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Chronic Ethanol Exposure and Withdrawal Impairs Synaptic GABA_A Receptor-Mediated Neurotransmission in Deep Layer Prefrontal Cortex

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Abstract

BACKGROUND: The prefrontal cortex (PFC) acts as an integrative hub for the processing of cortical and sub-cortical input into meaningful efferent signaling, permitting complex associative behaviors. PFC dysfunction is consistently observed with ethanol dependence and is a core component of the pathology of alcohol use disorders in current models of addiction. While intracortical GABAergic neurotransmission is understood to be essential for maintaining coordinated network activity within the cortex, relatively little is known regarding functional GABAergic adaptations in PFC during ethanol dependence.

METHODS: In the present study, male and female (>PN60) Sprague-Dawley rats were administered ethanol (5.0 g/kg; intragastric gavage) for 14–15 consecutive days. 24 hours after the final administration, animals were sacrificed and brains extracted for electrophysiological recordings of isolated GABA_A receptor-mediated currents or analysis of GABA_A receptor subunit protein expression in deep layer prefrontal cortical neurons.

RESULTS: Chronic ethanol exposure significantly attenuated activity-dependent spontaneous GABA_A receptor-mediated inhibitory post-synaptic current (IPSC) frequency with no effect on amplitude. Furthermore, analysis of IPSC decay kinetics revealed a significant enhancement of IPSC decay time that was associated with decrements in expression of the $\alpha 1$ GABA_A receptor subunit, indicative of further impaired phasic inhibition. These phenomena occurred irrespective of neuron projection destination and sex. Based on previous observations by our laboratory of an epigenetic mechanism for ethanol-induced changes in cortical GABA_A receptor subunit

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expression, the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) was administered to water- and ethanol-exposed animals, and prevented ethanol-induced changes in sIPSC frequency, IPSC decay kinetics, and GABA_A receptor subunit expression.

CONCLUSIONS: Taken together, these results demonstrate that chronic ethanol exposure impairs synaptic inhibitory neurotransmission in deep layer pyramidal neurons of the medial prefrontal cortex in both male and female rats. These maladaptations occur in neurons projecting to numerous regions implicated in the sequelae of ethanol dependence, offering a mechanistic link between the manifestation of PFC dysfunction and negative affective states observed with extended consumption.

Keywords

Alcohol dependence; prelimbic mPFC; slice electrophysiology

Introduction

Alcohol use disorders (AUDs) represent a major public health hazard with current estimates indicating nearly 6% of preventable fatalities worldwide are alcohol-related and costing nearly 3% of gross domestic product in high income countries (Rehm, 2011, World Health Organization, 2014). Curbing the prevalence of AUDs has proven to be an intractable problem due in part to an incomplete understanding of the alcohol-induced neurobiological mechanisms that drive maladaptive behaviors characteristic of AUDs. Chief among these behaviors is a loss of inhibitory control that is strongly associated with dysregulation in the prefrontal cortex (PFC). The PFC acts as a nexus that integrates sensory, motivational, and hedonic stimuli into meaningful efferent signaling across the brain, and is critical for the manifestation of complex goal-directed behaviors. Impaired activity within this region is linked to a variety of cognitive and behavioral deficits including impaired working memory and response inhibition (Unterrainer and Owen, 2006, Bokura et al., 2001). For these reasons, PFC dysfunction has become a core component in the pathophysiology of current models of alcohol addiction (Goldstein and Volkow, 2002, Koob, 2013).

The PFC is composed of stratified layers of excitatory pyramidal neurons and GABAergic inhibitory interneurons that collectively assemble into networks that permit the integration of cortical and sub-cortical input into meaningful efferent signaling to downstream brain areas including the central nucleus of the amygdala (CeA). Deep-layer pyramidal neurons (e.g. Layers IV/V/VI) are generally understood to constitute the primary source of efferent signaling in these networks, with locally nested interneurons serving to tune their excitability (Kubota et al., 2015, Hattori et al., 2017). Indeed, reports demonstrate that cortical interneurons are essential for maintaining network activity (Roux and Buzsaki, 2015), and clinical studies have observed significant correlations between chronic alcohol use and impaired PFC network coherence that conform to preclinical observations showing elevated PFC activity with extended drug use (Kamarajan et al., 2004, Seo et al., 2013). Collectively, these studies implicate dysregulated GABAergic inhibition within the PFC after extended alcohol intake, meriting further investigation.

Consistent with these findings are converging reports from our laboratory and others that demonstrate substantial changes in the transcription, translation, and trafficking of GABA_A receptor subunits in both cortical neuronal cultures and hippocampal slices after chronic ethanol exposure. Specifically, chronic ethanol exposure reduces transcription and promotes internalization of $\alpha 1$ -containing receptors while enhancing transcription and surface expression of $\alpha 4$ -containing receptors, resulting in diminished phasic inhibition indicated by faster current decay and reduced charge transfer (Kumar et al., 2003, Kumar et al., 2010, Werner et al., 2011, Olsen and Liang, 2017). Additional studies using knockout mice lacking the $\alpha 1$ subunit observed behavioral phenotypes consistent with alcohol dependence including tremor, elevated seizure susceptibility, and diminished zolpidem loss of righting reflex (Kralic et al., 2005, Kralic et al., 2002). Likewise, elevated synaptic enrichment of $\alpha 4$ -containing receptors has been linked to these behaviors, specifically seizure susceptibility (Gonzalez and Brooks-Kayal, 2011). More recently, we have also shown that such alterations in GABA_A subunit expression are at least partially governed by epigenetic mechanisms, as chronic ethanol treatment results in diminished Gabra1 expression that correlates with diminished acetylation of the Gabra1 promoter. Further, treatment with histone deacetylase (HDAC) inhibitors has been shown effective at preventing these ethanol-induced phenomena (Bohnsack et al., 2018). While compelling, a number of questions remain, namely: 1. Do these changes manifest functionally in the medial PFC, specifically in the deep-layer, predominantly efferent-projecting neurons? 2. Are these changes sex- or circuit-specific? 3. Can HDAC inhibition preclude these alcohol-induced effects within the medial PFC?

