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Ethanol produces corticotropin releasing factor receptor-dependent enhancement of spontaneous glutamatergic transmission in the mouse central amygdala

Yuval Silberman, PhD¹, Tracy L. Fetterly, BA¹, Elias K. Awad, BA¹, Elana J. Milano, BS¹, Ted B. Usdin, MD PhD², and Danny G. Winder, PhD^{1,*}

¹Molecular Physiology and Biophysics, Neuroscience Program, Vanderbilt University School of Medicine

²Section Fundamental Neuroscience, National Institute of Mental Health, National Institutes of Health

Abstract

Background—Ethanol modulation of Central Amygdala (CeA) neurocircuitry plays a key role in the development of alcoholism via activation of the corticotropin releasing factor (CRF) receptor system. Previous work has predominantly focused on ethanol/CRF interactions on the CeA GABA circuitry; however our lab recently showed that CRF enhances CeA glutamatergic transmission. Therefore, this study sought to determine if ethanol modulates CeA glutamate transmission via activation of CRF signaling.

Methods—The effects of ethanol on spontaneous excitatory postsynaptic currents (sEPSCs) and basal resting membrane potentials were examined via standard electrophysiology methods in adult male C57BL/6J mice. Local ablation of CeA CRF neurons (CRF^{CeAhDTR}) was achieved by targeting the human diphtheria toxin receptor (hDTR) to CeA CRF neurons with an adeno-associated virus. Ablation was quantified post-hoc with confocal microscopy. Genetic targeting of the diphtheria toxin active subunit to CRF neurons (CRF^{DTA} mice) ablated CRF neurons throughout the CNS, as assessed by qRT-PCR quantification of CRF mRNA.

Results—Acute bath application of ethanol significantly increased sEPSC frequency in a concentration dependent manner in CeA neurons, and this effect was blocked by pretreatment of co-applied CRF receptor 1 and CRF receptor 2 antagonists. In experiments utilizing a CRF-*tomato* reporter mouse, ethanol did not significantly alter the basal membrane potential of CeA CRF neurons. The ability of ethanol to enhance CeA sEPSC frequency was not altered in CRF^{CeAhDTR} mice despite a ~78% reduction in CeA CRF cell counts. The ability of ethanol to enhance CeA sEPSC frequency was also not altered in the CRF^{DTA} mice despite a three-fold reduction in CRF mRNA levels.

Conclusion—These findings demonstrate that ethanol enhances spontaneous glutamatergic transmission in the CeA via a CRF receptor dependent mechanism. Surprisingly, our data suggest that this action may not require endogenous CRF.

Keywords

Central nucleus of the amygdala; CRF reporter mice; whole-cell patch-clamp electrophysiology; diphtheria toxin; selective deletion

Introduction

Ethanol modulation of central amygdala (CeA) neurocircuitry plays a key role in the anxiolytic effects of ethanol and is postulated to play roles in the development of alcoholism (Koob, 2009; Koob and Volkow, 2010; Gilpin and Roberto, 2012; Silberman and Winder, 2015). In the CeA, ethanol enhances GABAergic transmission via activation of corticotropin releasing factor (CRF) receptors (Nie et al., 2004; Roberto et al., 2004a; Nie et al., 2009), presumably by increasing local CRF release (Roberto et al., 2010). However, CRF receptor 1 (CRFR1) deletion from glutamatergic synapses has been shown to produce more robust decreases in anxiety like behaviors than CRFR1 deletion from GABAergic synapses (Refojo et al., 2011). Therefore, ethanol modulation of CeA glutamatergic transmission may be of more relevance to modulation of anxiety like behaviors during the development of alcoholism.

