HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2018 March 05.

Published in final edited form as:

Neuropharmacology. 2017 October ; 125: 197–206. doi:10.1016/j.neuropharm.2017.07.017.

P/Q-type voltage-gated calcium channels mediate the ethanol and CRF sensitivity of central amygdala GABAergic synapses

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Abstract

The central amygdala (CeA) GABAergic system is hypothesized to drive the development of alcohol dependence, due to its pivotal roles in the reinforcing actions of alcohol and the expression of negative emotion, anxiety and stress. Recent work has also identified an important role for the CeA corticotropin-releasing factor (CRF) system in the interaction between anxiety/stress and alcohol dependence. We have previously shown that acute alcohol and CRF each increase action potential-independent GABA release in the CeA via their actions at presynaptic CRF type 1 receptors (CRF_1s); however, the shared mechanism employed by these two compounds requires further investigation. Here we report that acute alcohol interacts with the CRF/CRF_1 system, such that CRF and alcohol act via presynaptic CRF_1s and P/Q-type voltage-gated calcium channels to promote vesicular GABA release and that both compounds occlude the effects of each other at these synapses. Chronic alcohol exposure does not alter P/Q-type voltage-gated calcium channel membrane abundance or this CRF_1/P/Q-type voltage-gated calcium channel mechanism of acute alcohol-induced GABA release, indicating that alcohol engages this molecular mechanism at CeA GABAergic synapses throughout the transition to dependence. Thus, P/Q-type voltage-gated calcium channels, like CRF_1s, are key regulators of the effects of alcohol on GABAergic signaling in the CeA.

Keywords

Alcohol/ethanol; Central amygdala; Corticotropin-releasing factor (CRF); Corticotropin-releasing factor type 1 receptor (CRF_1); GABA; P/Q-type voltage-gated calcium channel; Alcohol dependence

1. Introduction

The central amygdala (CeA) is hypothesized to drive the development of alcohol dependence, due to its pivotal roles in the reinforcing actions of alcohol and the expression of negative emotion, anxiety and stress (Gilpin et al., 2015; Koob and Volkow, 2010). Alcoholics often cite anxiety and stress as strong motivators for drinking (Litman et al., 1977, 1983; Ludwig and Wikler, 1974; Sinha, 2009), and both cue-elicited craving and intoxication increase the amygdalar activity of alcohol-dependent patients (Koob and

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Volkow, 2010). The CeA comprises an interconnected network of γ-aminobutyric acid (GABA) interneurons and GABA projection neurons (Haubensak et al., 2010; Lopez de Armentia and Sah, 2004; Marek et al., 2013), and this inhibitory drive regulates the escalated alcohol intake and anxiety-like behavior of alcohol-dependent rats (Gilpin et al., 2015; Koob and Volkow, 2010; Rassnick et al., 1993).

Recent work has also identified an important role for the CeA corticotropin-releasing factor (CRF) system in the interaction between anxiety/stress and alcohol dependence (Gilpin et al., 2015; Rassnick et al., 1993; Roberto et al., 2010b). Notably, CRF is co-released with GABA in the CeA (Partridge et al., 2016), typically in response to neuronal burst firing (Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998), and the expression levels of CRF and its type 1 receptor (CRF_1), as well as the basal concentration of GABA, are increased in the CeA of alcohol-dependent rats (Roberto et al., 2010b). Moreover, CeA-specific CRF_1 antagonism reduced the alcohol intake (Funk et al., 2006; Varodayan et al., 2017b) and anxiety-like behavior (Rassnick et al., 1993) of alcohol-dependent rats. Critically, we have previously shown that acute alcohol and CRF each increase action potential-independent GABA release in the CeA via their actions at presynaptic CRF_1s (Roberto et al., 2010b); however, the shared mechanism employed by these two compounds to activate CeA GABAergic synapses is not fully understood.

GABA release is strictly controlled by calcium influx through voltage-gated calcium channels, with the different channel subtypes displaying distinct distribution patterns based on their physiological roles (Catterall and Few, 2008). P/Q- and N-type voltage-gated calcium channels couple to presynaptic vesicles to promote neurotransmitter release, while L-type voltage-gated calcium channels are primarily somatodendritic (Hell et al., 1993; Sinnegger-Brauns et al., 2009). Previous studies have implicated voltage-gated calcium channels in several alcohol-related behaviors, including alcohol consumption and withdrawal syndrome (Newton et al., 2004; Varodayan et al., 2017b; Walter and Messing, 1999; Watson and Little, 2002). Multiple groups have also reported that alcohol both inhibits (Belia et al., 1995; Maldve et al., 2004; Mullikin-Kilpatrick and Treistman, 1993; Pietrzykowski et al., 2013; Xiao et al., 2005; Zucca and Valenzuela, 2010) and enhances (Belia et al., 1995; Pietrzykowski et al., 2013; Simasko et al., 1999) voltage-gated calcium channel activity, and alcohol's actions on voltage-gated calcium channels regulate GABA release in several brain regions (Hirono et al., 2009; Varodayan et al., 2017b; Zucca and Valenzuela, 2010). Similarly, the CRF system can inhibit (Tao et al., 2008, 2009, 2006) or enhance (Yu and Shinnick-Gallagher, 1998) voltage-gated calcium channel activity to modulate CeA synaptic transmission (Krishnan et al., 2010; Pollandt et al., 2006).

Given the critical role of the CeA in the reinforcing actions of alcohol and the transition to dependence, a clearer understanding of the shared neurobiological mechanisms driving its activation by acute alcohol and the CRF/CRF_1 system may provide insight into the development of this disease and promote therapeutic strategies for alcohol use disorders. Here we investigated the hypothesis that voltage-gated calcium channels may represent novel mechanisms by which acute alcohol and CRF co-stimulate CeA GABAergic synapses, and explored their potential neuroadaptation in the transition to alcohol dependence.