To address this, male and female (>PN60) Sprague-Dawley rats were administered water or ethanol (5.0 g/kg, 25% v/v; intragastric gavage) for 14–15 consecutive days, as has previously been shown to reliably elicit neurobiological adaptations consistent with ethanol dependence (Devaud et al., 1997, Cagetti et al., 2003, Patten et al., 2014). We then performed whole-cell electrophysiological recordings and protein expression analysis to determine the effects of chronic ethanol exposure on deep-layer inhibitory signaling in the prelimbic aspect of the medial PFC, a region particularly important for alcohol relapse and drug reinstatement (Willcocks and McNally, 2013, McGlinchey et al., 2016). We hypothesized that chronic alcohol-exposed rats would exhibit significantly attenuated GABA_A receptor-mediated signaling, allowing for investigation of the synaptic mechanisms underlying the loss of GABA inhibition, the sex-dependency of these effects, the specificity of these phenomena in a model mPFC-to-CeA projection, as well as the efficacy of the HDAC inhibitor Trichostatin A in preventing alcohol-induced GABAergic adaptations.

Materials and Methods

Animal Use and Treatment

Male and female (>PN60) Sprague-Dawley rats were used for all experiments, ranging in weight from 319–418 g for males and 213–276 g for females with a mean weight of 358.8 ± 4.75 g and 247.6 ± 3.21 g respectively, and all procedures were performed in accordance with guidelines specified by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Animals were pair housed in a temperature

and humidity controlled facility under a 12hr light/dark cycle and given ad libitum access to food and water. For chronic ethanol treatment, rats were given a single water or ethanol dose (5 g/kg, 25% v/v) via intragastric gavage once daily during the light cycle for 14–15 days. As shown in Supplementary Figure 3, this method of administration elicits peak blood ethanol concentrations of ~250 mg/dL in both males and females of comparable age, consistent with binge-like intoxication levels (Patten et al., 2014). 24 hours after the final exposure, animals were sacrificed by rapid decapitation for electrophysiological and biochemical experiments. To determine the effect of TSA on ethanol-induced adaptations, animals received an injection of vehicle (10% DMSO in 0.9% saline, i.p.) or TSA (2 mg/kg, in 10% DMSO in 0.9% saline i.p.; Selleck Chem Inc.) 2 hours prior to gavage on the last 2 days of treatment as well as a third injection two hours prior to sacrifice, consistent with previously described methods (Pandey et al., 2008, Sakharkar et al., 2012, Bohnsack et al., 2018). While any potential effect of TSA on ethanol metabolism or clearance was not explicitly evaluated in this study, the fact that all recordings occurred 24 hours post-administration and that total ethanol clearance is typically observed 8–10 hours after administration with the gavage model make it unlikely that residual ethanol was present during recordings.

Stereotaxic Surgery

To determine whether ethanol-induced changes in GABAergic signaling were projection-dependent, animals underwent stereotaxic surgery using isoflurane anaesthesia for implantation of retrogradely-transported red fluorescent microspheres (Lumafluor Inc.; Durham, NC). A 300 nL volume of retrobeads was bilaterally injected using coordinates approximating the CeA (posterior from bregma: -2.3 mm; lateral ± 3.8 mm; ventral -8.2 mm: adapted from atlas coordinates) (Paxinos and Watson, 1998). Subjects were given 7–10 days of post-surgery recovery before beginning water/ethanol treatment. Injection sites were inspected following completion of electrophysiological experiments, and only recordings from tissue with verified “hits” were included for analysis of labeled neurons (approximate injection sites shown in Figure 2D).

Electrophysiology

Following brain extraction, 300 μ m thick coronal slices containing mPFC were prepared using a Leica VT1000S vibratome (Buffalo Grove, IL) containing oxygenated ice-cold sucrose cutting solution (in mM: 200 sucrose, 1.9 KCl, 1.2 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, 0.4 ascorbate, 10 glucose, 25 NaHCO₃, osmolarity adjusted to ~310 mOsm) to minimize cell death. Slices were then transferred to a continuously oxygenated (95% O₂, 5% CO₂) holding chamber containing artificial cerebrospinal fluid (aCSF) (in mM: 125 NaCl, 2.5 KCl, 1.5 NaH₂PO₄, 1.3 MgCl₂, 2.6 CaCl₂, 10 Glucose, 25 NaHCO₃, 0.4 Ascorbate, osmolarity adjusted to ~310 mOsm with sucrose) warmed to 37° Celsius for 30 min. Following incubation, the holding chamber was allowed to cool to room temperature for 1 hour, after which recordings were conducted. Whole-cell recordings were performed using an Axon Instruments Multiclamp 700A amplifier (San Jose, CA) sampling at 10 kHz and filtered at 2 kHz. Deep-layer (Layer V) pyramidal neurons were visually selected using an Olympus BX50WI microscope (Center Valley, PA) equipped with infrared differential interference contrast imaging. GABA_A receptor-mediated currents were isolated by