Previous studies investigating ethanol actions on glutamatergic transmission in CeA have examined NMDA receptors (Roberto et al. 2004b; Roberto et al., 2006) but not spontaneous glutamatergic transmission. We recently showed that CRF enhances spontaneous glutamatergic transmission in the CeA (Silberman and Winder, 2013). Given that EtOH may induce CRF release in the CeA (Roberto et al. 2010), we sought to determine if EtOH alters spontaneous glutamatergic transmission in the CeA via a CRF dependent mechanism. In these studies, we found that: 1) acute application of ethanol can increase presynaptic glutamate release in the CeA *ex vivo*; 2) the ethanol induced increase in CeA glutamate release is blocked by pretreatment with CRF receptor antagonists; 3) ethanol does not produce large changes in basal electrical activity of CRF-producing neurons in the CeA; and 4) substantial ablation of CRF-producing neurons either within the CeA or in the whole animal does not alter the ability of ethanol to enhance CeA glutamatergic transmission.

Materials and Methods

Animals

Adult male mice (>7 weeks) of three genetic lines were used for these studies. All mouse lines were originally purchased from the Jackson Laboratory. Wild-type C57BL/6J mice, purchased as needed, were used for initial studies examining the effects of ethanol on CeA neurocircuitry. Analysis of ethanol effects of CeA CRF neuron activity was made in CRF-*tomato* mice (Silberman et al., 2013). These mice were produced by mating CRF-*ires-cre* (strain B6(Cg)-Crhtm1(cre)Zjh/J; back-crossed for at least 1 generation onto a C57BL/6J breeder line) with Ai9 reporter mouse line where *tdtomato* is specifically targeted to *cre*

expressing neurons (strain B6.Cg-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze>/J}). CRF-*tomato* mice were also used to create CRF^{CeAhDTR} mice as described below. CRF-DTA mice were produced by mating back-crossed CRF-*ires-cre* mice with a ROSA-DTA mouse line (strain B6.129P2-Gt(ROSA)26Sor^{tm1(DTA)Lky/J}). All mice were housed in groups of two to five for the duration of the studies. Food and water were available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University (Nashville, TN).

Diphtheria Toxin mediated deletion of CRF neurons in CRF^{CeAhDTR} mice

On the day of surgery, CRF-*tomato* mice were anesthetized with Isoflurane (3% initial, 1.5% for maintenance) and placed in a stereotaxic apparatus (my NeuroLab, Leica). Angle Two software was used for setting injection targets in the CeA (coordinates were 1.34 mm posterior to bregma, 2.78 mm lateral to the midline, and 5.35 mm below the skull surface with a needle path angled 20° from the vertical plane). Other details about the surgical procedure have been described previously (Kash et al., 2008). A 33 gauge needle of a 10 µl syringe (Hamilton) was heat sterilized immediately before back filling with AAV8-FLEX-hDTR virus (Dimitrov et al., 2013) to express the human form of the diphtheria toxin receptor (hDTR) in *cre* expressing neurons in the CeA. 300nL of virus was injected unilaterally into the CeA at 50nL/min using a UltraMicroPump II and Micro4-controller (World Precision Instruments). Five minutes later, the syringe was withdrawn and the scalp wound was sutured closed. Postsurgical care included immediate subcutaneous saline (1.0 ml per 20 g of body weight) and analgesic (ketoprofen, 5 mg/kg, s.c.) followed by additional ketoprofen injections every 24 h for 3 days. Animals were monitored for health concerns including: loss of body weight >20%, signs of uncontrolled pain, stress, or dehydration. No animals displayed these signs and therefore none were removed from further studies. Beginning one week after surgery, mice were treated with diphtheria toxin (50ng/kg I.P.) three times over a 10-day period. Subsequent electrophysiological studies began a minimum of 3 days after the last diphtheria toxin injection. AAV8-FLEX-hDTR virus injected and non-injected CeA slices used for electrophysiology were immersed in 4% paraformaldehyde in PBS for 1–3 hours following recordings, cryoprotected with 30% sucrose in PBS at 4°C until prepared for imaging (typically 7–10 days later), and imaged with a Zeiss LSM 710 inverted confocal microscope.

