehicle) for 4h. Experiments were stopped by placing cell-culture plates on ice, cells were then washed twice with ice-cold DPBS, and then lysed in either Trizol or 0.32M sucrose homogenization buffer. Subcellular fractionation of cultured neurons was performed as previously described (Bohsnack et al., 2016).

**RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)**

Neurons were rinsed with ice-cold PBS then homogenized in Trizol (Ambion) and RNA was extracted and purified according to manufacturer's instructions. RNA was quantified and quality controlled using a Nanodrop (all 260/280, and 230/260 values ≥ 1.8, Fisher Scientific). Two μg of purified RNA was reversed transcribed to a cDNA library using High Capacity RNA to DNA kit (Applied Biosystems) following manufacturer's instructions. DNA (10 ng per reaction) was then subjected to qPCR analysis using
TaqMan gene expression probes and Taq Gene Expression MasterMix. Reactions were run in duplicate on a StepOnePlus RT-PCR system (Applied Biosystems) using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as a loading control (Bohnsack et al., 2016). Data was analyzed using the ΔΔCT method and expressed as fold control. Catalogue numbers of TaqMan probes: Gabra1, Rn00788315_m1; Hdac1, Rn01519308_g1; Hdac2, Rn01193634_g; Hdac3, Rn00584926_m1. Gapdh, Rn01775763_g1.

**Western Blot Analysis**

Western blots were performed as previously described, with some modifications (Bohnsack et al., 2016). Blots were blocked for 1h (room temperature) in Licor Blocking Buffer + PBS (1:1 v/v). Primary antibodies were added to the blot in Licor Blocking Buffer + PBST (1:1 v/v, 0.1% Tween 20) and incubated overnight at 4°C. Blots were washed 3× with PBST, then incubated for 1 h (room temperature) with 2° antibodies conjugated to a fluorophore. Blots were then washed 3× with PBST, then 1× with PBS to remove excess Tween 20. Bands were visualized using the Odyssey Classic Imaging System (Licor). Results were normalized to β-actin, GAPDH to account for discrepancies in loading and transfer. Loading controls were also evaluated for changes in expression. Loading control normalized values were then expressed as percent control values. Antibodies used were: GABA\textsubscript{A}-α1 (Millipore, Cat# AB5592-200); β-Actin (Novus, Cat# NB600-501) HDAC1 (Abcam, ab7028); HDAC2 (Abcam, Cat# ab7029); HDAC3 (Santa Cruz, Cat #sc-11417).
Chromatin Immunoprecipitations (ChIP)

ChIP assays were performed as previously described (Kennedy et al., 2013) with some modifications. Neurons were rinsed with ice-cold PBS, then cross-linked in 1% formaldehyde (Thermo Scientific) by agitation for 10 minutes at room temperature. The cross-linking reaction was quenched using 125 mM glycine for 5 minutes at room temperature. Samples were centrifuged at 1,600×g for 5 minutes at 4°C then washed with ice-cold PBS then spun again at 1,600×g for 5 minutes at 4°C. Samples were lysed using ChIP lysis buffer (10 mM Tris-HCl ph 8.0, 10 mM NaCl, 0.2% v/v NP-40 and 1 mM phenylmethylsulfonyl fluoride) on ice for 30 minutes and then homogenized and spun down at 2,400×g for 10 minutes, 4°C. Nuclei were lysed in nuclear lysis buffer (10 mM Tris-HCl, ph 8.0, 50 mM EDTA, 1%(v/v) SDS, and protease inhibitors) on ice for 10 minutes then sonicated to shear chromatin. Chromatin shearing of less than 500 bp was verified by running aliquots on 2% agarose gels. Following sonication, chromatin samples were spun for 18,000×g for 10 minutes at 4°C and DNA concentrations were determined using a Nanodrop (Fisher Scientific). Equal amounts of chromatin were incubated overnight at 4°C with antibody, and aliquot was set aside for input to ensure equal loading. PureProteome™ Protein G Magnetic beads (Millipore) were added to chromatin samples for 1h at 4°C, then complexes were washed, eluted in 1% (w/v) SDS, 0.75% (w/v) sodium bicarbonate buffer, and crosslinks were reversed overnight at 65°C. DNA was purified using QIAquick PCR Purification Kit (Qiagen) and analyzed using SYBR® Green Real-Time PCR Master Mixes (Thermo Scientific) following manufacturer’s instructions.
Primers used were *Gabra1* Prom Fw 5’ CCCCCAAAATAGAGGAATGC 3’; *Gabra1* Prom Rv 5’ AATAGGCGGTGACTTCATGC 3’. Antibodies used were: Anti-Acetyl-Histone H3 (pan) (Millipore, Cat #06-599); H3K9me3, (Abcam, Cat# ab8898).