supplementing the aCSF solution with 10 μ M CNQX, 50 μ M dl-AP5, and 10 μ M CGP-52432 to block AMPA, NMDA, and GABA_B receptor activity respectively. A number of studies have previously reported non-canonical excitatory effects of CNQX on interneuron function due in part to interactions of AMPA receptors with TARP accessory proteins (Menuz et al., 2007, Maclean and Bowie, 2011). To rule out a potential confounding effect of CNQX in our studies, we therefore performed control recordings with NBQX in lieu of CNQX (data not shown) to block AMPA receptor activity. Similar sIPSC frequencies and amplitudes were observed between the two conditions, suggesting this phenomenon is minimal or absent in cortex and do not underlie our findings. Nevertheless, explicit investigation of AMPA/TARP association after ethanol exposure could be of value. Recordings were obtained using thin-walled (OD = 1.5 mm, ID = 1.17 mm) borosilicate glass electrodes with tip resistances ranging from 2–5 M Ω filled with a cesium chloride-based internal solution (in mM: 140 CsCl, 2 MgCl₂, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.3 Na-GTP, 5 Phosphocreatine, 3 QX-314, pH 7.4, osmolarity adjusted to ~290 mOsm with sucrose). Cells were held at –70 mV and spontaneous inhibitory post synaptic currents (sIPSC) were recorded. Miniature IPSC recordings were obtained by supplementing aCSF with 1 μ M tetrodotoxin (TTX; Tocris; Minneapolis, MN). Spontaneous and miniature IPSC kinetics were analyzed offline using MiniAnalysis software (v6.0.7, Synaptosoft, Decatur, GA). In brief, 30–40 IPSC events per recording were visually selected for capture that exhibited a sharp downward falling phase, single peak, and decay back to baseline. Captured events were then used to construct a scaled average composite trace to which a double exponential curve was fit to determine total and component IPSC decay kinetics (i.e. – τ_1 = decay time from peak to 50%, τ_2 = decay time from 50% to baseline). Kinetic values were subsequently pooled for statistical analysis. Any recordings in which access resistance exceeded 25 M Ω were excluded from analysis.

Western Blotting

Sub-cellular fractionation and western blot procedures were adapted from previously-described methods (Devaud et al., 1997, Bohnsack et al., 2018). Briefly, animals were sacrificed 24 hours after final exposure by rapid decapitation and brains extracted and snap frozen. Tissue was then microdissected by hand for prelimbic mPFC and hand homogenized and sonicated in ice-cold hypotonic lysis buffer composed of the following (in mM): 50 Tris-HCl, 50 NaCl, 5 EDTA, 320 Sucrose, 1x HALT protease inhibitor (ThermoScientific; Waltham, MA), pH to 7.4 with NaOH. Cell and nuclear debris were removed by centrifugation at 1,000xg for 10 minutes at 4°C, and the supernatant retained for an additional centrifugation at 12,000xg for 30 minutes at 4°C. The resulting pellet containing the enriched membrane fraction was washed once with ice-cold PBS, resuspended in ice-cold lysis buffer containing 0.5% (v/v) Triton-X100, and protein concentration determined using a Pierce BCA assay (ThermoScientific; Waltham, MA).

18 μ g of protein was electrophoresed using 4–12% Bis-Tris polyacrylamide gels and transferred to nitro-cellulose membranes (Invitrogen; ThermoScientific; Waltham, MA). Membranes were blocked for 1–1.5 hours with 1:1 PBS/Odyssey Blocking Buffer (LI-COR; Lincoln, NE), then incubated overnight at 4°C with one of the following antibodies: anti-GABA_A α 1 (Millipore; AB5592–200; 1:2,000), anti-GABA_A α 4 (Abcam; ab117080;

1:500), or anti- β -actin (Novus; NB600–501; 1:10,000). Bands corresponding to previously established molecular weights (52 kDa, 64 kDa, and 42 kDa for $\alpha 1$, $\alpha 4$, and β -actin respectively) were subsequently visualized using the Odyssey Classic Imaging System and band intensity quantified using ImageStudio Lite (LI-COR; Lincoln, NE). Results were normalized to β -actin and all values expressed as percent of control.

Statistics

Statistical analyses including Student's t-test and standard two-factor ANOVA (with Tukey's post-hoc test) were performed using GraphPad Prism 6.0 software (San Diego, CA). For electrophysiological experiments, multiple neurons were typically recorded from each animal, with 6–9 animals generally comprising each group. Standard analysis using either individual neurons or averaged values for each animal as the unit of determination both require an untenable assumption regarding variance, precluding use of ANOVA for statistical testing. Specifically, defining an n as a neuron assumes independence between observations within an animal, while defining an n as the average of responses from an animal ignores potentially important between-neuron variation. We therefore opted to employ a linear mixed model for these analyses using the "proc mixed" procedure in SAS 9.4 software (SAS Institute; Cary, NC). This model is well suited to incorporate multiple levels of variation to address these limitations (e.g. between-neuron and between-animal) and furthermore has substantial capacity for modification that makes it well suited for analysis of slice electrophysiological data (see (DeHart and Kaplan, 2019) for recent discussion). For our purposes, fixed effects were evaluated according to traditional two-factor designs using individual neuron values as the unit of determination and inter-animal variance modeled as a random subject effect with variance component covariance matrix. Solutions for linear mixed models were estimated using restricted maximum likelihood (REML) and effective degrees of freedom determined by Satterthwaite's estimation method. Reported F values and approximate p values were evaluated using Type 1 tests of fixed effects, followed by post-hoc testing when appropriate. Initial ethanol effects shown in Figure 1 were analyzed with sex as a factor, and after observing no effect of sex on any of the metrics tested, male and female data were subsequently combined for analysis. For statistical analyses shown in Supplementary Figures 1 and 2, changes in holding current were assessed by a previously-described method wherein a normalized all-points histogram was constructed for baseline and treatment time periods (20–30 sec) and a Gaussian function was fit (Glykys and Mody, 2007, Herman et al., 2012). Bin center values corresponding to mean holding current for baseline and THIP/TTX treatment periods of individual cells were pooled and statistically compared using the linear mixed model procedure with THIP/TTX treatment as an added repeated effect. For all figures, a p value less than 0.05 was considered statistically significant (* < 0.05, ** < 0.01, *** < 0.001).

