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Chronic ethanol exposure decreases CB₁ receptor function at GABAergic synapses in the rat central amygdala

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Abstract

The endogenous cannabinoids (eCBs) influence the acute response to ethanol and the development of tolerance, dependence and relapse. Chronic alcohol exposure alters eCB levels and type 1 cannabinoid receptor (CB₁) expression and function in brain regions associated with addiction. CB₁ inhibits GABA release, and GABAergic dysregulation in the central nucleus of the amygdala (CeA) is critical in the transition to alcohol dependence. We investigated possible disruptions in CB₁ signaling of rat CeA GABAergic transmission following intermittent ethanol exposure. In the CeA of alcohol-naïve rats, CB₁ agonist WIN 55,212-2 (WIN) decreased the frequency of spontaneous and miniature GABA_A receptor-mediated inhibitory postsynaptic currents (s/mIPSCs). This effect was prevented by CB₁ antagonism, but not type 2 cannabinoid receptor (CB₂) antagonism. After 2–3 weeks of intermittent ethanol exposure, these WIN inhibitory effects were attenuated, suggesting ethanol-induced impairments in CB₁ function.

The CB₁ antagonist AM251 revealed a tonic eCB/CB₁ control of GABAergic transmission in the alcohol-naïve CeA that was occluded by calcium chelation in the postsynaptic cell. Chronic ethanol exposure abolished this tonic CB₁ influence on mIPSC, but not sIPSC, frequency. Finally, acute ethanol increased CeA GABA release in both naïve and ethanol exposed rats. Although CB₁ activation prevented this effect, the AM251- and ethanol-induced GABA release were additive, ruling out a direct participation of CB₁ signaling in the ethanol effect. Collectively, these observations demonstrate an important CB₁ influence on CeA GABAergic transmission and indicate that the CeA is particularly sensitive to alcohol-induced disruptions of CB₁ signaling.

Keywords

alcohol; amygdala; cannabinoid; endocannabinoid CB₁ receptor; GABA

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AUTHOR CONTRIBUTIONS

MR, PS and LHP were responsible for the study concept and design. FPV, NS, MB, GL and SGM contributed to the acquisition, analysis and interpretation of the data. FPV and MR wrote and edited the manuscript. All authors critically reviewed the manuscript and approved the final version for publication.

DISCLOSURE

The authors declare no conflict of interest.

INTRODUCTION

The central nucleus of the amygdala (CeA) plays an important role in stress- and addiction-related processes, and CeA dysregulation is a critical link in the etiology of aberrant stress reactivity and excessive alcohol consumption associated with alcohol dependence (Koob and Volkow, 2010). Endogenous cannabinoids (eCBs) provide an important constraint of stress responses and homeostatic control over emotional state, in part through influences on amygdala neurotransmission (Riebe and Wotjak, 2011; Ruehle et al., 2012). Unlike classical neurotransmitters, eCBs are lipidic neuromodulators that are not stored in vesicles and act retrogradely at central synapses. The type 1 cannabinoid receptor (CB₁) is one of the most abundant G-protein-coupled receptors in the brain. Its presynaptic activation by eCBs decreases GABAergic and glutamatergic transmission (Gerdeman and Lovinger, 2001; Heifets and Castillo, 2009; Katona and Freund, 2008; Szabo et al., 2002).

Within the past decade, a growing body of evidence has demonstrated that acute and chronic ethanol exposure interact with and alter the eCB system. Acute ethanol exposure increases eCB formation in human neuroblastoma cells and primary cultures of rodent neurons (Basavarajappa et al., 2003), and increases *in vivo* eCB levels in several rat brain regions (Alvarez-Jaimes et al., 2009; Caille et al., 2007; Ceccarini et al., 2013). Chronic alcohol exposure downregulates CB₁ expression and function in the rodent brain (Ceccarini et al., 2013; Mitirattanakul et al., 2007; Vinod et al., 2012), and disrupted CB₁ expression has been observed in *post-mortem* brain tissue from alcohol dependent patients (Vinod et al., 2005; Vinod et al., 2010). *In vivo* imaging studies in heavy drinking alcoholics revealed decreased CB₁ receptor availability that persists for one month of abstinence ((Ceccarini et al., 2014; Hirvonen et al., 2013), but see also (Neumeister et al., 2012)) and is followed by transient recovery (and perhaps upregulation) during protracted alcohol abstinence (Mitirattanakul et al., 2007; Vinod et al., 2006). Chronic alcohol exposure also disrupts gene expression for the primary eCB clearance enzymes (FAAH and MAGL) in a manner that is sensitive to the intermittent nature of alcohol exposure and post-alcohol abstinence period (Serrano et al., 2012). Collectively, there is substantial evidence that long-term alcohol exposure disrupts eCB processing and signaling mechanisms, leading to a hypothesized role for disrupted eCB signaling in the etiology of alcohol use disorders and alcoholism (Pava and Woodward, 2012; Pava, 2014; Serrano and Parsons, 2011).