2. Material and methods

All the procedures in this study were approved by The Scripps Research Institute (TSRI) Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1. Chronic intermittent ethanol exposure

Male Sprague Dawley rats (n = 63; 329.8 ± 9.3 g) were purchased from Charles River Laboratories (Raleigh, NC). Chronic intermittent ethanol (CIE) rats (n = 17) were exposed daily to ethanol vapor (14 h vapor/10 h air) for 5–7 weeks. We, and others, have previously shown that rats that experience CIE become physically alcohol-dependent, with increased alcohol-drinking behavior, anxiety-like behavior, and reward deficits (Gilpin et al., 2008; O'Dell et al., 2004; Roberto et al., 2010b). Blood alcohol levels (BALs) were measured twice weekly by tail-bleeding and immediately prior to sacrifice. The mean BAL for all animals across the study was 206 ± 8 mg/dL. The naive rats (n = 46) were treated similarly, but received continuous air. CIE rats were taken directly from the ethanol-filled vapor chambers for sacrifice. However, electrophysiology slice preparation occurred in ethanol-free solutions, so all recordings were performed during an acute *in vitro* withdrawal period (1–8 h).

2.2. Electrophysiology

Rats were anesthetized (3–5% isoflurane) and decapitated, and the brains placed in oxygenated (95% O_2/5% CO_2), cold high-sucrose solution (pH 7.3–7.4; in mM): 206.0 sucrose; 2.5 KCl; 0.5 $CaCl_2$; 7.0 $MgCl_2$; 1.2 NaH_2PO_4; 26.0 $NaHCO_3$; 5.0 glucose; 5.0 HEPES, as previously described (Herman et al., 2013; Herman and Roberto, 2016; Roberto et al., 2010b; Varodayan et al., 2016). The brains were coronally sliced (300 μm) and the tissue incubated (30 min at 37 °C, then 30 min at room temperature) in oxygenated artificial cerebrospinal fluid (aCSF; in mM): 130.0 NaCl; 3.5 KCl; 2 $CaCl_2$; 1.25 NaH_2PO_4; 1.5 $MgSO_4$; 24 $NaHCO_3$; 10 glucose.

We recorded from neurons located in the medial subdivision of the central amygdala (CeA) using infrared differential interference contrast (IR-DIC) optics, a w60 water immersion objective (Olympus BX51WI, Tokyo, Japan) and a CCD camera (EXi Aqua, QImaging, Surrey, BC, Canada). Whole-cell voltage-clamp recordings were performed in gap-free acquisition mode with a sampling rate per signal of 10 kHz and low-pass filtered at 10 k Hz, using a Multiclamp 700B amplifier, Digidata 1440A and pClamp 10 software (Molecular Devices, Sunnyvale, CA). Recording pipettes (3–7 MΩ; King Precision, Claremont, CA) were filled with potassium-chloride internal solution (in mM): 145.0 KCl; 5.0 EGTA; 5.0 $MgCl_2$; 10.0 HEPES; 2.0 Na+-ATP; 0.2 Na+-GTP. Miniature spontaneous $GABA_A$–mediated inhibitory postsynaptic currents (mIPSCs) were isolated with 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM), DL-2-amino-5-phosphonovalerate (AP-5, 30 μM), CGP 55845A (1 μM) and tetrodotoxin (TTX, 0.5 μM). TTX is a voltage-gated sodium channel blocker and so prevents action potential generation/propagation, allowing for the mechanistic study of synaptic transmission at isolated synapses and the identification of pre vs. postsynaptic drug effects. The neurons were clamped at −60 mV and experiments with a

series resistance >15 MΩ or a >20% change in series resistance, as monitored with a 10 mV pulse, were excluded. For all experiments involving altered aCSF calcium concentration, the aCSF magnesium levels were adjusted to compensate.

Recording frequencies, amplitudes and kinetics were analyzed over a 3 min interval using Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) and visually confirmed, with mIPSC events <5 pA excluded and cells with less than 60 events/3 min interval excluded. To control for cell-to-cell variation in baseline electrophysiology properties, drug effects were normalized to their own neuron's baseline prior to group analyses. Final values were analyzed (Prism 5.02; Graph-Pad, San Diego, CA) for independent significance using one-sample t-tests and compared using two-tailed t-tests. Data are presented as mean ± standard error of the mean (SEM), with the number of cells and animals used for each experiment reported in the figure legend.

2.3. Western blotting

Rats were anesthetized with isoflurane and decapitated. Naïve and CIE rat brains (n = 6 per group) were coronally sliced (2 mm) with a wire matrix and the CeA punched on a chilled stage with an 18-gage blunt needle. The 2 CeA from each animal were combined and the tissue stored at −80 °C.

The samples were homogenized with a rotor-stator homogenizer (Tissue Tearor, Cole-Parmer Instrument Co., Vernon Hills, IL) in 250 μL buffer A (in mM): 4.0 HEPES pH 7; 320.0 sucrose; 5.0 EDTA pH 8; phosphatase inhibitor cocktail (PhosSTOP; Roche Life Science, Basel, Switzerland); protease inhibitor cocktail (cOmplete, EDTA-free; Roche), and then enriched for membrane proteins ((Goebel-Goody et al., 2009), with slight modifications (Varodayan et al., 2017b)). Specifically, the homogenate was centrifuged twice (1000*g*, 10 min, 4 °C) and supernatant collected and combined. This total supernatant was then re-centrifuged (100,000*g*, 1 h, 4 °C) and the pellet re-suspended in 50 μL buffer A. 25 μg samples were loaded onto a 7.5% SDS polyacrylamide gel (Mini-PROTEAN TGX, Bio-rad Laboratories; Hercules, CA), electrophoresed (100 V, 2 h), and transferred to a PVDF membrane (100 mA, 22 h, 4 °C; Immobilon-P, EMD Millipore, Billerica, MA). The membranes were washed in Tris-buffered saline with 0.1% Tween-20 (TBST; Sigma-Aldrich, Inc., St. Louis, MO), blocked in 5% milk/TBST (2 h, room temperature), incubated in primary antibody (overnight, 4 °C; $Ca_v2.1$, 190 kDa, 1:500, Alomone Labs #ACC-001; Jerusalem, Israel) and incubated in HRP-conjugated secondary antibody (1 h, room temperature; donkey anti-rabbit, 1:5000, EMD Millipore AP182PMI). The protein was visualized using enhanced chemiluminescence (SuperSignal West Pico, Thermo Scientific Pierce, Pittsburgh, PA) and exposed to Hy-Blot CL film (Denville Scientific, South Plainfield, NJ). To obtain a loading control, the membranes were incubated for 15 min in 0.4% Coomassie stain (in 50% methanol, 10% acetic acid, 40% ddH_2O; Coomassie Brilliant Blue R-250, Bio-Rad), de-stained (50% ddH_2O, 43% methanol, 7% acetic acid) and dried (Lee et al., 2015). Digital images were acquired using light transmission (film) or reflective (membrane) scanning on a Scanjet G4050 (Hewlett-Packard Company, Palo Alto, CA).