Brain slice preparation and electrophysiology

Mice were decapitated under Isoflurane and brains were rapidly removed from the skull and placed in ice-cold oxygenated sucrose-based artificial cerebrospinal fluid (ACSF) cutting solution [containing (in mM): 183 sucrose, 20 NaCl, 0.5 KCl, 1 MgCl₂, 1.4 NaH₂PO₄, 2.5 NaHCO₃, and 1 glucose]. Two hundred and fifty µm thick brain slices containing the CeA (bregma, –1.22 to –1.78) were prepared from adult male mice with a VT1000 Vibratome (Leica) and then stored in a holding chamber with Modified ACSF [containing (in mM): 100 sucrose, 60 NaCl, 2.5 KCl, 1.4 NaH₂PO₄, 1.1 CaCl₂, 3.2 MgCl₂, 2 MgSO₄, 22 NaHCO₃, 20 glucose, 1 ascorbic acid] at 32°C for 20 min and then moved to a separate holding chamber with oxygenated normal ACSF [containing (in mM): 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10 glucose, 26 NaHCO₃] at 28°C for at least 30 min prior for use in electrophysiology experiments. For recordings, slices were transferred to the submersed

recording chamber where they were constantly perfused with normal ACSF at a rate of 2mL/min and allowed to equilibrate for at least 15minutes prior to recordings. All electrophysiology recordings were targeted to neurons in the lateral subdivision of the CeA. For whole-cell voltage-clamp recordings, AMPA receptor-mediated spontaneous EPSCs (sEPSCs) were recorded from a holding potential of -70 mV and pharmacologically isolated by adding $25\mu\text{M}$ picrotoxin to the ACSF. sEPSC recordings were acquired in 2 min gap-free blocks. Recording electrodes for voltage-clamp sEPSC experiments were filled with (in mM): 118 CsOH, 117 D-gluconic acid, 5 NaCl, 10 HEPES, 0.4 EGTA, 2 MgCl_2 , 5 tetraethylammonium chloride, 4 ATP, 0.4 GTP, pH 7.2–7.3, 285–295 mOsmol. For experiments in which the effects of antagonists were determined, slices were treated with antagonists for at least 20 min before application of the agonist and remained on for the duration of the experiment. For whole-cell current-clamp recordings, electrodes were filled with (in mM): 135 K^+ -gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP, pH 7.2, 285–295 mOsmol. These experiments were carried out at each neuron's initial resting membrane potential after allowing at least 3 min for the cell to equilibrate and for the resting membrane potential to stabilize. Current-clamp experiments analyzing the effect of ethanol on resting membrane potential were recorded continuously in gap-free mode. All electrophysiology recordings were made using Clampex 9.2 and analyzed using Clampfit 10.4 (Molecular Devices).

Statistical Analyses

Statistical analyses were performed using Microsoft Excel 2010 and GraphPad Prism 6. Specifically, when determining whether a compound had a significant effect, a student's paired t test was used, comparing the baseline value to the experimental value. To analyze the effects of different concentrations of drugs on sEPSCs, a one-way ANOVA was used followed by Tukey's post-test to determine the significance of specific comparisons. All values given for drug effects throughout the study are presented as average $\pm$ SEM.

qRT-PCR

Expression of CRF mRNA was evaluated using Taq-Man gene expression assays. For whole slice verification of CRF cell deletion, $\text{CRF}^{\text{CeAhDTR}}$ slices that had been used for electrophysiological recordings were used for RNA isolations. For region specific verification of CRF cell deletion in the CeA, $500\mu\text{m}$ tissue punches were taken using a 0.5 mm circular punch. For the CeA, bilateral punches were used for RNA isolations. A single punch was used for the paraventricular nucleus (PVN) of the hypothalamus. Tissue was added directly to TRIzol reagent (Invitrogen). RNA isolation was done using chloroform extraction followed by overnight isopropanol precipitation. Isolated RNA was converted to cDNA from $1\mu\text{g}$ of total RNA using the High Capacity Reverse Transcription Kit (Applied Biosystems). Changes in CRF (Mm01293920_s1) mRNA levels were measured using TaqMan Gene Expression Assay (Applied Biosystems) for quantitative real-time RT-PCR. GAPDH (4352932E) was used as the endogenous control. All samples were run in duplicate using the BioRad CFX96 system. The amplification program used was 20 s at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Analysis was performed using the 2^{-C_T} method (Livak and Schmittgen, 2001) and normalizing each value to the average fold change for the control population.