**Lentiviral shRNA knockdown**

Lentiviral experiments for HDAC1 knockdown experiments used shRNA from the RNAi Consortium library, provided by the UNC Lentiviral core. Target sequences for HDAC1 shRNAs were as follows:

- 5’ GCTTGGGTAATAGCAGCCATT 3’
- 5’ CCGGTATTTTGATGGCTTGTTT 3’
- 5’ CCCTACAATGACTACTTTGAA 3’
- 5’ GCCAGTCATGTCCAAAGTAAT 3’
- 5’ GCGTTCTATTCGCCAGATAA 3’

Lentivirus shRNA experiments for HDAC2 used shRNA plasmids from Origene (Cat# TL7118660). Target sequences for HDAC2 shRNAs were as follows:

- 5’ AGAAAGTGTGCTACTATTATGACGGTGAT 3’,
- 5’ GCTTGTGATGAAGAGTTCTCAGATTCTGA 3’,
- 5’ ACAACAGATCGCGTGATGACCGTCTCATT 3’,
- 5’ TCAAAGGTCACGCTAAATGTGTAGAAGTA 3’.

Control experiments used a virus containing a scrambled DNA (SHC002, Sigma Aldrich; 5’

CCGGCGTGATCTTCACCGACAAGATCTCGAGA TCTTGTCGGTGAAGATCACGTTTT
shRNA plasmids were transformed in DH5α cells, grown up overnight at 37°C, then purified using Maxiprep kits (Qiagen). DNA purity was checked with Nanodrop, and rejected if 260/280 or 230/260 values were below 1.8. Plasmids were then sequenced by the UNC genome sequencing facility. Lentivirus was packaged by the UNC Lentiviral core, then aliquoted before use. Neurons were seeded on plates for 24h before virus was added directly to media (MOI, > 1, DIV1). 4-5 shRNAs were pooled to achieve maximum knockdown. On DIV18, H2O or ethanol (50 mM) was added directly to the media for 4h then cells were harvested for western blot analysis.

**siRNA Transfections**

Small-interfering RNA transfections were performed as we have previously described with some modifications (Werner et al., 2011). Neurons were grown until DIV17 then transfected with 25 pmol Silencer® HDAC3 siRNA (Cat# 4390771, Thermo Scientific) or Silencer® Select Negative Control No. 1 siRNA (Cat# 4390843, Thermo Scientific) using Lipofectamine® RNAiMax Reagent (Thermo Scientific) following manufacturer’s instructions. After 20h, cells were exposed to 50 mM EtOH or H2O for 4h, then harvested for western blot analysis.

**sgRNA Production**

sgRNAs were designed in silico (crispr.mit.edu) based on experimentally determined algorithms described previously by (Hsu et al., 2013) to be targeted at the promoter region or exon 5 and areas measured by ChIP primers. BsmBI sites (fw = 5’ TCCC 3’; rev = 5’ AAAC 3’) were added to the CRISPR design in order to facilitate subcloning into
the inducible vector (FgH1tUTG was a gift from Marco Herold [Addgene plasmid # 70183] (Aubrey et al., 2015)). Oligos were annealed in a thermocycler starting at 95°C and then decreasing by 5°C every minute until 20°C. Oligos (100μM of both fw and rev) were then phosphorylated with T4 PNK ligase according to manufacturer’s instructions (NEB. Cat# M0201S). Golden gate cloning was used to insert sgRNA oligos into FgH1tUTG (100 ng) by digesting with BsmbI (Fermentas, Cat# ER0451) and annealing with T7 ligase (NEB, Cat# M0318S) in the thermocycler (37°C for 5 min then 23°C for 5 min, 15 cycles, hold at 4°C). The reaction was then transformed into homemade Sbtl3 cells by following manufacturer’s instructions, (Zymo Research, Cat# T3001 made from Thermo Scientific, Cat# C737303), plated on LB-Amp plates 37°C and grown overnight. Individual colonies were selected, grown overnight in LB-Amp media at 37°C. Plasmid DNA was extracted using Miniprep kits (Promega, Cat# A1340) then sequenced using the H1 primer. For sgRNA sequences see Table 1. Correctly sequenced clones where then packaged into lentivirus by the UNC Lentiviral Core.

Subcloning dCas9-P300 into the Lentiviral Expression Vector

We utilized a previously characterized dCas9-P300 construct (Hilton et al., 2015) for experiments. The dCas9-P300 construct was in a vector driven by the CMV promoter, which has poor expression in neurons, therefore we subcloned the dCas9-P300 constructs (Addgene # 61357 and 61358, a generous gift from Dr. Charles Gersbach) into a lentiviral expression vector with an RFP promoter (Addgene # 17619). dCas9-P300 was amplified out of the vector using primers designed with 20bp overhangs for Gibson assembly using PCR then extracted using PCR cleanup kits. The lentiviral
vector was cut using EcoRV (NEB, Cat# R0195S), and the resultant fragments purified using Qiagen Gel Extract kit (Qiagen, Cat# 28704). The two fragments were incorporated using Gibson assembly following manufacturer’s instructions (NEB, Cat# E2611S). Clones were sequenced by Eton Biosciences then packaged into lentivirus by the UNC Lentiviral Core. Primers for cloning:

fw = 5’ GCTGGCTAGGTAAGCTTGATATGGACTACAAAGACCATGA 3’;
rev = 5’ TAGGGCTGCAGGAATTCGATAGAAGCGTAGTCCGGAACGT 3’.