Results

Chronic ethanol exposure decreases GABAergic inhibition of deep-layer prelimbic mPFC pyramidal neurons.

Voltage-clamp recordings ($V_m = -70\text{mV}$) of baseline spontaneous inhibitory post-synaptic currents (sIPSC) revealed a significant attenuation (Figure 1C) of event frequency following

chronic ethanol exposure that occurred independent of the sex of the animal (water-exposed = 2.914 ± 0.15 Hz; ethanol-exposed = 2.366 ± 0.12 Hz: $F_{(1,29.1)}$ interaction = 0.44, $p = 0.5104$; $F_{(1,27.6)}$ treatment = 8.401, $p = 0.0086$; $F_{(1,28.6)}$ sex = 0.13, $p = 0.7214$; $n = 18$ – 23 cells from 8–9 animals per group). Chronic alcohol exposure did not, however, alter sIPSC amplitude in male or female rats (water-exposed = 89.19 ± 5.24 pA; ethanol-exposed = 89.14 ± 4.12 pA: $F_{(1,33.4)}$ interaction = 0.13, $p = 0.7184$; $F_{(1,31)}$ treatment = 0.01, $p = 0.9335$; $F_{(1,32.9)}$ sex = 1.63, $p = 0.2108$; $n = 18$ – 23 cells from 8–9 animals per group). Summary values of sIPSC frequency, amplitude, decay time, and membrane characteristics between groups are shown in Table 1. Analysis of sIPSC kinetics revealed a significant reduction in current decay time (Figure 1E) in ethanol-exposed animals compared to controls indicative of reduced charge transfer per event. Weighted analysis (Figure 1G–H) further revealed that this observation was largely driven by reductions in the “fast” tau1 component (water-exposed = 11.19 ± 0.45 ms; ethanol-exposed = 8.64 ± 0.39 ms: $F_{(1,24.4)}$ interaction = 2.33, $p = 0.1395$; $F_{(1,23)}$ treatment = 16.93, $p = 0.0004$; $F_{(1,23.9)}$ sex = 0.87, $p = 0.3613$; $n = 18$ – 23 cells from 8–9 animals per group). No significant change in the tau2 component was observed (water-exposed = 23.14 ± 0.91 ms; ethanol-exposed = 21.0 ± 0.76 ms $F_{(1,29.2)}$ interaction = 0.92, $p = 0.3462$; $F_{(1,27.9)}$ treatment = 3.05, $p = 0.0918$; $F_{(1,28.7)}$ sex = 0.02, $p = 0.8812$; $n = 18$ – 23 cells from 8–9 animals per group). We note that in all analyses, no effect of sex was observed between water- or ethanol-exposed animals.

Preliminary experiments investigating ethanol-induced changes in GABA_A tonic current shown in Supplementary Figures 1 and 2 did not yield any significant effects. Specifically, bath application of the δ subunit-containing selective GABA_A agonist 4,5,6,7-tetrahydroisoxadolo(5,4-c)pyridine-3-ol (THIP; 1 μ M) (S1) elicited robust enhancement of GABA_A receptor-mediated current that did not differ significantly between water- and alcohol-exposed groups ($F_{(1,52.9)}$ interaction = 0.01, $p = 0.9183$; $F_{(1,52.9)}$ THIP treatment = 5.63, $p = 0.0213$; $F_{(1,14.7)}$ treatment = 0.02, $p = 0.8867$; $n = 13$ – 16 cells from 8–10 animals per group). Additionally, noise after THIP application (S1 D) did not differ between water and ethanol exposed animals ($F_{(1,50.2)}$ interaction = 0.30, $p = 0.589$; $F_{(1,50.2)}$ THIP treatment = 1.17, $p = 0.2839$; $F_{(1,12.3)}$ treatment = 0.04, $p = 0.8423$; $n = 13$ – 16 cells from 8–10 animals per group). S1 E and F respectively show representative histograms with overlaid Gaussian functions from water- and alcohol-exposed neurons before and after THIP. We similarly measured the effect of TTX on holding current (S2), and while TTX significantly reduced holding current and noise, there was no apparent difference between animals administered water or alcohol (holding current; $F_{(1,27.4)}$ interaction = 0.04, $p = 0.8417$; $F_{(1,27.4)}$ TTX treatment = 4.28, $p = 0.0482$; $F_{(1,10.4)}$ treatment = 1.51, $p = 0.246$; noise two-way repeated measures ANOVA; noise; $F_{(1,26.4)}$ interaction = 1.31, $p = 0.2633$; $F_{(1,26.4)}$ TTX treatment = 20.76, $p = 0.0001$; $F_{(1,10.8)}$ treatment = 0.01, $p = 0.9725$; $n = 10$ – 13 cells from 7–9 animals per group). Representative histograms with overlaid Gaussian functions from water- and alcohol-exposed neurons before and after TTX application are shown in S2 B and C respectively.