Reagents

NBI27914 and Astressin2B were purchased from Tocris. All other reagents were purchased from Sigma-Aldrich unless otherwise noted in the text.

Results

Ethanol increases glutamate release in the Central Amygdala through a CRFR-mediated mechanism

Ethanol is known to enhance CRFR signaling in the CeA (Roberto et al., 2010) and we have previously shown that CRFR activation enhances CeA glutamate transmission (Silberman and Winder, 2013). Therefore, we hypothesized that ethanol may enhance glutamatergic transmission in the CeA via a CRFR dependent mechanism. We performed whole-cell voltage clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs) in neurons from the lateral subdivision of the CeA in C57BL/6J adult male mice (Fig 1). 10 min bath application of 100mM ethanol significantly enhanced sEPSC frequency ($32.01 \pm 8.38\%$ increase from baseline, $n=8$, $p<0.05$) and caused small but significant decreases in sEPSC rise time ($-8.57 \pm 2.9\%$, $p<0.05$) and decay time ($-11.32 \pm 3.48\%$, $p<0.05$) (Fig 1A–B). Ethanol significantly enhanced the frequency of sEPSCs in a concentration dependent manner (5–100m M; one-way ANOVA, $F_{(4,21)} = 3.596$, $p<0.05$, $EC_{50} = 18$ mM) with significant effects of ethanol at concentrations of 20mM and above (Fig 1C). There were no significant inhibitory effects of ethanol on sEPSC rise time or decay time at any concentration lower than 100mM ($p>0.05$). Similarly, there were no significant effects of ethanol on sEPSC amplitude or area at any concentration ($p>0.05$).

To determine if the ability of ethanol to enhance sEPSC frequency was mediated via CRFR activity, CeA slices were pretreated with a CRFR1 antagonist (1 μ M NB 127914), a CRFR2 antagonist (100nM Astressin 2B), or co-application of both antagonists for at least 20 min prior to sEPSC recordings (Fig 2). 100 mM ethanol significantly enhanced sEPSC frequency in CeA slices that were pretreated with the CRFR1 antagonist alone ($57.4 \pm 17.8\%$ increase from baseline, $n=5$, $p<0.05$) or with the CRFR2 antagonist alone ($71.2 \pm 13.1\%$ increase from baseline, $n=11$, $p<0.05$) (Fig 2C). The ability of 100mM ethanol to enhance sEPSC frequency was attenuated when the CRFR1 and CRFR2 antagonists were co-applied. ($15.43 \pm 7.68\%$ decrease from baseline; $n=8$; $p>0.05$) (Fig 2B). Under these conditions, 100mM ethanol also significantly decreased sEPSC amplitude ($22.02 \pm 3.26\%$ decrease from baseline, $p<0.05$) and sEPSC area ($17.77 \pm 3.39\%$ decrease from baseline, $p<0.05$) while causing no significant changes to sEPSC rise time or decay time ($p>0.05$). A one way ANOVA revealed a significant difference in the ability of 100 mM ethanol to enhance sEPSC frequency between control conditions and CRFR antagonist pretreatment conditions ($F_{(3,27)} = 11.2$, $p<0.0001$). Posthoc analysis with the Bonferroni's multiple comparison test revealed no significant difference in the ability of 100mM ethanol to enhance sEPSC frequency between control CeA slices and CeA slices were pretreated with CRFR antagonists individually ($p>0.05$) while the effect of ethanol in slices pretreated with both CRFR1 and CRFR2 antagonists was significantly reduced ($p<0.05$, Fig 2C).