Lentiviral Production of dCas9-VP64 and dCas9-KRAB

Plasmids containing dCas9-VP64 (Addgene # 53192) and dCas9-KRAB (Addgene #71237) were a generous gift from Dr. Charles Gersbach. dCas9-VP64 was characterized previously (Kabadi et al., 2014). dCas9-KRAB was characterized previously (Thakore et al., 2015). Plasmids were sequenced by Eton Biosciences then packaged into lentivirus by the UNC Lentiviral Core.

Transduction of Cortical Neurons and Ethanol Exposure

Equal volumes of three different lentiviruses with three different sgRNAs were mixed together with an equal volume of dCas9 vector (P300, P300<sup>D1399Y</sup>, VP64, or KRAB) and transduced into neurons on DIV15 with 10 μg of polybrene (Sigma Aldrich, Cat# H9268). On DIV16, doxycycline in H<sub>2</sub>O was added to cell culture medium (final concentration = 1 μg/mL) in order to induce sgRNA expression (Aubrey et al., 2015). On DIV18, 50 mM EtOH or equivalent volume ddH<sub>2</sub>O was added for 4h then cells were harvested for qPCR, ChIP or western blot analysis.
Statistics. All groups were randomly assigned. Two-way ANOVAs were performed to determine significance for all experiments (ANOVA details for each experiment are given in the Figure legends). Bonferroni’s post-hoc test was used to perform multiple comparisons between groups in order to determine significance. Significance was set at \( p < 0.05 \). Biological replicates (n) were performed by running the same experiment in different plates from a new cohort of animals.

Results

*Histone deacetylase inhibitors prevent decreases in Gabra1 expression caused by ethanol exposure.*

We have previously shown that ethanol exposure (50 mM for 4h) in cultured cortical neurons causes a decrease in \( \alpha 1 \) expression in a crude membrane fraction (Kumar et al., 2010). We next evaluated whether 50 mM ethanol exposure for 4h also caused a decrease in *Gabra1* transcription (Fig. 1). Our results indicate that there is a robust decrease in *Gabra1* expression similar to what is seen *in vivo* (Devaud et al., 1995b). We next evaluated several different histone deacetylase inhibitors in order to determine if this would prevent decreases in *Gabra1* expression after 4h of 50 mM ethanol exposure (Fig. 1). TSA (500 nM) co-exposure prevented decreases in *Gabra1* expression caused by ethanol exposure. Similarly, co-exposure to SAHA (3 \( \mu \)M) also prevented decreases in *Gabra1* expression. Since both inhibitors are broad spectrum and inhibit all Zn-dependent histone deacetylases, we next evaluated whether certain isoforms were responsible using pharmacological tools. We used the HDAC3 selective
inhibitor RGFP966 (Malvaez et al., 2013) at two different concentrations (24 nM, and 80 nM) and found that co-exposure of this compound prevented decreases in Gabra1 expression caused by ethanol exposure. In contrast, the class II selective inhibitor MC1568, failed to prevent ethanol-induced decreases in Gabra1 expression. Finally, the broad spectrum HDAC inhibitor, sodium butyrate, did not prevent decreases in Gabra1 expression caused by ethanol exposure.

Knockdown of class I HDACs prevents decreases in α1 expression caused by ethanol exposure.

Since MC1568 exposure failed to prevent ethanol-induced decreases in Gabra1 expression we hypothesized that inhibition of class I HDACs (1-3) would prevent ethanol-induced decreases in Gabra1 expression. In order to test this hypothesis, we used a genetic strategy to target HDAC1-3 (Fig. 2A and 2B). Knockdown of HDAC1 via a lentiviral strategy produced a decrease in HDAC1 expression (Fig. 2C, 58.78 ± 3.41% control). Western blot analysis of the P2 membrane fraction revealed that knockdown of HDAC1 prevented the decrease of α1 expression after ethanol exposure (Fig. 2D).

We tested for specificity of the knockdown of HDAC1 versus HDAC2 and HDAC3; qPCR analysis revealed that knockdown of HDAC1 did not decrease Hdac2 (0.96 ± 0.1 fold control) or Hdac3 (0.82 ± 0.08 fold control) (data not shown). Knockdown of HDAC2 using the same lentiviral strategy caused a decrease in HDAC2 expression (Fig. 2E, 48.96 ± 6.25% control) and also prevented the decrease of α1 expression after ethanol exposure (Fig. 2F). We next tested for specificity of the knockdown of HDAC2; qPCR analysis revealed that knockdown of HDAC2 did not decrease Hdac1 (1.26 ± 0.14 fold control).
control) or Hdac3 (0.83 ± 0.09 fold control) (data not shown). Long-term knockdown of HDAC3 using a lentiviral strategy caused unviable neuronal cultures, so we utilized a siRNA strategy. Knockdown of HDAC3 with siRNA transfection caused a decrease in HDAC3 expression (Fig. 2G, 64.16 ± 3.21% control) and also prevented decreases in α1 expression caused by ethanol exposure (Fig. 2H). Knockdown of HDAC3 did not cause any change in Hdac1 (0.99 ± 0.08 fold control) or Hdac2 (1.18 ± 0.11 fold control).