Several studies have investigated ethanol-induced eCB signaling and reported eCB-induced disinhibition of VTA dopamine cells (via suppressed GABA release) (Melis and Pistis, 2012), inhibition of hippocampal glutamate release (Basavarajappa et al., 2008) and facilitation of GABA release from cerebellar Purkinje neurons (Kelm et al., 2008) upon ethanol exposure. CB₁ is highly expressed in the CeA (Ramikie et al., 2014), where it mediates tonic inhibition of evoked GABAergic transmission, as we previously reported (Roberto et al., 2010a). Furthermore, we observed that CB₁ activation prevents acute ethanol induction of evoked CeA GABAergic transmission. However, despite strong evidence implicating CeA GABAergic dysregulation in the transition to alcohol dependence (Gilpin and Roberto, 2012; Roberto et al., 2010b; Roberto et al., 2004), the effects of chronic ethanol exposure on CB₁ signaling in the CeA have not been investigated.

In the present study, our hypothesis was that disruptions in CeA eCB signaling participate in the transition to ethanol dependence. Much of our prior work on alcohol-induced neuroadaptations in the CeA used long-term (5–7 weeks) exposure to intermittent ethanol vapor inhalation. This approach leads to ethanol dependence defined by the emergence of somatic, affective and motivational indices (Gilpin et al., 2008; O'Dell et al., 2004). This dependent state is associated with significant increases in baseline CeA GABA release without tolerance to ethanol-induced increases in GABAergic signaling, and these disruptions are believed to significantly contribute to withdrawal-related emotional disturbances and excessive alcohol consumption (Gilpin and Roberto, 2012; Roberto et al., 2010b; Roberto et al., 2004). Based on the hypothesized involvement of eCB signaling dysregulation in the transition to the dependent state, the present studies evaluated the effects of a shorter period (2–3 weeks) of intermittent ethanol exposure on CB₁ modulation of GABAergic signaling in the CeA. Our results demonstrate that this period of ethanol exposure decreased CB₁ influence on GABAergic transmission in the CeA, supporting the hypothesis that disrupted CeA eCB signaling contributes to the neuropathology underlying the progression to alcohol dependence.

MATERIALS AND METHODS

All procedures were approved by The Scripps Research Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Whole-cell voltage clamp electrophysiology

Slice preparation—We prepared slices from 128 adult male Sprague Dawley rats (280–350 g), as previously reported (Cruz et al., 2012; Gilpin et al., 2011; Roberto et al., 2010b; Roberto et al., 2008; Roberto et al., 2004). Rats were briefly anesthetized (3–5% isoflurane), followed by rapid decapitation and removal of the brain to an ice-cold high-sucrose solution (pH 7.3–7.4; in mM): sucrose 206; KCl 2.5; CaCl₂ 0.5; MgCl₂ 7; NaH₂PO₄ 1.2; NaHCO₃ 26; glucose 5; HEPES 5. Brains were cut into coronal sections (300 µm) on a vibrating microtome (Leica VT1000S, Leica Microsystems, Buffalo Grove, IL) and placed in an oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (aCSF) solution (in mM): NaCl 130; KCl 3.5; CaCl₂ 2; NaH₂PO₄ 1.25; MgSO₄ 1.5; NaHCO₃ 24; glucose 10. Slices were incubated for 30 minutes at 35–37 °C, followed by 30 minutes equilibration at room temperature. For each experiment, a single slice was transferred to a recording chamber mounted on the stage of an upright microscope (Olympus BX50WI, Tokyo, Japan) and perfused at 2–5 ml/min with continuously oxygenated aCSF at room temperature. Recordings were performed 1–8 hours after slice preparation.

Electrophysiological recording—We recorded from medial subdivision CeA neurons, visualized using infrared differential interference contrast (IR-DIC) optics and a CCD camera (EXi Aqua and ROLERA-XR, QImaging, Surrey, BC, Canada) (Cruz et al., 2012; Gilpin et al., 2011). A w60 or w40 water immersion objective (Olympus) was used to identify and approach neurons. Recordings were performed in a gap-free acquisition mode with a sampling rate per signal of 10 kHz and low-pass filtered at 10kHz, using a

Multiclamp 700B amplifier, a Digidata 1440A and pClamp 10 software (all Molecular Devices, Sunnyvale, CA). Patch pipettes (3–6 M Ω) were pulled from borosilicate glass (Warner Instruments, Hamden, CT and King Precision, Claremont, CA) and filled with an internal solution (in mM): KCl 145; EGTA 5; MgCl₂ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. To isolate the spontaneous inhibitory postsynaptic currents mediated by GABA_A receptors (sIPSCs), recordings were performed in the presence of the glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μ M) and DL-2-amino-5-phosphonovalerate (AP-5, 30 μ M) and the GABA_B receptor antagonist CGP55845A (1 μ M). GABAergic miniature IPSCs (mIPSCs) were recorded in the presence of 1 μ M tetrodotoxin (TTX). Drugs were constituted in aCSF and applied by bath superfusion. All 196 cells were clamped at –60 mV and experiments with >20% change in series resistance (<15 M Ω), as monitored with a 10 mV pulse, were excluded. The frequency, amplitude and kinetics of s/mIPSCs were analyzed using a semi-automated threshold-based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ) and visually confirmed. To accurately determine the s/mIPSC amplitude, only s/mIPSCs that were >5 pA were accepted for analysis. Averages of s/mIPSC characteristics were based on a minimum time interval of 3–5 minutes.