Ethanol does not alter CRF neuron excitability in the CeA

The above data suggest that ethanol enhances presynaptic glutamate release in the CeA via a CRFR dependent mechanism. Previous work suggests that ethanol induces CRF release to produce its effects on CeA circuitry (Roberto et al. 2010). Since the CeA contains a large population of CRF neurons that could be a source of extracellular CRF in this brain region, we next wanted to determine if the activity of CRF producing neurons in the CeA is modulated by acute ethanol application. We recorded from *tomato*-positive neurons in the CeA of CRF-*tomato* reporter mice in the current-clamp mode to assess if ethanol modulates the membrane potential of these neurons (Fig 3). 10 min bath application of 100mM ethanol did not significantly alter CeA CRF neuron membrane potential from their baseline levels (baseline = -71.38 ± 1.44 mV; 100mM EtOH = -71.49 ± 1.68 mV; $n=10$; $p>0.05$) (Fig 3B). We further examined the ability of 100 mM ethanol to modulate input resistance or action potential firing. 100 mM ethanol did not significantly alter the input resistance of these neurons ($-3.21 \pm 8.57\%$ change from baseline, $n=8$, $p>0.05$) nor did 100 mM ethanol significantly alter action potential firing latency, current injection required for first action potential, or action potential threshold, rise tau, decay tau or half-width ($n=8$, $p>0.05$; see Table 1). 100mM ethanol induced a small but significant decrease in action potential amplitude (105.1 ± 4.1 mV vs 100.5 ± 4.6 mV, $n=8$, $p=0.047$).

Virally mediated ablation of CeA CRF neurons does not alter ethanol enhancement of glutamatergic transmission in the CeA

We next sought to locally ablate CRF neurons in the CeA to determine if these neurons are necessary for the ability of ethanol to enhance CeA glutamatergic transmission. To that end, we microinjected a viral construct (AAV8-FLEX-hDTR), that targets the expression of the human diphtheria toxin receptor (hDTR) specifically to *cre* expressing neurons (Dimitrov et al., 2013), unilaterally into the CeA of CRF-*tomato* mice. These CRF^{CeAhDTR} mice were then exposed to diphtheria toxin (3 injections over 10 days; 50ng/kg I.P.) to ablate CRF neurons in the virus-injected side and were subsequently used for electrophysiology recordings 3–5 days after the last injection (Fig 4A–C). *Post hoc* analysis of brain slices from CRF^{CeAhDTR} mice used for electrophysiology showed that there was a significant reduction in CRF neuron cell counts in the injected side of CRF^{CeAhDTR} mice compared to the CeA of the non-injected side (CRF neuron counts: non-injected side = 23.6 ± 5.91 cells; injected side = 4.0 ± 1.79 cells; $n=6$; $p<0.05$; Figure 4B–C). The effect of 100mM ethanol on sEPSC frequency was tested in both the injected and non-injected CeA slices of CRF^{CeAhDTR} mice. 10 min bath application of 100mM ethanol significantly increased sEPSC frequency in the non-injected side ($59.42 \pm 6.41\%$ increase from baseline; $n=6$; $p<0.05$; Fig 4D) of CRF^{CeAhDTR} mice to a similar level as that previously seen in control wild-type mice. 10 min bath application of 100mM ethanol also significantly increase sEPSC frequency in the virus-injected side of CRF^{CeAhDTR} mice ($58.07 \pm 11.32\%$ increase from baseline; $n=6$; $p<0.05$; Fig 4E) to a similar level as control wild-type mice. There was no significant difference in the ability of ethanol to enhance sEPSC frequency in the injected vs. non-injected sides of CRF^{CeAhDTR} mice ($p>0.05$). We also correlated the effect of 100mM ethanol on sEPSC frequency in CRF^{CeAhDTR} slices to the number of CRF neurons counted *post hoc* and found no significant correlation ($r^2=0.01$, $p=0.74$, Fig 4F).

Whole animal ablation of CRF neurons does not alter ethanol enhancement of glutamatergic transmission in the CeA