Protein band optical densities (OD) and Coomassie staining were measured using Image Studio Lite (Li-Cor Biosciences, Lincoln, NE). To control for protein loading variation, each

protein OD was normalized to its own lane's Coomassie staining (50–150 kDa) (Welinder and Ekblad, 2011). To allow for sample comparison across blots, Coomassie-normalized protein values were expressed relative to the mean value for naïve rats on the same membrane. The final values were compared using two-tailed t-tests in Prism 5.02. Data are presented as mean ± SEM.

2.4. Drugs and chemicals

We purchased ω-Agatoxin TK, AP-5, CGP 55845A and DNQX from Tocris (Bristol, UK); 1,2-Bis(2-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), Nifedipine and TTX from Sigma (St. Louis, MO); ω-Conotoxin GVIA from AnaSpec (Fremont, CA); and ethanol from Remet (La Mirada, CA). CRF was synthesized by Dr. Jean Rivier at the Salk Institute for Biological Studies, and R121919 was synthesized by Dr. Kenner Rice at the Drug Design and Synthesis Section of the National Institute on Drug Abuse. Drugs were dissolved in aCSF and applied locally by Y-tubing (Murase et al., 1989) or bath perfusion.

3. Results

3.1. Acute ethanol increased GABA release via P/Q-type voltage-gated calcium channels

We first assessed the effects of acute alcohol on GABA transmission in the CeA of naïve rats by recording action potential-independent mIPSCs in the presence of the voltage-gated sodium channel blocker TTX (across the entire study the mean baseline mIPSC frequency = 0.34 ± 0.02 Hz, amplitude = 48.4 ± 1.7 pA, rise time = 2.40 ± 0.06 ms and decay time = 5.67 ± 0.26 ms). As previously reported (Roberto et al., 2004; Varodayan et al., 2016), here we found that application of a maximal effective concentration of ethanol (44 mM EtOH; see (Nie et al., 2009; Roberto et al., 2003, 2004) for concentration-effect curves on GABA transmission in the CeA) significantly increased the mIPSC frequency to 133.3 ± 7.5% of baseline ([$t(13) = 4.46$, $p < 0.001$ by one-sample *t*-test]; Fig. 1A). There were no significant changes in the mean mIPSC amplitude or kinetics, but ethanol increased mIPSC amplitudes in 3/14 neurons (to 130.7 ± 5.9% of baseline; [$t(2) = 5.20$, $p < 0.05$ by one-sample *t*-test]), with no effect in the remaining 11/14 neurons (similar to (Herman et al., 2013; Roberto et al., 2004; Varodayan et al., 2016)). As mIPSCs are action potential-independent, increased frequencies indicate higher GABA release probabilities and altered amplitudes/kinetics reflect changed $GABA_A$ receptor sensitivity (De Koninck and Mody, 1994; Otis et al., 1994). Therefore, acute ethanol increased GABA release at CeA synapses, without significantly affecting the region's $GABA_A$ receptor composition or expression.

Since calcium promotes synaptic vesicle fusion and neurotransmitter release (Catterall and Few, 2008), we suspected that ethanol's actions on GABA release may involve changes in local calcium signaling. To investigate whether calcium in the postsynaptic recording cell mediates ethanol's effects, we added the calcium chelator BAPTA (10 mM) to the recording pipette. Ethanol continued to increase the mIPSC frequency in these BAPTA-loaded neurons (147.2 ± 17.6%; [$t(8) = 2.68$, $p < 0.05$ by one-sample *t*-test]), indicating that its actions on GABA release do not require calcium signaling in the postsynaptic cell (Fig. 1B). We next assessed if ethanol-induced GABA release requires presynaptic neuronal calcium influx, by altering the extracellular calcium concentration (from the normal artificial cerebrospinal

fluid (aCSF) calcium concentration of 2 mM to low (0.5 mM) or high (5 mM) calcium). Ethanol increased the mIPSC frequency in high Ca^{2+} aCSF (122.2 ± 4.9%; [t(5) = 4.57, p < 0.01 by one-sample t-test]), but had no effect in low Ca^{2+} aCSF (93.7 ± 3.9%; [t(6) = 1.61, p = n.s. by one-sample t-test]), revealing a role for extracellular calcium in ethanol-induced GABA release (Fig. 1C). There were no other ethanol- or calcium concentration-induced effects on mIPSC characteristics in these experiments. Thus, we find that ethanol requires presynaptic neuronal calcium influx in order to enhance CeA GABA release.

As calcium influx through voltage-gated calcium channels can directly stimulate neurotransmitter release (Catterall and Few, 2008), we investigated the possible role that voltage-gated calcium channels may play in ethanol's enhancement of CeA GABA release. P/Q-type voltage-gated calcium channel blockade with ω-Agatoxin TK (500 nM) prevented ethanol's effects on the mIPSC frequency (96.0 ± 6.5; [t(9) = 0.63, p = n.s. by one-sample t-test]), while L- and N-type voltage-gated calcium channel blockers had no effect (10 μM Nifedipine; [t(5) = 4.96, p < 0.01 by one-sample t-test] and 1 μM ω-Conotoxin GVIA; [t(5) = 6.13, p < 0.01 by one-sample t-test], respectively; Fig. 1D). ω-Agatoxin TK also had a *per se* effect on the mIPSC frequency (across the study it increased the mIPSC frequency to 134.4 ± 10.6% in nine cells; [t(8) = 3.25, p < 0.05 by one-sample t-test]), which was surprising given the role of these channels in regulating baseline GABA release (Catterall and Few, 2008; Hell et al., 1993; Sinnegger-Brauns et al., 2009). Of note, in three of these nine cells, ω-Agatoxin TK had no *per se* effect on the mIPSC frequency (105.0 ± 4.1%), but still prevented the ethanol/CRF facilitation (100.7 ± 1.4%; see section 3.2 for CRF facilitation), suggesting a lack of ceiling effect. Additionally, all three voltage-gated calcium channel blockers had no effects on mIPSC amplitudes or kinetics throughout the experiment. Collectively, these data reveal that acute ethanol acts via P/Q-type voltage-gated calcium channels to stimulate action potential-independent GABA release at naïve rat CeA synapses.