As shown in Figure 2, recordings conducted in pyramidal neurons projecting to the CeA recapitulated observations shown in Figure 1, suggesting that ethanol-induced impairments of GABA_A receptor-mediated neurotransmission onto deep-layer cortical pyramidal neurons occur irrespective of projection destination in the prelimbic mPFC. Figures 2E–F

demonstrate that ethanol blunts spontaneous IPSC frequency onto these neurons with no significant effect on IPSC amplitude (sIPSC frequency $F_{(1,14)} = 4.61$, $p = 0.0499$, $n = 8$ –11 cells; sIPSC amplitude $F_{(1,14.6)} = 0.82$, $p = 0.3802$; $n = 8$ –11 cells from 7 animals per group). As shown in Figure 2G, the tau1 decay component was similarly attenuated with no concomitant reduction in tau2 as observed in Figures 1G–H (tau1 $F_{(1,17)} = 6.00$, $p = 0.0254$; tau2 $F_{(1,12.9)} = 3.18$, $p = 0.0982$). Furthermore, we also note that GABA_A receptor-mediated neurotransmission on CeA-projecting pyramidal neurons displayed characteristics (e.g., event frequency, amplitude, and kinetics) similar to the broader population of deep layer pyramidal neurons, indicating that projection-specific subpopulations do not exhibit apparent innate differences in GABAergic tone.

In order to determine whether the reduction in spontaneous IPSC frequency was due to a change in excitability or action potential-dependent release probability of the presynaptic neuron, we recorded IPSCs before and after application of TTX (1 μ M). We again observed a significantly reduced IPSC frequency on deep-layer pyramidal neurons following chronic ethanol administration (Figure 3A); however, after application of TTX there was no significant difference in IPSC frequency, indicative of an activity-dependent change in presynaptic neuron excitability following alcohol exposure (Linear Mixed Model with Type 1 test of fixed effects and Tukey's post-test: $F_{(1,48)}$ interaction = 1.29, $p = 0.2622$; $F_{(1,48)}$ TTX treatment = 32.17, $p < 0.0001$; $F_{(1,48)}$ treatment = 8.10, $p = 0.0065$; $n = 12$ –14 cells from 7–10 animals per group: % change in frequency by TTX (mean $\pm$ SEM); water = -44.63 ± 6.29 , ethanol = -39.09 ± 7.77). As shown in Figure 3B, we did not observe any significant ethanol-induced change in IPSC amplitude pre- or post-TTX application (% change in amplitude by TTX (mean $\pm$ SEM); water = -28.27 ± 5.72 , ethanol = -22.68 ± 6.67). While we observed a significant reduction in total miniature IPSC decay time (Figure 3C; unpaired one-tailed t-test; $t_{(24)} = 1.712$, $p = 0.0499$), weighted analysis could not resolve the underlying component governing this observation (Figure 3D–E; unpaired one-tailed t-test: tau1 $t_{(24)} = 1.302$, $p = 0.103$; tau2 $t_{(24)} = 1.644$, $p = 0.0567$). Miniature IPSC kinetic values are shown in Table 2.

Effects of chronic ethanol exposure on GABA_A receptor mediated-neurotransmission are prevented by the HDAC inhibitor Trichostatin A.

As we and others have consistently observed ethanol-induced changes in GABA_A receptor subunits $\alpha 1$ and $\alpha 4$ following chronic ethanol exposure (Kumar et al., 2003, Kumar et al., 2010, Cagetti et al., 2003), we next probed the mPFC for changes in protein expression of these subunits. Furthermore, we similarly sought to determine if administration of TSA, an HDAC inhibitor, could prevent changes in expression as has previously been reported (Bohnsack et al., 2018). As shown in Figures 4A and 4C, ethanol exposure significantly reduces tau1 decay time and $\alpha 1$ subunit expression, and these changes are prevented by administration of TSA (tau1; Linear Mixed Model with Type 1 test of fixed effects and Tukey's post-hoc test: $F_{(1,51.4)}$ interaction = 2.71, $p = 0.106$; $F_{(1,51)}$ TSA treatment = 5.19, $p = 0.0269$; $F_{(1,51.7)}$ treatment = 9.19, $p = 0.0038$; $n = 29$ –42 cells from 13–18 animals per group: $\alpha 1$ expression; two-way ANOVA with Tukey's post-hoc test: $F_{(1,39)}$ interaction = 3.694, $p = 0.0619$; $F_{(1,39)}$ TSA treatment = 4.861, $p = 0.0334$; $F_{(1,39)}$ treatment = 3.762, $p = 0.0597$; $n = 10$ –12 animals). No significant effects of TSA or water/ethanol treatment were

observed on tau2 (Figure 4B) (Linear Mixed Model with Type 1 test of fixed effects and Tukey's post-hoc test: $F_{(1,55)}$ interaction = 0.59, $p = 0.4472$; $F_{(1,54.5)}$ TSA treatment = 3.48, $p = 0.0674$; $F_{(1,55.3)}$ treatment = 0.24, $p = 0.6226$; $n = 29$ –42 cells from 13–18 animals per group). In addition, we did not observe any significant effect of alcohol administration or TSA treatment on $\alpha 4$ subunit expression (Figure 4D; two-way ANOVA: $F_{(1, 40)}$ interaction = 0.188, $p = 0.667$; $F_{(1, 40)}$ TSA treatment = 0.236, $p = 0.63$; $F_{(1, 40)}$ treatment = 1.013, $p = 0.3201$; $n = 10$ –12 animals). Representative blots of $\alpha 1$ and $\alpha 4$ subunit expression between groups are shown in Figure 4E and 4F respectively.