CRF neurons in the CeA might not be the only source of endogenous CRF in this area as other CRF-containing brain regions, like the parabrachial nucleus, may innervate the CeA. Therefore, we mated CRF-*ires-cre* mice with ROSA-DTA mice to produce the CRF-DTA line in which expression of the active subunit of diphtheria toxin (DTA) is targeted to *cre* positive neurons resulting in ablation of CRF neurons in the whole animal (Fig 5A). Punches from the CRF rich paraventricular nucleus (PVN) of the hypothalamus and CeA of CRF-DTA mice were used to verify loss of CRF mRNA expression. Significant decreases in CRF mRNA expression were observed in both brain regions compared with punches from littermate control mice (CeA= $70.6 \pm 3.6\%$ decrease, $p < 0.05$; PVN= $83.0 \pm 5.7\%$ decrease, $p < 0.05$; Fig 5B–C). CeA slices from CRF-DTA mice were used for analysis of sEPSCs. Bath application of 100mM ethanol significantly increased sEPSC frequency in neurons recorded from the CeA of CRF-DTA mice to a similar level as previously seen in wild-type control mice ($52.34 \pm 12.95\%$ increase from baseline, $n=8$, $p < 0.05$, Fig 5D). CeA slices used in these experiments were saved for post-hoc analysis of CRF mRNA and were found to have significant reductions in CRF mRNA expression compared to CeA slices from littermate control mice ($68.4 \pm 1.2\%$ decrease, $p < 0.05$, Fig 5E). One-way ANOVA revealed no significant difference in the ability of 100mM ethanol to increase sEPSC frequency in any CRF ablated mice line compared to wild-type C57BL/6J mice ($F_{(2,19)} = 1.524$, $p > 0.05$, Fig. 6). Brown-Forsythe test also revealed no significant difference in the standard deviation of the effect of ethanol between groups ($F_{(2,19)} = 0.9724$, $p > 0.05$).

Discussion

The results of these studies show that ethanol increases spontaneous glutamate transmission in the CeA, and that this effect requires CRFR activation. In contrast, ethanol does not alter the excitability of CeA CRF neurons suggesting that ethanol modulation of CRF signaling may not require activity dependent CRF release. Further consistent with this idea, ablation of CRF-producing neurons either within the CeA or in the whole animal does not alter the ability of ethanol to enhance CeA glutamatergic transmission (Fig. 6). Together, these data uncover a previously uncharacterized mechanism of ethanol action in the CeA.

It is surprising that ethanol enhanced glutamatergic transmission in the CeA at physiologically relevant concentrations as the overwhelming majority of studies indicate that acute ethanol exposure inhibits glutamatergic transmission in most brain regions (Möykkö and Korpi, 2012). To our knowledge, only one other study to date has shown that acute ethanol application can increase glutamatergic transmission in an *ex vivo* brain slice preparation, in this case via modulation of dopamine neurons in the VTA (Xiao et al., 2008). Indeed, previous studies of the effects of ethanol on CeA glutamatergic transmission show that acute ethanol inhibits CeA glutamate responses, predominantly through an NMDA receptor dependent mechanism (Roberto et al., 2004b; Roberto et al., 2006; Zhu et al., 2007). The discrepancy between these prior studies and the current findings may be due to differences in the type of synaptic transmission examined or species differences. While the previous studies tested the effects of ethanol on evoked and/or miniature glutamate

transmission in multiple rat strains, the current study focused on spontaneous glutamate transmission in lines of mice based on the C57Bl/6J background. Overall, the data presented here may suggest that ethanol has a unique ability to alter activity/action-potential dependent glutamate release in the CeA that would not have been examined in previous studies with miniature EPSCs (Zhu et al., 2007) which focus on action potential independent forms of glutamate release. The effects of ethanol on action-potential dependent glutamate release may also have been occluded by electrical stimulation protocols in previous studies. Supporting the hypothesis that ethanol has divergent effects on CeA glutamate transmission based on the type of recording method used, we have previously shown that CRF has similar dissociative effects on spontaneous vs. evoked glutamatergic transmission in this brain region (Silberman and Winder, 2013). Since the effect of ethanol reported here is dependent on CRFR activation, these findings suggest that ethanol/CRFR interactions likely occur on presynaptic sites upstream from axon terminals and modulate the activity of glutamatergic axons in the CeA.