**Design of inducible CRISPR dCas9-P300 system in order to study acetylation at the Gabra1 promoter**

Since HDACs catalyze the removal of acetylation from histones (Wang et al., 2009) we hypothesized that ethanol was causing a decrease in Gabra1 expression and α1 expression by decreasing acetylation at the Gabra1 promoter. To test this idea, we utilized a previously described CRISPR dCas9 that is fused with the P300 acetyltransferase and specific sgRNAs in order to increase acetylation the Gabra1 promoter (Fig. 3A, 3B) (Hilton et al., 2015). sgRNAs were designed to target the region of -50bp to +250bp since we found decreased histone acetylation associated with the Gabra1 promoter (data not shown) and others have shown that this region has promoter activity (Hu et al., 2008). We also designed an sgRNA targeting exon 5 because in vivo experiments demonstrated that ethanol did not changes histone acetylation in this region, and this region is known to have low baseline acetylation (Wang et al., 2008). Transduction of neurons on DIV 15 with both lentiviruses, followed by doxycycline administration on DIV 16 produced lentiviral constructs co-localized to the same
neurons (Fig. 3C and D). Targeting of dCas9-P300 to the Gabra1 promoter caused a 349.8 ± 64.3% increase in histone acetylation of the Gabra1 promoter region and prevented the ethanol-induced decrease in histone acetylation at the Gabra1 promoter (Fig. 4A. mock ethanol: 22.0 ± 6.7% control; dCas9-P300 + ethanol: 412.4 ± 117.5% control). Changes in histone acetylation correlated with a decrease in Gabra1 expression after ethanol (Fig. 4B. 0.22 ± 0.02 fold control) and an increase in Gabra1 expression in the absence (Fig. 4B. 6.22 ± 0.26 fold control) and presence of ethanol (Fig. 4B. 6.60 ± 1.04 fold control) with dCas9-P300 targeted to the Gabra1 promoter region. Western blot analysis of α1 expression reveals that dCas9-P300 targeted to the promoter region prevents the decrease of α1 expression caused by ethanol exposure (Fig. 4C). We next utilized dCas9-P300(D1399Y), with no histone acetyltransferase activity, in order to determine if histone acetyltransferase activity was necessary for preventing decreases caused by ethanol exposure. qPCR analysis revealed that targeting dCas9-P300(D1399Y) to the Gabra1 promoter fails to prevent the decrease in Gabra1 expression caused by ethanol exposure (Fig. 4D). We then targeted dCas9-P300 to exon 5 of the Gabra1 gene in order to determine if acetylation of another region of the Gabra1 gene is sufficient to prevent decreases in Gabra1 expression after ethanol exposure. qPCR analysis revealed that targeting dCas9-P300 to exon 5 of the Gabra1 gene fails to prevent decreases in Gabra1 expression caused by ethanol exposure (Fig. 4E). We then tested whether targeting another dCas9 construct (dCas9-VP64) (Thakore et al., 2015) that is known to activate gene transcription via the Gabra1 promoter would prevent decreases in α1 expression caused by ethanol exposure.
Western blot analysis revealed that dCas9-VP64 targeted at the *Gabra1* promoter is no different than mock transduced neurons in the presence of ethanol (Fig. 4F).

**Targeted H3K9me3 at the Gabra1 promoter using dCas9-KRAB fails to prevent decreases in acetylation at the Gabra1 promoter caused by ethanol exposure**

Histone PTMs often work in conjugation or in opposition with one another to regulate gene transcription (Wang et al., 2008). Therefore, we examined whether changes in histone acetylation were due to interactions with another repressive histone PTM. We utilized a dCas9 construct that is fused to the KRAB domain (Kabadi et al., 2014) in order to increase repressive H3K9me3 at the *Gabra1* promoter (Fig. 5A). ChIP analysis revealed that ethanol did not increase repressive H3K9me3 (Fig. 5B. 129.89 ± 18.03% control). Targeting dCas9-KRAB to the *Gabra1* promoter increased H3K9me3 associated with the *Gabra1* promoter in both the absence (Fig. 5B. 393.76 ± 21.71% control) and presence of ethanol (Fig. 5B. 432.05 ± 132.84% control). We next analyzed acetylation associated with the *Gabra1* promoter after dCas-KRAB was targeted to the *Gabra1* promoter in the presence and absence of ethanol. Only ethanol exposure caused a decrease of histone acetylation (Fig. 5C. mock ethanol = 41.44 ± 4.26% control; dCas9-KRAB + EtOH 33.77 ± 10.34% control). Western blot analysis suggested that both dCas9-KRAB transduction (dCas9-KRAB + H2O: 66.87±15.27% control) and ethanol exposure (no dCas9-KRAB + EtOH 71.28±4.25% control) cause a decrease in α1 expression (Fig. 5D), but there was no further decrease in α1 expression when both dCas9-KRAB and EtOH were present (74.54 ± 9.48% control).
However, these changes failed to reach statistical significance when analyzed by two-way ANOVA.