Discussion

In the present study we examined whether chronic ethanol exposure and withdrawal elicit GABAergic adaptations within the medial PFC, and furthermore whether such adaptations occur in a specific CeA-projecting neuronal population. The data demonstrate that GABA_A receptor-mediated neurotransmission within the prelimbic mPFC onto deep layer pyramidal neurons is significantly impaired after chronic ethanol exposure via both pre- and post-synaptic mechanisms in both male and female rats. These findings indicate a broad adaptation within deep-layer mPFC that promotes enhanced efferent excitatory signaling and are consistent with clinical reports of elevated prefrontal cortical activity in human alcoholics (Kamarajan et al., 2004, Seo et al., 2013). Our observations of diminished post-synaptic GABA_A receptor function and attenuated presynaptic GABA release join a growing body of preclinical evidence implicating altered GABA_A receptor-mediated neurotransmission in the pathophysiology of alcohol use disorders.

Among our findings, we observed that chronic alcohol exposure significantly blunted basal spontaneous IPSC frequency onto deep-layer pyramidal neurons (Figure 1), indicative of decreased presynaptic release. Spontaneous vesicular release from presynaptic boutons is governed by a variety of mechanisms including stochastic fusion of ready-release vesicles, spontaneous calcium influx through voltage-gated calcium channels, as well as spontaneous action potential-driven vesicular release. As shown in Figure 3, application of the sodium channel blocker TTX resulted in significant reductions in frequency and amplitude of IPSCs to equivalent levels between alcohol and water exposed animals, indicating that ethanol-induced impairments in spontaneous IPSC frequency reflect altered action potential-dependent vesicular release (Figure 3). This phenomenon is consistent with previous work from (Pleil et al., 2015) showing that mice that received chronic intermittent ethanol exposure displayed similar decrements in sIPSC frequency onto Layer 2/3 pyramidal neurons of mPFC. While the discrete mechanism(s) governing our observations remains under investigation, the recording conditions employed in this study (i.e. in the presence of the NMDAR and AMPAR antagonists DL-AP5 and CNQX, respectively) argue this adaptation is likely not driven by altered glutamatergic signaling.

Cortical interneurons are known to exhibit vast heterogeneity both in their intrinsic properties as well as projection targets, and efforts are currently underway to localize our observations to a specific interneuron sub-population (Hattori et al., 2017). In the deep layers of cortex, parvalbumin-positive basket/chandelier interneurons and somatostatin-positive Martinotti cells appear to predominate, comprising >80% of total interneurons in

approximately equal frequency (Rudy et al., 2011). While Martinotti interneurons generally distribute their projections back into the superficial cortical layers, basket/chandelier interneurons preferentially target the soma/peri-soma of cis-layer pyramidal neurons and for this reason are the most likely source of GABAergic events recorded in this study (Holmgren et al., 2003, Rudy et al., 2011, Hattori et al., 2017). Importantly, basket/chandelier interneurons typically innervate 100's to 1000's of different pyramidal neurons; thus, while ethanol's effects on spontaneous release (~20% reduction) may appear modest, the aggregate effect on cortical function can be substantial (Holmgren et al., 2003, Hattori et al., 2017).

The post-synaptic GABA_A receptor adaptations presented herein after chronic alcohol exposure generally conform to previous findings in the literature across a number of brain regions including whole cortex, cultured cortical neurons, and hippocampus (Kumar et al., 2003, Kumar et al., 2010, Cagetti et al., 2003). Consistent among all these studies are reliable decrements in $\alpha 1$ subunit expression following chronic ethanol exposure that correlate with enhanced IPSC decay kinetics. We extend these observations by demonstrating similar adaptations in the medial PFC as well as showing that the adaptations occur irrespective of the sex of the animal. Functionally, the loss of $\alpha 1$ subunit expression and resultant enhancement of IPSC decay kinetics diminishes charge transfer per event, thereby blunting the efficacy of released GABA to shunt excitatory current. This phenomenon in tandem with diminished GABA release frequency reflects a state biased toward excitation, permitting heightened excitatory output to downstream targets. While we did not observe an effect of alcohol treatment on current injection-evoked spiking in deep-layer pyramidal neurons (data not shown), consistent with other reports (Trantham-Davidson et al., 2017), this does not necessarily conflict with the above interpretation. A prominent feature of the neocortex is relatively quiescent synaptic activity at rest, such that spontaneous depolarizations of pyramidal neurons are uncommon (Waters and Helmchen, 2006). As the electrode is generally placed on the soma near the axon initial segment, current-injection evoked spiking poorly models local network activity-induced neuronal firing when coordinated phasic GABAergic activity would likely be most efficacious. Thus, this method is likely insufficient to resolve the true impact of diminished GABA inhibition on excitability. Additional studies explicitly investigating how blunted phasic GABA tone impacts deep-layer pyramidal neuron excitability during network activity is therefore warranted.