3.2. CRF increased GABA release via P/Q-type voltage-gated calcium channels

Ethanol's actions in the CeA have previously been linked to the CRF_1 (Roberto et al., 2010b), and so we next investigated the influence of CRF at CeA GABAergic synapses. Similar to acute ethanol, CRF (200 nM; see (Nie et al., 2009; Roberto et al., 2010b) for concentration-effect curves on GABA transmission in the CeA) significantly increased the mIPSC frequency to 163.2 ± 19.5% of baseline in CeA neurons from naïve rats ([t(7) = 3.24, p < 0.05 by one-sample t-test]; Fig. 2A). As before, there were no significant changes in the mean mIPSC amplitude or kinetics, although CRF did increase mIPSC amplitudes in 3/8 cells (to 126.7 ± 5.7% of baseline; [t(2) = 4.67, p < 0.05 by one-sample t-test]; similar to (Herman et al., 2013)). CRF continued to enhance the mIPSC frequency in BAPTA-loaded neurons (145.7 ± 10.7%; [t(5) = 4.27, p < 0.01 by one-sample t-test]; Fig. 2B) and in the presence of high Ca^{2+} aCSF (180.0 ± 27.4%; [t(5) = 2.92, p < 0.05 by one-sample t-test]), but not in low Ca^{2+} aCSF (95.0 ± 6.1%; [t(6) = 0.81, p = n.s. by one-sample t-test]; Fig. 2C). Moreover, ω-Agatoxin TK prevented CRF's enhancement of the mIPSC frequency (100.2 ± 7.5%; [t(4) = 0.02, p = n.s. by one-sample t-test]; Fig. 2D). There were no additional effects on mIPSC characteristics in these experiments. Thus, CRF acts similarly to acute ethanol by employing P/Q-type voltage-gated calcium channels to induce CeA GABA release in naïve rats.

3.3. Acute ethanol interacts with the CRF/CRF_1 system to enhance GABA release

In order to clarify the relationship between ethanol and the CRF/CRF_1 system in terms of their respective effects on GABA release in naïve rats, we next performed a series of interaction studies. We found that CRF prevented ethanol-induced GABA release ([t(5) = 1.94, p = n.s. by two-tailed paired t-test]; Fig. 3A). Specifically, the mIPSC frequency was potentiated by CRF to 132.1 ± 7.2% of baseline ([t(5) = 4.46, $p < 0.01$ by one-sample t-test]), and after the subsequent addition of ethanol (in the presence of CRF) it remained at 140.2 ± 10.2% of baseline ([t(5) = 3.92, $p < 0.05$ by one-sample t-test]). Similar to this interaction, ethanol occluded the effects of CRF on GABA release ([t(8) = 0.72, p = n.s. by two-tailed paired t-test]; Fig. 3B); ethanol increased the mIPSC frequency to 144.7 ± 10.1% of baseline ([t(8) = 4.43, $p < 0.01$ by one-sample t-test]), and CRF (in the presence of ethanol) maintained it at 154.6 ± 10.2% of baseline ([t(8) = 5.36, $p < 0.001$ by one-sample t-test]). Finally, the CRF_1-specific antagonist R121919 (1 μM) prevented ethanol's enhancement of the mIPSC frequency (96.4 ± 5.5%; [t(6) = 0.66, p = n.s. by one-sample t-test]; Fig. 3C), with no additional effects on mIPSC characteristics. Thus, alcohol interacts with the CRF/CRF_1 system to enhance CeA GABA release in naïve rats.

3.4. Alcohol dependence does not alter the effects of acute ethanol at CeA GABAergic synapses

To investigate whether chronic alcohol exposure alters the response of CeA GABAergic synapses to acute ethanol, we next exposed rats to chronic intermittent alcohol exposure (CIE) to induce physical dependence (O'Dell et al., 2004; Roberto et al., 2010b). We found that CeA neurons from CIE rats had a significantly higher baseline mIPSC frequency vs. naïve rats ([t(103) = 3.05, $p < 0.01$ by unpaired two-tailed t-test] (Roberto et al., 2004)), with a mean CIE baseline mIPSC frequency = 0.46 ± 0.03 Hz, amplitude = 49.7 ± 1.7 pA, rise time = 2.35 ± 0.06 ms and decay time = 5.64 ± 0.21 ms. CIE rats also displayed a similar increase in the mean mIPSC frequency (139.8 ± 9.7%; [t(5) = 4.12, $p < 0.01$ by one-sample t-test]; Fig. 4A) after acute ethanol application as observed in naïve rats, with no significant changes in the mean mIPSC amplitude or kinetics (though acute ethanol increased mIPSC amplitudes in 2/6 cells to 117.7 ± 4.2% of baseline; [p=n.s. by one-sample t-test]; similar to (Roberto et al., 2004)). Therefore, chronic alcohol exposure does not produce tolerance to ethanol's acute actions at CeA GABAergic synapses.