Discussion (1500 words)

Ethanol exposure decreases α1 protein expression both in vitro and in vivo and is thought to underlie GABA\(_A\)-R hypofunction and ethanol withdrawal symptoms in rodents (Devaud et al., 1995b; Devaud et al., 1997; Liang et al., 2004; Liang et al., 2007; Kumar et al., 2009). Decreases in α1 protein expression has been shown to occur at the same time that there are decreases in \(\text{Gabra1}\) gene expression (Devaud and Morrow, 1995; Devaud et al., 1997), suggesting that a transcriptional mechanism might be involved. Our results reveal that ethanol controls \(\text{Gabra1}\) gene expression through a histone deacetylation mechanism that can be prevented by interventions on either the pharmacological, genetic, or epigenetic level. Histone deacetylation associated with the \(\text{Gabra1}\) promoter is likely driven by class I HDACs, as knockdown of HDAC1-3 prevented ethanol-induced decreases of α1 protein expression.

Previous studies have found that HDAC inhibitors prevent decreases in α1 protein expression after chronic ethanol exposure (Arora et al., 2013) or via the α1(A322D) gene mutation that decreases α1 expression found in epilepsy (Di et al., 2013). However, neither of these studies determined if transcriptional mechanisms via epigenetic modulation were involved. Our results indicate that ethanol exposure causes a decrease in α1 protein expression through histone deacetylation associated with the \(\text{Gabra1}\) promoter. Deacetylation of histone associated with promoters often correlates with decreases in gene transcription, although there are cases in which the gene
silenced appears to preclude changes in histone acetylation (Wang et al., 2008). Importantly, until recent advances in gene targeting technology, most studies have only examined correlations between gene transcription and histone acetylation. We utilized several strategies to determine that histone acetylation associated with the Gabra1 promoter was contributing to decreases in Gabra1 expression. First, ethanol exposure caused a decrease in histone acetylation associated with the Gabra1 promoter and this was prevented by targeting histone acetylation to this location. Second, targeting a generic Cas9 activator (dCas9-VP64) to this region did not prevent decreases in α1 expression caused by ethanol exposure. Third, increasing repressive H3K9me3 at this region did not interfere with the ethanol-induced decrease in H3 acetylation. Together, these results suggest that histone deacetylation associated with the Gabra1 promoter regulates the decrease in α1 expression in response to ethanol and elucidates a new regulatory mechanism for ethanol’s control of α1 expression. This does not preclude the possibility that histone acetylation is also regulating trafficking and/or folding in response to ethanol and that HDAC inhibitors are working to prevent changes on that level. However, our results appear to suggest that a transcriptional mechanism is likely driving the changes as targeted acetylation of the Gabra1 promoter prevents changes in GABA<sub>A</sub>-R α1 protein expression.

The region of the Gabra1 promoter where we found increased histone acetylation contains 797 putative transcription factor binding motifs (Mathelier et al., 2016). Of these transcription factors, cAMP response element binding (CREB) and CREB binding protein sites were identified, which have previously been implicated in controlling Gabra1 transcript and surface expression in animal models of epilepsy (Hu et al., 2008;
Grabenstatter et al., 2012). Deficits in CREB binding protein have been implicated in models of alcohol dependence in the amygdala (Pandey et al., 2005; Pandey et al., 2017), an area that shows evidence of GABA<sub>A</sub> receptor hypofunction (Herman et al., 2013; Herman et al., 2016). Thus, decreases in histone acetylation at the Gabra1 promoter may initiate signaling cascades that are involved in decreasing GABA<sub>A</sub>-R α1 expression and function. Future experiments should consider the transcription factors involved to specifically determine ethanol effects on Gabra1 gene transcription due to changes in histone acetylation.