Previous work examining ethanol/CRFR interactions on CeA circuitry suggests that ethanol likely induces CRF release from local or extrinsic sources to promote changes in GABA transmission (Roberto et al., 2010). The data presented in this study indicate that although ethanol requires CRFR activation to enhance CeA glutamate transmission this mechanism of action may not require CRF itself. Three pieces of evidence led to this conclusion: 1) ethanol did not modulate CeA CRF neuron excitability; 2) the ability of ethanol to increase sEPSC frequency was maintained in CRF^{CeAhDTR} mice in which local CRF-producing neurons were ablated, and 3) the ability of ethanol to increase sEPSC frequency was maintained in CRF-DTA mice in which CRF neurons were ablated in the whole animal. Together these findings appear to rule out the possibility that ethanol induces CRF release from either local neuronal populations or from sources outside the CeA to enhance glutamatergic transmission. In light of the current findings, the putative role of CRF release in ethanol modulation of CeA GABAergic transmission should be directly tested in future studies.

Overall the current data indicate a novel yet unresolved mechanism of ethanol action in the CeA. Since ethanol modulation of CeA glutamate transmission appears to be activity dependent and requires CRFR, one possibility is that ethanol may induce the release of other endogenous CRFR ligands in the CeA, such as Urocortins. Urocortins are a family of related peptides (Urocortin 1, 2, and 3) that act as endogenous ligands of CRFR1 and CRFR2 and have been recently implicated as important mediators of many alcohol directed behaviors (Ryabinin et al., 2012). For instance, mice that are naturally or selectively bred for alcohol-preference show elevated Urocortin 1 neuron numbers and ethanol-induced activity in the cortical projecting Edinger-Westphal nucleus (the main source of Urocortin 1 in the brain) than in non-preferring mouse lines (Bachtell et al., 2002; Bachtell et al., 2003), with similar findings in alcohol-preferring vs. non-preferring rat lines (Turek et al., 2005; Fonareva et al., 2009). Ethanol can induce FOS expression in the Edinger-Westphal nucleus and lesioning this brain region is also associated with decreased ethanol preference. The cortical projecting Edinger-Westphal nucleus has been shown to send Urocortin positive efferent fibers to the CeA (Weitemier et al., 2005) suggesting that Urocortin 1 could be released in CeA brain slices during acute ethanol application. Urocortin 1 has similarly high affinity for CRFR 1

and CRFR 2 (Ryabinin et al., 2012) and therefore makes an attractive target for the combined CRFR 1 and CRFR 2 mediated effects seen here. Urocortin 2 and Urocortin 3 could also play a role in ethanol related behaviors (Sharpe and Phillips, 2009; Lowery et al., 2010), although the role of these peptides in CeA mediated ethanol behaviors is less well established. Future research will be needed to delineate these possibilities.

In a previous study, we observed basal actions of a CRFR antagonist on CeA glutamatergic transmission, suggesting these receptors might be constitutively active at CeA glutamatergic synapses (Silberman and Winder, 2013). While the current studies showed a trend for increased basal sEPSC frequency (data not shown) in CeA slices pretreated with CRFR1+R2 antagonists, these studies were not designed to directly examine the effects of CRFR antagonists on CeA glutamatergic transmission or the possibility of constitutive CRFR activity. Future studies will be needed to test the potential alternative hypothesis that ethanol may somehow modulate constitutive activity of CRF receptors to promote increased presynaptic glutamate release in the CeA. Another formal alternative hypothesis is that since CRF neuron ablation was not 100% effective in the CRF-DTA and CRF^{CeAhDTR} mouse lines, residual CRF production could account for the lack of differences in the effect of ethanol in these mice compared to the wild-type animals. While this hypothesis cannot be ruled out at this time, the data presented here showing no change in CRF-*tomato* neuron excitability in response to ethanol, no change in ethanol effects on sEPSC frequency across mouse lines which had varying levels of CRF reduction, and no significant difference in the deviation of individual data points between groups makes this hypothesis unlikely.

In conclusion, the data presented here suggest a novel mechanism of ethanol action. Acute ethanol enhances presynaptic glutamate release in the CeA and is dependent on CRFR signaling but does not rely on CRF as a critical ligand. Instead ethanol may utilize other CRFR ligands, such as Urocortins, or utilize another uncharacterized mechanism to enhance CeA glutamate release. The precise behavioral role of acute ethanol mediated increases in CeA glutamate or how this system is altered by chronic ethanol exposure is not currently understood but will be important to investigate in future studies.