Although tonic extrasynaptic GABA_A signaling can exert much more profound inhibitory tone relative to phasic signaling, null results from preliminary experiments investigating tonic GABA_A receptor-mediated current shown in Supplementary Figures 1 and 2 lead us to conclude that alcohol preferentially impairs phasic GABA inhibition. Given the critical role of cortex in processes like pattern recognition and signal integration that require the exquisite temporal precision phasic signaling offers, observing selective effects of ethanol on synaptic GABA_A neurotransmission is perhaps unsurprising. Nevertheless, others have shown that adolescent alcohol exposure can alter THIP-sensitive tonic current in mPFC, however these observations did not correlate with any apparent change in δ subunit protein expression (Centanni et al., 2017). It is worth noting as well that in contrast to other reports we did not observe any compensatory increase in $\alpha 4$ subunit expression as observed by

others (Bohnsack et al., 2016, Bohnsack et al., 2018). Given that $\alpha 4$ subunits seem to exhibit widespread extrasynaptic distribution this supports our conclusion of minimal effects of chronic ethanol exposure on tonic GABA_A-mediated currents (Bohnsack et al., 2016). Nevertheless, attenuated $\alpha 1$ subunit expression with no concomitant reduction in overall IPSC amplitude argues for compensatory upregulation of another GABA_A receptor subunit(s), and a more complete survey of α subunit expression would therefore be of value.

The clinical relevance of our findings becomes further apparent when one considers that models of alcoholism and alcohol dependence are increasingly integrating the concept of the “dark side” of addiction, in which negative affective states experienced during withdrawal drive continued consumption (Koob, 2013). As shown in Figure 2, we observed a robust sub-population of deep-layer pyramidal neurons that project to the CeA, a brain region critical for the expression of stress and anxiety-like behaviors that is significantly dysregulated following alcohol exposure (Roberto et al., 2004, Gilpin et al., 2015, Herman and Roberto, 2016). Recordings from these neurons displayed similar decrements in pre- and post-synaptic GABA_A receptor-mediated neurotransmission following chronic ethanol exposure as the general population of deep-layer pyramidal neurons. That these results are consistent with other reports showing elevated CeA activity following chronic ethanol exposure and withdrawal offers a potential mechanism for these observations (Roberto et al., 2004, George et al., 2012, Pleil et al., 2015). Specifically, a disinhibited state in CeA-projecting pyramidal neurons could precipitate elevated glutamatergic signaling to the CeA during acute withdrawal, thereby promoting negative affect and, ultimately, alcohol craving. Such a scenario is consistent with previous findings from our laboratory that showed elevations in the expression of anxiety-like behaviors in rats that underwent an alcohol exposure paradigm identical to the one employed in this study (Bohnsack et al., 2018). In addition, that study further showed that treatment with TSA prevented both the alcohol-induced changes in $\alpha 1$ subunit expression, replicated in the present study, as well as showing significant reductions in anxiety-like behavior during the acute withdrawal period investigated herein. In context with other observations demonstrating mPFC connectivity to additional limbic regions that contribute to dependence symptomology, the functional adaptations in GABAergic signaling described herein offer a tenable mechanistic link between cortical dysregulation and dependence-related symptoms (Vertes, 2004, Pleil et al., 2015, Vranjkovic et al., 2017). Further, the data offer a foundation for future studies to evaluate epigenetically-driven adaptations during various phases in the transition to, and recovery from, alcohol dependence that may yield new strategies to treat intractable alcohol use disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Chronic ethanol exposure attenuates GABA release on deep-layer medial prefrontal cortical pyramidal neurons. **A**) Schematic depicts alcohol exposure paradigm, in which animals are administered ethanol (5 g/kg, 25% ethanol) via intragastric gavage (i.g.), followed by a 24 hour withdrawal (adapted from (Bohnsack et al., 2018)). **B**) Recordings were conducted from deep-layer pyramidal neurons in the superior aspect of the prelimbic mPFC. **C**) Chronic alcohol treatment induced a significant reduction in spontaneous inhibitory post-synaptic currents in both male and female animals [$F_{(1,29.1)}$ interaction = 0.44, $p = 0.5104$; $F_{(1,27.6)}$ treatment = 8.01, $p = 0.0086$; $F_{(1,28.6)}$ sex = 0.13, $p = 0.7214$]. **D**) Chronic ethanol exposure did not significantly alter sIPSC amplitude. **E**) Chronic ethanol exposure significantly reduced total sIPSC decay time [$F_{(1,29.2)}$ interaction = 0.01, $p = 0.9358$; $F_{(1,27.5)}$ treatment = 8.55, $p = 0.0068$; $F_{(1,28.7)}$ sex = 0.07, $p = 0.7977$]. **F**) Representative scaled composite traces demonstrating effect of chronic ethanol on sIPSC decay. **G-H**)

Weighted analysis of decay time revealed that ethanol predominantly attenuated the “fast” tau1 decay component with no discernable effect on tau2 [tau1 $F_{(1,24.4)}$ interaction = 2.33, $p = 0.1395$; $F_{(1,23)}$ treatment = 16.93, $p = 0.0004$; $F_{(1,23.9)}$ sex = 0.87, $p = 0.3613$].