Pharmacological and knockdown experiments suggested that class I HDACs were responsible for ethanol-induced changes in Gabra1 expression. Class I HDACs include HDAC(1-3) and are typically localized to the nucleus (Haberland et al., 2009). HDAC1 and HDAC2 are nearly identical and are often found in the same repressive complexes (Haberland et al., 2009). However, HDAC2 has been suggested to be involved in the development of alcohol dependence in rodent models (Arora et al., 2013; Moonat et al., 2013; López-Moreno et al., 2015) and another study has shown that HDAC2 is up-regulated by ethanol at 4h in a neuronal cell line and that this effect can be blocked by TSA (Agudelo et al., 2011). However, none of the previous studies reported evaluating HDAC1 or HDAC3 expression. Interestingly, HDAC2 knockdown in the hippocampus causes a decrease in excitatory transmission and increase in inhibitory transmission, but had no effect on α1 expression (Hanson et al., 2013). Other experiments have shown that HDAC2, but not HDAC1 overexpression regulate memory formation and synaptic plasticity (Guan et al., 2009), while another study has suggested that HDAC1, but not HDAC2 regulate locomotor effects of cocaine (Kennedy et al.,
Finally, MS-275 which inhibits class I HDACs prevented cocaine-induced increases in Gabra1 expression (Kennedy et al., 2013). Interestingly, HDAC knockdown did not cause compensatory changes other HDAC isoform expression that we measured via qPCR, and this effect has been previously reported (Kennedy et al., 2013; Kuzmochka et al., 2014), however this doesn’t preclude compensatory changes that could occur or that we may not have observed due to the temporal and spatial constraints of our study.

Neurons were cultured from mixed sex (50% F/M) rat cerebral cortex. Thus, it is noteworthy that ethanol induced the same changes in Gabra1 and GABA<sub>A</sub>-R α1 subunit expression in these neurons as previously reported in various in vivo studies in male rat cortex following chronic ethanol exposure that produced ethanol dependence (Montpied et al., 1991; Mhatre and Ticku, 1992; Devaud et al., 1995b; Devaud et al., 1997; Cagetti et al., 2003; Sanna et al., 2003; Liang et al., 2004), but distinct from one study in female rats (Devaud et al., 1999), where no change in GABA<sub>A</sub>-R α1 subunit expression was observed. This comparison raises the possibility that ethanol effects on histone deacetylation may also involve sex differences that should be explored in future studies.

Histone deacetylase inhibitors have been suggested as a promising treatment for alcohol use disorders (Pandey et al., 2008; Arora et al., 2013; Warnault et al., 2013; Simon-O’Brien et al., 2015). The present results further support the use of class I histone deacetylase inhibitors for the treatment of alcohol use disorders. GABA<sub>A</sub>-Rs have long been known to be down-regulated after chronic ethanol use in both humans (Lingford-Hughes et al., 1997; Lingford-Hughes et al., 1998; Lingford-Hughes et al., 2000; Taylor et al., 2008) and rodents (Morrow et al., 1991; Liang et al., 2007) and this
downregulation causes benzodiazepine cross-tolerance (Lingford-Hughes et al., 1997; Lingford-Hughes et al., 1998; Lingford-Hughes et al., 2000; Cagetti et al., 2003; Liang et al., 2007; Taylor et al., 2008). Interventions that effect GABA\textsubscript{A}-R expression on a transcriptional level may bypass problems associated with using benzodiazepines in acute alcohol withdrawal syndrome as benzodiazepines have high abuse potential, are potentially lethal when combined with alcohol, and show cross-tolerance with ethanol (Hollister, 1990). Conversely, HDAC inhibitors have been shown to decrease ethanol and cocaine self-administration (Romieu et al., 2008; Simon-O’Brien et al., 2015) making them less likely to be abused. Additionally, human alcohol use disorder patients exhibit changes in a number of different genes (Farris et al., 2014) that may be related to changes in the epigenome (Ponomarev et al., 2012). Our results indicate that careful selection of the proper pharmacological inhibitors for preventing epigenetic changes in AUDs is required as sodium butyrate failed to prevent changes in \textit{Gabra1} expression, although this inhibitor has been shown to prevent drinking \textit{in vivo} (Simon-O’Brien). Sodium butyrate is a GABA analogue and may have agonist activity at receptors leading to activity dependent down-regulation of these receptors. Thus, interventions for alcohol dependence may require targeting molecular components that control acetylation mechanisms rather than individual genes as demonstrated in the current study using dCas9-P300. However, this system has useful experimental value because it allows for determination of specific epigenetic mechanisms associated with specific genes.

In conclusion, we present a new signaling pathway activated by chronic ethanol exposure and withdrawal that involves histone deacetylation associated with the \textit{Gabra1}
promoter which drives decreases in *Gabra1* transcription in cortical cultured neurons.

Finally, we suggest that histone deacetylase inhibitors may be a useful therapeutic intervention for the treatment of alcohol use disorders and possibly other diseases where α1 expression is dysregulated.

**Authorship Contributions**

*Participated in research design:* Bohnsack and Morrow

*Conducted experiments:* Bohnsack and Patel

*Contributed new reagents or analytical tools:* n/a

*Performed data analysis:* Bohnsack, Patel, and Morrow

*Wrote or contributed to writing of the manuscript:* Bohnsack and Morrow
References


Liang J, Cagetti E, Olsen RW and Spigelman I (2004) Altered pharmacology of synaptic and extrasynaptic GABA$_\alpha$ receptors on CA1 hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence. *J Pharmacol Exp Ther* 310:1234-1245.