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Figure 1. Ethanol enhances sEPSC frequency in the CeA

A) Example traces of sEPSC recordings from CeA neuron during baseline and after bath application of 100mM ethanol. B) Summary of the effect of 100mM ethanol on sEPSCs in CeA neurons normalized to control conditions. Dashed line indicates normalized control value. * Indicates a significant effect of ethanol compared to control. C) Concentration response curve of ethanol effects on sEPSC frequency. Dotted line indicates approximate EC_{50} values.

Figure 2. CRF receptor antagonists block the ability of ethanol to enhance sEPSC frequency
 A) Example traces of sEPSC recordings from a CeA neuron during baseline conditions with CRFR1+2 antagonist pretreatment and after subsequent bath application of 100mM ethanol.
 B) Summary of the effect of 100mM ethanol on sEPSCs in CeA neurons normalized to control conditions. Dashed line indicates normalized control value. * Indicates a significant effect of ethanol compared to control. C) Summary of the effect of 100mM ethanol in absence or presence of CRFR antagonist pretreatment. * Indicates a significant difference increase from baseline.

Figure 3. Ethanol does not alter CeA CRF neuron membrane potentials

A) Example trace of membrane potential recordings from a CeA CRF neuron. Solid bar indicates duration of ethanol application. B) Summary of the effect of 100mM ethanol on membrane potential of CeA CRF neurons. Gray line and connected data points indicate one CeA CRF neuron tested under basal conditions and after ethanol.

Figure 4. Deletion of CeA CRF neurons does not alter ethanol enhancement of sEPSC frequency

A) Workflow schematic for utilizing AAV-FLEX-DTR virus of CRF neurons in the CeA. B) Example confocal images taken from non-injected and injected CeA slices used for cell counts and electrophysiology recordings in subsequent panels. Note that these images are of the virus-injected and non-injected CeA slices from the same mouse. Green is false colored *tdtomato* expression on DIC background false colored red. C) Counts of *tdtomato* positive cell bodies from virus-injected or non-injected CeA slices that were used for electrophysiology recordings. * Indicates a significant difference between groups. D–E)

Summary of the effect of 100mM ethanol on sEPSCs from non-injected and virus-injected CeA slices. * Indicates a significant effect of ethanol compared to baseline. F) Distribution of the number of *tdtomato* positive neurons counted in CeA slices used for recordings compared to the effect of 100mM ethanol on sEPSC frequency in the corresponding slice. Note no significant correlation between the number of *tdtomato* positive neurons and the ability of ethanol to modulate sEPSC frequency.

Figure 5. Deletion of CRF neurons in the whole animal does not alter ethanol enhancement of sEPSC frequency

A) Schematic for crossing DTA mice with CRF-ires-Cre mice to produce whole-animal ablation of CRF-producing neurons. B-C) Relative fold change in CRF mRNA levels in CeA and PVN punches from CRF-DTA mice and control littermates. D) Summary of the effect of 100mM ethanol on CeA sEPSCs in CRF-DTA mice. E) Relative fold change in CRF mRNA levels in CRF-DTA CeA slices used for recordings and CeA slices from control littermates.

Figure 6. Summary of ethanol effects on sEPSC frequency across experimental groups
 Bar graph and data points showing the effects of 100mM ethanol on sEPSC frequency across all experimental groups utilized in these studies, expressed as a percent of normalized baseline value (% control). Dashed line indicates normalized control level.

Table 1

Effects of 100mM ethanol on measurements of CeA CRF neuron excitability.

	Current injection for first action potential (pA)	Action potential threshold (mV)	Action potential peak amplitude (mV)	Action potential rise tau (ms)	Action potential decay tau (ms)	Action potential half-width (ms)	Action potential latency (ms)
Basal	127.5 ± 9.96	-34.09 ± 1.02	105.1 ± 4.11	0.84 ± 0.13	2.92 ± 0.46	2.53 ± 0.18	957.4 ± 167.0
Ethanol	142.5 ± 14.36	-32.92 ± 1.46	100.5* ± 4.63	0.82 ± 0.09	2.550 ± 0.4298	2.617 ± 0.2482	736.5 ± 154.3

* indicates a significant difference between groups.