Similar to its effects in the naïve rats, acute ethanol enhanced the mIPSC frequency in CIE rat CeA neurons that were loaded with BAPTA (143.2 ± 16.5; [t(5) = 2.61, $p < 0.05$ by one-sample t-test]; Fig. 4B) or exposed to high Ca^{2+} aCSF (139.6 ± 8.7%; [t(5) = 4.58, $p < 0.01$ by one-sample t-test]), but not low Ca^{2+} aCSF (113.2 ± 10.3%; [t(5) = 1.28, p = n.s. by one-sample t-test]; Fig. 4C). Moreover, as in the naïve rats, ω-Agatoxin TK had a *per se* effect on the mIPSC frequency (138.4 ± 10.3% in 5 cells from 3 CIE rats; [t(4) = 3.75, $p < 0.05$ by one-sample t-test]), and prevented ethanol's enhancement of the mIPSC frequency in CIE rats (102.4 ± 7.3; [t(4) = 0.33, p = n.s. by one-sample t-test]; Fig. 4D). In accordance with these findings, western blotting studies revealed that chronic alcohol exposure did not change the CeA membrane abundance of P/Q-type voltage calcium channels ($Ca_V2.1$; [t(10) = 1.18, p = n.s. by unpaired two-tailed t-test]; Fig. 4E). Finally, CRF_1 antagonism by R121919 blocked ethanol's actions on the mIPSC frequency (102.9 ± 5.0%; [t(5) = 0.58, p =

n.s. by one-sample *t*-test]; Fig. 4F). There were no additional effects on mIPSC characteristics in these experiments. Thus, acute ethanol acts similarly in the CeA of both naïve and alcohol-dependent rats by employing CRF_1s and P/Q-type voltage-gated calcium channels to induce GABA release.

4. Discussion

Collectively, these data indicate that acute alcohol interacts with the CRF/CRF_1 system to increase CeA GABA release, via P/Q-type voltage-gated calcium channel activity. As the CeA is primarily GABAergic, alcohol- and CRF-induced inhibition of local interneurons can lead to the disinhibition of CeA inhibitory projection neurons and thus, the inhibition of downstream brain regions (e.g. the bed nucleus of the stria terminalis (BNST), hypothalamus, midbrain and brainstem (Alheid, 2003; Herman and Roberto, 2016)). Chronic alcohol exposure does not alter P/Q-type voltage-gated calcium channel membrane abundance or this CRF_1/P/Q-type voltage-gated calcium channel mechanism of acute alcohol-induced GABA release, so alcohol's stimulation of CeA synaptic function with each and every alcohol exposure may contribute to the region's over-activation during dependence (Gilpin et al., 2015; Koob and Volkow, 2010).

Notably, basal GABA release was enhanced in the CeA of CIE rats compared to naïve rats, suggesting greater local inhibition of CeA neurons in alcohol-dependent rats. CRF_1 and P/Q-type voltage-gated calcium channel activity did not mediate this change, as there were no differences in the *per se* effects of their respective antagonists on CeA GABA transmission in naïve vs. alcohol-dependent rats. Additionally, both alcohol and CRF increased CeA GABA release, and in a subset of CeA neurons, enhanced $GABA_A$ receptor function. While we identified a shared presynaptic mechanism in this study, future work is needed to elucidate whether alcohol and CRF's postsynaptic actions occur within the same neuronal populations and share common mechanisms of action. Finally, acute alcohol increased CeA GABA release in alcohol-dependent rats to a similar magnitude as in naïve rats, indicating a lack of functional tolerance to acute alcohol's actions at CeA GABAergic synapses (Roberto et al., 2004, 2010b). Others have reported alcohol-induced GABA release in the basolateral amygdala (BLA), brainstem, cerebellum, hippocampus, substantia nigra and ventral tegmental area (VTA) of naïve rodents (Hirono et al., 2009; Kelm et al., 2011; Qi et al., 2010; Theile et al., 2009; Weiner and Valenzuela, 2006). Multiple mechanisms likely govern these effects, but only a few intracellular pathways have been identified. Specifically, alcohol increased GABA release in cerebellar interneurons through protein kinase A (PKA), protein kinase C (PKC) and intracellular calcium pathways (Hirono et al., 2009; Kelm et al., 2011) and in VTA neurons via 5-hydroxytryptomine-2C (5HT-2C) receptors and intracellular calcium stores (Theile et al., 2009). Therefore, alcohol has widespread effects on GABA transmission throughout many brain regions, but only a few intracellular pathways have been identified and these mechanisms appear to be region-specific.

Here we report that CRF_1 antagonism prevented alcohol-induced GABA release in the CeA of naïve rats (as previously described in (Herman et al., 2013)) and alcohol-dependent rats. Moreover, pretreatment with either acute alcohol or CRF occluded the effect of the other compound in naïve rats, indicating a clear interaction between alcohol and the CRF/CRF_1

system. While the direct site of alcohol's actions remains unknown, the most parsimonious explanation is that alcohol activates CRF_1s to induce CeA GABA release. Alternatively, alcohol may activate intracellular pathways (see below), or may induce CRF release from the lateral subdivision of the CeA (Veening et al., 1984) (though the lateral CeA CRF input to the medial CeA seems to be minor in rats (Pomrenze et al., 2015)) or from distal inputs from other known CRF-expressing regions, such as the BNST or paraventricular hypothalamic nucleus (Gafford et al., 2012; Merchenthaler, 1984; Wang et al., 2011). Other G protein-coupled receptors (GPCRs) have also been implicated in alcohol-induced CeA GABA release, including the type 1 cannabinoid receptor (CB_1) (Roberto et al., 2010a; Varodayan et al., 2016), δ-opioid receptor (Kang-Park et al., 2009) and neuropeptide Y receptor (Gilpin et al., 2011). Additionally, CB_1 and $GABA_B$ receptors mediate alcohol's potentiation of GABA release in the BLA and cerebellum (Kelm et al., 2011; Talani and Lovinger, 2015; Varodayan et al., 2017a), while in the VTA, μ-opioid and 5HT-2C receptors are involved (Theile et al., 2009). Therefore, alcohol's actions on CeA GABA release via CRF_1 do not occur in isolation, and future studies must identify which alcohol-induced GPCR pathways interact and predominate, and whether they are dysregulated after chronic ethanol exposure.