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Figure 2:

Chronic ethanol exposure attenuates synaptic GABA_A signaling onto deep-layer CeA-projecting cortical pyramidal neurons. **A–C)** Schematic demonstrates approximate injection site of retrogradely-transported fluorescent microspheres, subsequent location of recordings from labeled pyramidal neurons, and general morphology of labeled neurons (red = fluorescent microspheres, blue = DAPI). **D)** Approximate sites and spread of retrobead injections for data shown in **E–G**. **E)** sIPSC frequency is significantly attenuated in labeled neurons from ethanol exposed animals [$F_{(1,14)} = 4.61$, $p = 0.0499$; $n = 8–11$ cells]. **F)** Ethanol treatment did not significantly alter sIPSC amplitude in labeled neurons [$F_{(1,14.6)} = 0.82$, $p = 0.3802$; $n = 8–11$ cells]. **G)** τ_1 was significantly reduced following chronic ethanol treatment, with no significant change observed on τ_2 [τ_1 $F_{(1,17)} = 6.00$, $p = 0.0254$; τ_2 $F_{(1,12.9)} = 3.18$, $p = 0.0982$].

Figure 3:

Effect of tetrodotoxin (1 μ M) on GABA_A receptor-mediated neurotransmission. **A–B)** A main effect of TTX application was observed on both frequency and amplitude. Post-hoc analysis revealed that post-TTX frequency did not differ significantly between water and ethanol exposed groups, indicative of an activity-dependent mechanism underlying ethanol attenuation of baseline frequency [Linear Mixed Model with Type 1 test of fixed effects and Tukey's post-hoc test; $F_{(1,48)}$ Interaction = 1.29, $p = 0.2622$; $F_{(1,48)}$ TTX treatment = 32.17, $p < 0.0001$; $F_{(1,48)}$ treatment = 8.10, $p = 0.0065$; % change in frequency by TTX (mean $\pm$ SEM); water = -44.63 ± 6.29 , ethanol = -39.09 ± 7.77]. **C)** Reduced decay time after chronic ethanol administration was similarly observed in miniature IPSCs, consistent with spontaneous IPSC observations [unpaired one-tailed t-test; $df = 24$, $t = 1.712$, $p = 0.0499$]. **D–E)** Weighted analysis of decay kinetics could not resolve the underlying component

governing this attenuation [tau1 unpaired one-tailed t-test; $df = 24$, $t = 1.302$, $p = 0.103$; tau2 unpaired one-tailed t-test; $df = 24$, $t = 1.644$, $p = 0.0567$].

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Figure 4:

Treatment with the HDAC inhibitor Trichostatin-A prevents ethanol-induced deficits in synaptic GABA_A-mediated neurotransmission. **A)** Diminished decay tau1 time by ethanol treatment is rescued by TSA (2 mg/kg) [$F_{(1,51.4)}$ interaction = 2.71, $p = 0.106$; $F_{(1,51)}$ TSA treatment = 5.19, $p = 0.0269$; $F_{(1,51.7)}$ treatment = 9.19, $p = 0.0038$; Tukey's post-hoc test]. **B)** No significant effects of TSA or water/ethanol treatment were observed on tau2 [$F_{(1,55)}$ interaction = 0.59, $p = 0.4472$; $F_{(1,54.5)}$ TSA treatment = 3.48, $p = 0.0674$; $F_{(1,55.3)}$ treatment = 0.24, $p = 0.6226$; Tukey's post-hoc test]. **C)** TSA treatment rescues ethanol-induced decreases in alpha1 subunit protein expression [two-way ANOVA; $F_{(1,39)}$ interaction = 3.694, $p = 0.0619$; $F_{(1,39)}$ TSA treatment = 4.861, $p = 0.0334$; $F_{(1,39)}$ treatment = 3.762, $p = 0.0597$; Tukey's post-hoc test]. **D)** Neither alcohol exposure nor TSA treatment significantly altered alpha4 subunit protein expression. **E-F)** Images show representative immunoblots for alpha1 and alpha4 respectively (green = $\alpha 1/\alpha 4$, red = β -Actin).

Table 1:

Summary of functional characteristics of deep-layer medial prefrontal cortical pyramidal neurons.

Treatment	sIPSC Frequency (Hz)	sIPSC Amplitude (pA)	Membrane Capacitance (pF)	Membrane Resistance (M Ω)
Water - Male	3.029 $\pm$ 0.239	82.83 $\pm$ 5.09	95.58 $\pm$ 2.77	75.68 $\pm$ 3.74
Water - Female	2.82 $\pm$ 0.202	94.44 $\pm$ 8.55	93.57 $\pm$ 3.80	81.04 $\pm$ 3.09
Ethanol - Male	2.294 $\pm$ 0.186**	86.35 $\pm$ 5.04	92.11 $\pm$ 3.63	87.83 $\pm$ 4.12
Ethanol - Female	2.366 $\pm$ 0.171**	91.93 $\pm$ 6.60	93.89 $\pm$ 8.18	92.28 $\pm$ 8.75

Table 2:

Summary of miniature IPSC kinetics between water and ethanol exposed groups.

Treatment	mIPSC Amplitude (pA)	mIPSC Rise Time (ms)	mIPSC Unweighted Decay (ms)	mIPSC Decay tau1 (ms)	mIPSC Decay tau2 (ms)
Water Treated (n = 14)	68.14 ± 3.20	0.843 ± 0.05	41.42 ± 3.76	9.53 ± 0.67	31.89 ± 3.42
Ethanol Treated (n = 12)	72.99 ± 2.05	0.836 ± 0.05	32.91 ± 2.87*	8.27 ± 0.66	24.64 ± 2.37