Lingford-Hughes AR, Acton PD, Gacinovic S, Busatto GF, Costa DC, Boddington S, Marshall JE, Ell PJ, Kerwin RW and Pilowsky LS (1997) Reduced levels of the


Footnotes

This work was supported by the National Institute of Alcohol Abuse and Alcoholism grant AA11605 to ALM and the Bowles Center for Alcohol Studies.
Legends for Figures

Figure 1. Pharmacological inhibition of class I HDACs prevents decrease in 

\textit{Gabra1} expression caused by ethanol exposure. Co-exposure to TSA (500 nM) and 

ethanol (50 mM) prevents the ethanol-induced decrease in \textit{Gabra1} expression [Two-

way ANOVA interaction, \(F_{1,20} = 25.68, p < 0.0001, n = 6\)]. Co-exposure to SAHA (3 \(\mu\)M) 

and ethanol (50 mM) prevents decrease in \textit{Gabra1} expression [Two-way ANOVA 

interaction, \(F_{1,15} = 7.831, p = 0.0135, n = 4-5\)]. Co-exposure to HDAC3 specific inhibitor 

and ethanol (50 mM) at two concentrations of RGFP966 (24 nM and 80 nM) prevents 

decreases in \textit{Gabra1} expression. RGFP966 (24 nM) [Two-way ANOVA interaction, \(F_{1,8} 

= 26.47, p = 0.0009, n = 3\)], RGFP966 (80 nM) [Two-way ANOVA interaction, \(F_{1,20} = 

7.758, p = 0.0114, n = 6\)]. Co-exposure of class II HDAC inhibitor MC1568 (1 \(\mu\)M) with 

ethanol does not prevent decreases in \textit{Gabra1} expression [Effect of ethanol, two-way 

ANOVA, \(F_{1,20} = 64.49, p < 0.0001, n = 6\)]. Exposure to sodium butyrate (1 mM) ± 

ethanol (50mM) causes a decrease in \textit{Gabra1} expression [Two-way ANOVA interaction, 

\(F_{1,20} = 17.16, p = 0.0005, n = 6\)]. Statistical significance is determined by two-way 

ANOVAs followed by Bonferroni post-hoc tests. * = \(p < 0.05\), ** = \(p < 0.01\), *** = \(p < 

0.001\), **** = \(p < 0.0001\). Each bar represents mean ± S.E.M.

Figure 2. Knockdown of HDAC1-3 prevents decreases in \(\alpha1\) expression caused 

by ethanol exposure. (A) Schematic demonstrating the experimental outline for 

knockdown of HDAC1 and HDAC2 using shRNA delivered via lentiviral transduction. 

(B) Schematic demonstration of the experimental outline for knockdown of HDAC3 

using siRNA via transfection. (C) Quantification of HDAC1 expression after HDAC1
knockdown [Effect of HDAC1 knockdown, two-way ANOVA, $F_{1,20} = 105.1, p < 0.0001, n = 6$]. (D) Quantification of HDAC2 expression after HDAC2 knockdown [Effect of HDAC2 knockdown, two-way ANOVA, $F_{1,19} = 10.24, p = 0.0047, n = 5-6$]. (E) Quantification of HDAC3 expression after HDAC3 knockdown [Effect of HDAC3 knockdown, two-way ANOVA, $F_{1,20} = 47.41, p < 0.0001, n = 6$]. (F) Representative western blots and quantification of $\alpha 1$ expression after HDAC1 knockdown [Effect of HDAC1 knockdown, two-way ANOVA, $F_{1,20} = 20.84, p = 0.0002, n = 6$]. (G) Representative western blots and quantification of $\alpha 1$ expression after HDAC2 knockdown [Two-way ANOVA interaction, $F_{1,17} = 7.521, p = 0.0139, n = 4-6$]. (H) Representative western blots and quantification of $\alpha 1$ expression after HDAC3 knockdown [Two-way ANOVA interaction, $F_{1,19} = 9.403, p = 0.0064, n = 5-6$]. Each bar represents the mean ± S.E.M. Statistical significance is determined by two-way ANOVAs followed by Bonferroni post-hoc tests.

* = $p < 0.05$, ** = $p < 0.01$. SC = scrambled virus or siRNA + H$_2$O exposure. SE = scrambled virus or siRNA + EtOH exposure. KC = knockdown of HDAC + H$_2$O exposure. KE = knockdown of HDAC + EtOH exposure.

Figure 3. Experimental strategy for dCas9-P300 use in probing histone acetylation associated with the Gabra1 promoter following ethanol exposure. (A) Plasmid maps demonstrating the vector for dCas9-P300 with the dsRed reporter and the inducible sgRNA constructs with eGFP reporters. (B) Experimental strategy to determine if preventing ethanol-induced histone deacetylation at the Gabra1 promoter...
prevents decreases in *Gabra1* expression. (C) Schematic demonstrating experimental timeline for experiments. dCas9 constructs in lentiviral particles are transduced at DIV15, sgRNA production is induced on DIV16 by doxycycline, on DIV18 50mM EtOH is added to neurons for 4h. (D) Representative epifluorescent image showing co-localization of dsRed and eGFP to demonstrate transduction of cortical neurons with dCas9-P300 and sgRNA, respectively.