CRF_1s also regulate P/Q-type voltage-gated calcium channel activity (Kuryshev et al., 1996; Ritchie et al., 1996), and here we demonstrated that both alcohol- and CRF-induced GABA release were mediated by P/Q-type voltage-gated calcium channels. P/Q-type voltage-gated calcium channels closely interact with synaptic vesicle fusion machinery proteins so that their activation produces a calcium influx that can directly trigger GABA release (Catterall and Few, 2008). CRF_1 activity can also induce the adenylyl cyclase (AC)/PKA and phospholipase C/PKC pathways (Blank et al., 2003; Gutknecht et al., 2009; Riegel and Williams, 2008), and our laboratory has previously reported a role for AC7, PKA and PKCε in alcohol- and CRF-induced GABA release in the rodent CeA (Bajo et al., 2008; Cruz et al., 2011, 2012). These 2nd messenger systems have significant crosstalk, and both can interact with voltage-gated calcium channels (Catterall, 2000; Catterall and Few, 2008; Cens et al., 2006; Dai et al., 2009). Therefore, alcohol's site of action is difficult to determine in the present study, as it may directly act upon any combination of these signaling molecules. Nonetheless, the most likely scenario from the present work is that acute alcohol acts on CRF_1s to produce downstream changes in PKA/PKC signaling that modulate P/Q-type voltage-gated calcium channel activity leading to action potential-independent GABA release.

Interestingly, we recently reported that acute alcohol also enhances action potential-dependent GABA release in the CeA of naïve rats, but this effect occurs via an L-type voltage-gated calcium channel mechanism (Varodayan et al., 2017b). It is important to note that action potential-dependent neurotransmitter release results from neuronal activity across the entire synaptic network to produce classical neural communication, whereas the addition of the voltage-gated sodium channel blocker TTX (as in the current study) blocks action potential generation/propagation to reveal action potential-independent neurotransmitter release that can maintain homeostasis and mediate plasticity at mature synapses (Kavalali, 2015). Critically, P/Q-type voltage-gated calcium channel blockade did not alter alcohol-induced action potential-dependent GABA release in the naïve rat CeA (Varodayan et al.,

2017b), while in the present study L-type voltage-gated calcium channel blockade did not alter alcohol-induced action potential-independent GABA release. Therefore, these two types of GABA release represent different forms of CeA neurotransmission that are regulated by alcohol via distinct voltage-gated calcium channel mechanisms. Action potential-dependent and -independent neurotransmission are also differently regulated by several other molecules/compounds (e.g. presynaptic metabotropic glutamate receptors (Glitsch, 2006), nitric oxide species (Pan et al., 1996), cholesterol (Wasser et al., 2007), antimalarial drugs (McArdle et al., 2006), γ-secretase (Pratt et al., 2011) and methyl CpG binding protein 2 (MeCP2) (Nelson et al., 2006, 2008)), leading to the growing consensus that different vesicle populations govern action potential-dependent and -independent neurotransmitter release, possibly via distinct vesicle fusion machinery, spatial segregation of the vesicle and/or retrograde signaling (Kavalali, 2015).

The CeA integrates emotionally salient sensory information about fearful and anxiety-inducing stimuli to produce the appropriate behavioral and physiological responses (Gilpin et al., 2015). Its activity promotes alcohol drinking and anxiety-like behaviors, and its over-activation is considered a hallmark of the transition to alcohol dependence (Gilpin et al., 2015; Koob and Volkow, 2010). Collectively, our work highlights the diversity of acute alcohol's actions on CeA voltage-gated calcium channels (P/Q-vs. L-type voltage-gated calcium channels), revealing its intricate control over different types of GABA release (action potential-independent vs. -dependent release). Moreover, acute alcohol interacts with the CRF/CRF_1 system to produce action potential-independent CeA GABA release. This CRF_1/P/Q-type voltage-gated calcium channel mechanism can still be engaged after chronic alcohol exposure, indicating that alcohol uses this molecular mechanism at CeA GABAergic synapses throughout the transition to dependence. Therefore, our data identify P/Q-type voltage-gated calcium channels, like CRF_1s, as critical regulators of acute alcohol's actions on CeA synaptic transmission, presenting a novel locus for therapeutic development to ameliorate alcohol use disorders.

Acknowledgments

This is manuscript number 29493 from The Scripps Research Institute. We thank Dr. Matthew Buczynski, Maury Cole and Dr. Miranda Staples for their technical support and Dr. Dean Kirson for valuable comments on the manuscript. CRF was synthesized by Dr. Jean Rivier at the Salk Institute for Biological Studies, and R121919 was synthesized by Dr. Kenner Rice at the Drug Design and Synthesis Section of the National Institute on Drug Abuse. This work was supported by the National Institutes of Health [AA015566, AA021491, AA017447, AA006420, AA013498, AA021802]. The authors declare no competing financial interests.

Abbreviations

5HT-2C	5-hydroxytryptomine-2C
aCSF	artificial cerebrospinal fluid
AC	adenylyl cyclase
AP-5	DL-2-amino-5-phosphonovalerate
BLA	basolateral amygdala

BAL	blood alcohol level
BAPTA	1,2-Bis(2-Aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid
$Ca_v2.1$	P/Q-type voltage-gated calcium channel
CB_1	type 1 cannabinoid receptor
CeA	central amygdala
CIE	chronic intermittent ethanol vapor exposed
CRF	corticotropin-releasing factor
CRF_1	corticotropin-releasing factor type 1 receptor
DNQX	6,7-dinitroquinoxaline-2,3-dione
EtOH	ethanol
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor
mIPSC	miniature $GABA_A$–mediated inhibitory postsynaptic current
OD	optical density
PKA	protein kinase A
PKC	protein kinase C
SEM	standard error
TTX	tetrodotoxin
VTA	ventral tegmental area

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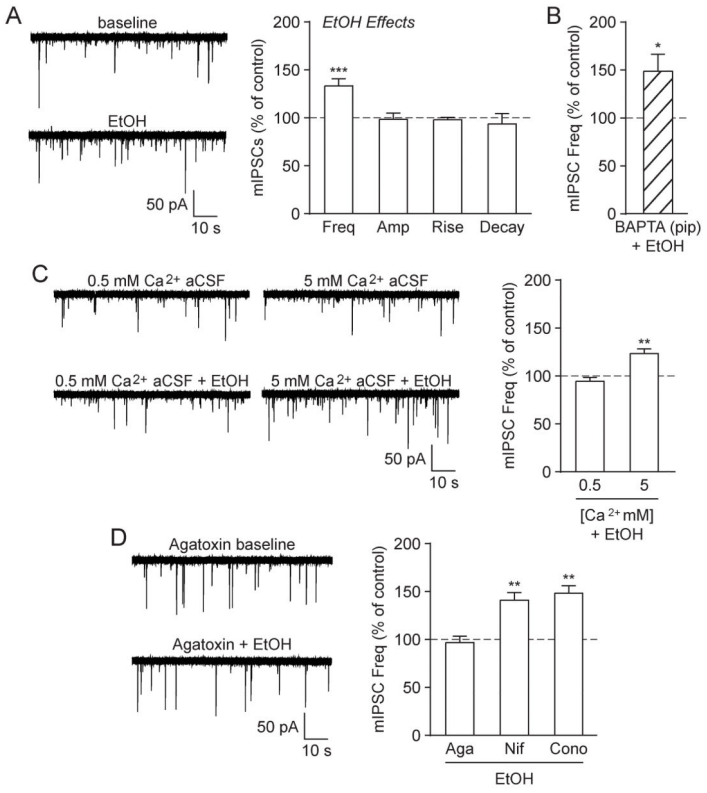

Fig. 1.

Acute alcohol increased GABA release in the naïve rat CeA via P/Q-type voltage-gated calcium channel activity. **A: (Left)** Representative mIPSC traces from a naïve rat CeA neuron in baseline conditions and during acute alcohol (44 mM EtOH) superfusion. **(Right)** EtOH significantly increased the mIPSC frequency, but had no effect on the mIPSC amplitude or kinetics (14 cells from 10 rats). **B:** EtOH significantly increased the mIPSC frequency in CeA neurons that were pre-loaded with 10 mM BAPTA (9 cells from 3 rats). **C: (Left)** Representative mIPSCs in low (0.5 mM) and high (5 mM) Ca^{2+} aCSF and during subsequent EtOH superfusion. **(Right)** EtOH significantly increased the mIPSC frequency in CeA neurons exposed to high Ca^{2+} aCSF, but not low Ca^{2+} aCSF (normalized to pre-alcohol baseline). For these extracellular calcium experiments, 6–7 cells from a minimum of 5 rats were used for each experimental group. **D: (Left)** Representative mIPSCs in the P/Q-type voltage-gated calcium channel blocker ω-Agatoxin TK (500 nM Aga) and during subsequent EtOH superfusion. **(Right)** EtOH's enhancement of the mIPSC frequency was blocked in the presence of Aga (10 cells from 4 rats), but was unchanged by the L-type calcium channel blocker Nifedipine (10 μM Nif; 6 cells from 4 rats) or the N-type calcium channel blocker ω-Conotoxin GVIA (1 μM Cono; 6 cells from 5 rats).

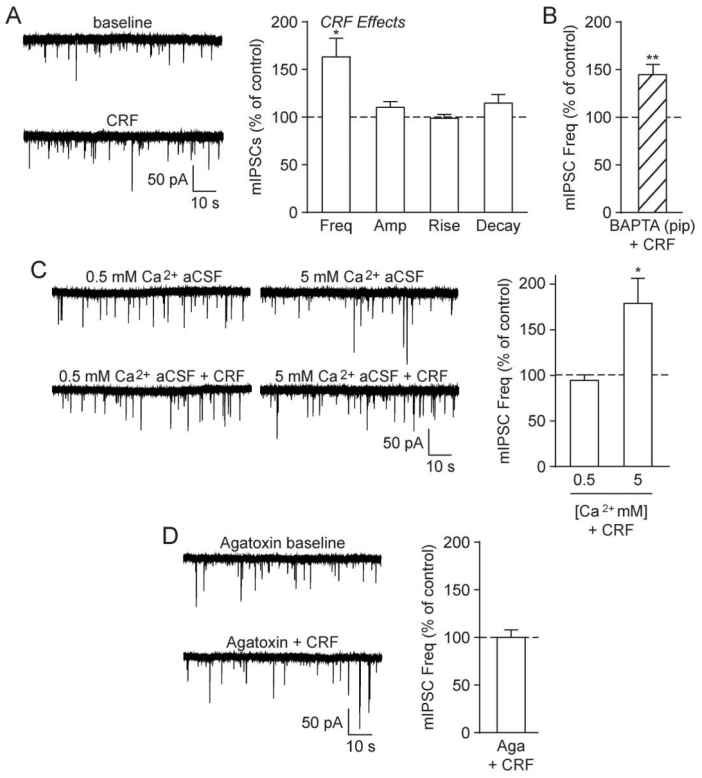

Fig. 2.

CRF increased GABA release in the naïve rat CeA via P/Q-type voltage-gated calcium channel activity. **A: (Left)** Representative mIPSCs from a naïve rat CeA neuron in baseline conditions and during CRF (200 nM) superfusion. **(Right)** CRF significantly increased the mIPSC frequency, but had no effect on the mIPSC amplitude or kinetics (8 cells from 5 rats). **B:** CRF significantly increased the mIPSC frequency in CeA neurons that were pre-loaded with 10 mM BAPTA (6 cells from 3 rats). **C: (Left)** Representative mIPSCs in low (0.5 mM) and high (5 mM) Ca^{2+} aCSF and during subsequent CRF superfusion. **(Right)** CRF significantly increased the mIPSC frequency in CeA neurons exposed to high Ca^{2+} aCSF, but not low Ca^{2+} aCSF (normalized to pre-CRF baseline). For these extracellular calcium experiments, 6–7 cells from a minimum of 4 rats were used for each experimental group. **D: (Left)** Representative mIPSCs in the P/Q-type voltage-gated calcium channel blocker ω-Agatoxin TK (500 nM Aga) and during subsequent CRF superfusion. **(Right)** CRF's enhancement of the mIPSC frequency was blocked in the presence of Aga (5 cells from 3 rats).