**Figure 4.** dCas9-P300 prevents deacetylation of the *Gabra1* promoter and decreases in *Gabra1* expression caused by ethanol exposure. (A) Quantitative chromatin immunoprecipitations using a pan-acetyl H3 antibody reveal that targeting the *Gabra1* promoter with dCas9-P300 increases acetylation at the *Gabra1* promoter [Effect of dCas9-P300, two-way ANOVA, *F*\(_{1,8}\) = 22.10, *p* = 0.0015]. (B) qPCR analysis reveals that targeting the *Gabra1* promoter with dCas9-P300 increases *Gabra1* expression [Effect of dCas9-P300, two-way ANOVA, *F*\(_{1,8}\) = 18.81, *p* = 0.0025]. Asterisk's shown are for two-way ANOVA significance. (C) Western blot analysis reveals that targeting the *Gabra1* promoter with dCas9-P300 prevents the decrease in GABA\(_\text{A}\)-R \(\alpha_1\) expression [Two-way ANOVA, *F*\(_{1,8}\) = 14.32, *p* = 0.0054]. (D) Targeting the *Gabra1* promoter with a mutant dCas9-P300 that lacks histone acetyltransferase activity fails to prevent the decrease in *Gabra1* expression caused by ethanol [Two-way ANOVA, ANOVA *F*\(_{1,8}\) = 91.08, *p* < 0.0001]. (E) Targeting *Gabra1* exon 5 with dCas9-P300 fails to prevent decreases in *Gabra1* expression caused by ethanol exposure [Two-way ANOVA, *F*\(_{1,8}\) = 415.0, *p* < 0.0001]. (F) Generic activation with a VP64 domain also fails to prevent decreases in \(\alpha_1\) expression caused by ethanol exposure [Significant effect of
ethanol, two-way ANOVA $F_{1,8} = 12.16$, $p = 0.0082$. Each bar represents mean ± S.E.M. $n = 3$ independent experiments. Statistical significance is determined by two-way ANOVAs followed by Bonferroni post-hoc tests. * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$.

**Figure 5.** Increasing repressive H3K9me3 does not alter decreases in *Gabra1* expression caused by deacetylation. (A) Schematic of the experimental strategy to use dCas9-KRAB targeted at the *Gabra1* promoter to increase H3K9me3. (B) ChIP reveals that targeting dCas9-KRAB to the *Gabra1* promoter increases H3K9me3 [Effect of dCas9-KRAB, two-way ANOVA, $F_{1,8} = 18.81$, $p = 0.0025$]. (C) ChIP reveals that H3 acetylation at the *Gabra1* promoter is only decreased in the presence of ethanol [Effect of ethanol, two-way ANOVA, $F_{1,8} = 21.05$, $p = 0.0018$]. (D) Western blot analysis reveals that both dCas9-KRAB and EtOH trend toward decreased α1 expression, but dCas9-KRAB does not alter the effect of ethanol. [Interaction between dCas9-KRAB and ethanol, two-way ANOVA, $F_{1,8} = 2.21$, $p = 0.18$]. Each bar represents mean ± S.E.M. of 3 independent experiments. Statistical significance is determined by two-way ANOVAs followed by Bonferroni post-hoc test. * = $p < 0.05$. MC = mock transduction ± H$_2$O. ME = mock transduction + EtOH. KC = dCas9-KRAB transduction ± H$_2$O. KE = dCas9-KRAB transduction + EtOH.
Table 1: sgRNA sequences

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence 5 -&gt; '3</th>
<th>PAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabra1 Promoter #1</td>
<td>TAATACGTCCCAGCGCAAAC</td>
<td>CGG</td>
</tr>
<tr>
<td>Gabra1 Promoter #2</td>
<td>ATTTACATCCGGTTTGCCT</td>
<td>TGG</td>
</tr>
<tr>
<td>Gabra1 Promoter #3</td>
<td>TTTCACATCCGGTTTGCCT</td>
<td>GGG</td>
</tr>
<tr>
<td>Gabra1 Exon 5 #1</td>
<td>TGCCATCTCTCTGATACGC</td>
<td>AGG</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing the comparison of different treatments on gene expression](image-url)

**Graph Key**
- Vehicle
- Ethanol (EtOH, 50 mM)
- HDAC Inhibitor
- HDAC Inhibitor + EtOH

**Compounds**
- TSA (500 nM)
- SAHA (3 μM)
- RGFP966 (24 nM)
- RGFP966 (99 nM)
- MC1568 (1 μM)
- Sodium Butyrate (1 mM)

**Categories**
- Hydroxamates: broad spectrum
- Carboxamide: HDAC3 selective
- Hydroxylamine (Class II selective)
- Fatty Acid (broad spectrum)
Figure 2.
Figure 3.
Figure 4.
Figure 5.