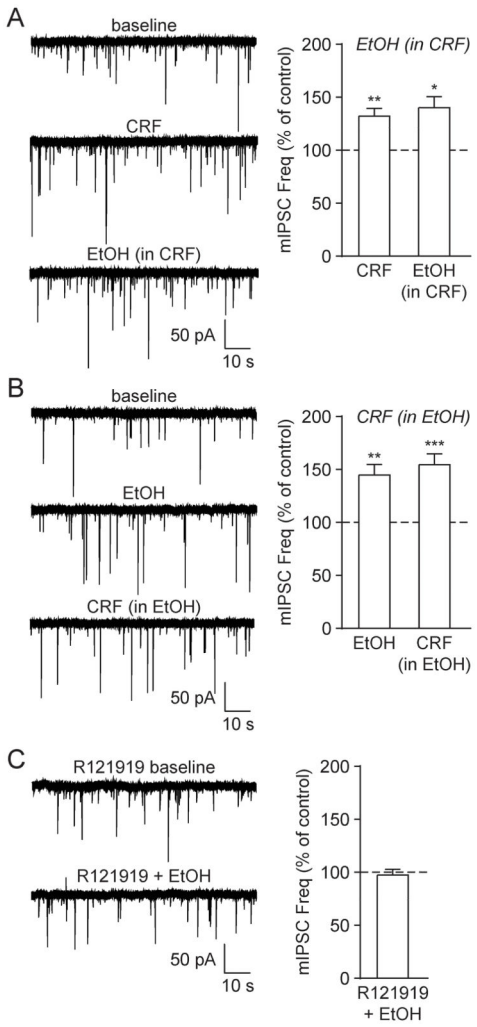

Fig. 3.

Acute alcohol interacts with the CRF/CRF_1 system to enhance GABA release. **A: (Left)** Representative mIPSCs from a naïve rat CeA neuron in baseline conditions, during CRF (200 nM) superfusion and following acute alcohol (44 mM EtOH) co-application in the continued presence of CRF. **(Right)** CRF significantly increased the mIPSC frequency, and EtOH (in CRF) had no further effect (6 cells from 4 rats). **B: (Left)** Representative mIPSCs in baseline conditions, during EtOH superfusion and following CRF + EtOH co-application. **(Right)** EtOH significantly increased the mIPSC frequency, and CRF (in EtOH) had no further effect (9 cells from 4 rats). **C: (Left)** Representative mIPSCs in the CRF_1 antagonist R121919 (1 μM) and during subsequent EtOH superfusion. **(Right)** EtOH's enhancement of the mIPSC frequency was blocked in the presence R121919 (7 cells from 3 rats).

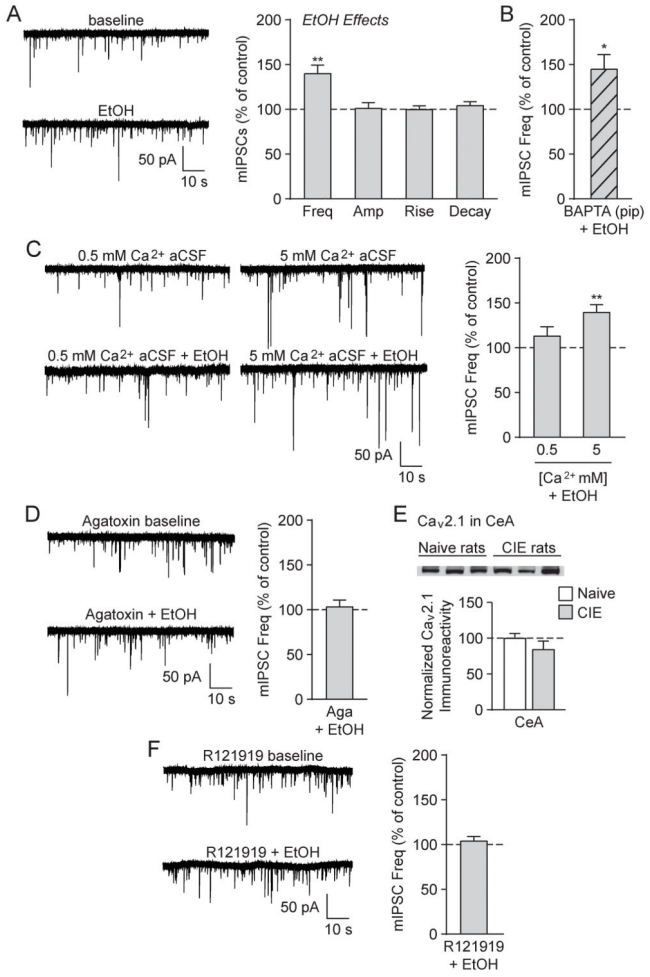

Fig. 4.

Alcohol dependence does not alter the effects of acute alcohol at CeA GABAergic synapses. **A: (Left)** Representative mIPSCs from a CIE rat CeA neuron in baseline conditions and during acute alcohol (44 mM EtOH) superfusion. **(Right)** EtOH significantly increased the mIPSC frequency, but had no effect on the mIPSC amplitude or kinetics (6 cells from 5 rats). **B:** EtOH significantly increased the mIPSC frequency in CeA neurons that were pre-loaded with 10 mM BAPTA (6 cells from 3 rats). **C: (Left)** Representative mIPSCs in low (0.5 mM) and high (5 mM) Ca^{2+} aCSF and during subsequent EtOH superfusion. **(Right)** EtOH significantly increased the mIPSC frequency in CIE CeA neurons exposed to high Ca^{2+} aCSF, but not low Ca^{2+} aCSF (normalized to pre-alcohol baseline). For these extracellular calcium experiments, 6 cells from a minimum of 4 rats were used for each experimental group. **D: (Left)** Representative mIPSCs in the P/Q-type voltage-gated calcium channel blocker ω-Agatoxin TK (500 nM Aga) and during subsequent EtOH superfusion. **(Right)** EtOH's enhancement of the mIPSC frequency was blocked in the presence of Aga (5 cells from 3 rats). **E: (Top)** Representative western blot image of P/Q-type voltage-gated calcium channel ($Ca_V2.1$) membrane abundance from the CeA of naïve and CIE rats. **(Bottom)** Quantification revealed no difference in CeA $Ca_V1.2$ membrane expression in CIE vs. naïve rats (6 rats were used for each experimental group). **F: (Left)** Representative

mIPSCs in the CRF_1 antagonist R121919 (1 μM) and during subsequent EtOH superfusion. **(Right)** EtOH's enhancement of the mIPSC frequency was blocked in the presence of R121919 (6 cells from 3 rats).