Chronic Intermittent Ethanol Exposure—Male Sprague Dawley rats (n=45) were housed in standard cages in separate, sealed, clear plastic chambers into which ethanol vapor or air was intermittently injected. Rats were exposed to chronic intermittent ethanol (CIE) vapor for 14 hours, with 10 hours of air exposure between each ethanol vapor exposure, for a period of 2–3 weeks. Blood alcohol levels (BALs) were determined 1–2 times weekly by tail-bleeding and terminal BALs were determined at the time of sacrifice. The mean BAL of all ethanol-exposed animals was 173.0 $\pm$ 21 mg/dL. Ethanol naive rats were treated in a similar way, except that they were exposed to continuous air. On electrophysiology experiment days, rats were maintained in the chambers filled with ethanol vapor until sacrifice and the preparation of the CeA slices in ethanol-free aCSF.

Drugs—We purchased CGP 55845A, DL-AP5, picrotoxin, bicuculline and BAPTA (1,2-Bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) from Sigma (St. Louis, MO), tetrodotoxin from Biotum (Hayward, CA); DNQX from Tocris (Ellisville, MO) and ethanol from Remet (La Mirada, CA). WIN (WIN55,212-2; [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-2,4-dichloro phenyl)-4-methyl-1H-pyrazole-3-carboxamide), and SR144528 (5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide) were purchased from Cayman Chemical (Ann Arbor, MI). WIN, AM251, and SR144528 were dissolved in dimethylsulfoxide (final concentrations of 0.05–0.1%), which had no effect on the studied synaptic responses in control experiments. WIN, SR144528, and AM251 were applied only once on the same CeA slice.

Data analysis and statistics—We used MiniAnalysis 5.1 software (Synaptosoft, Leonia, NJ) to analyze the s/mIPSCs and GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) for all statistical analyses of results. The s/mIPSC results were evaluated

with cumulative probability analysis, and statistical significance was determined using the Kolmogorov-Smirnov, non-parametric two-sample test (Van der Kloot, 1991) with $p < 0.05$ considered significant. We used t-test analyses for individual means comparisons, and within-subject one-way repeated measures (RM) ANOVA to compare s/mIPSCs within a group (naïve and ethanol exposed). When appropriate, the Student Newman-Keuls *post hoc* test was used to assess significance between treatments, with $p < 0.05$ considered significant. To assess differences resulting from treatment (naïve and ethanol exposed) and drug interaction between groups (WIN, AM251 and ethanol), we used two-way RM ANOVA. When appropriate, the Bonferroni *post hoc* test was used to assess significance between treatments. All data are presented as mean $\pm$ SEM and n refers to the number of cells. One to five neurons were recorded per animal and all electrophysiological measures were obtained by pooling data from at least four animals in each group.

RESULTS

Chronic ethanol exposure does not alter CeA GABA release—Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in medial CeA neurons from either ethanol-exposed (CIE; BAL 173.0 $\pm$ 21 mg/dL) or naïve rats. Baseline properties were similar between the two groups, with comparable average sIPSC frequencies (naïve: 0.66 $\pm$ 0.05 Hz, n=51 and CIE: 0.61 $\pm$ 0.06 Hz, n=46; Fig. 1A) and no differences in the mean sIPSC amplitudes and kinetics (Fig. 1B and C).

Miniature IPSCs (mIPSCs), assessed with TTX, also showed similar average frequencies (naïve: 0.44 $\pm$ 0.06 Hz, n=26 and CIE: 0.47 $\pm$ 0.05 Hz, n=28; Fig. 1D). However, CeA mIPSC amplitudes (45 $\pm$ 2.5 pA) from CIE rats were significantly ($p < 0.05$, unpaired t-test) lower than those (53 $\pm$ 3 pA) from naïve rats (Fig. 1E). We did not find differences in mIPSC kinetics between the two groups (Fig. 1F). Collectively, these data suggest that chronic ethanol exposure does not affect spontaneous GABA release, but may alter GABA_A receptor function.

The CB₁ agonist WIN 55,212-2 decreases CeA GABA release and chronic ethanol exposure attenuates this effect—We first assessed the effect of 2 μ M WIN55,212-2 [WIN; (Roberto et al., 2010a)], a potent cannabinoid receptor agonist, on spontaneous GABAergic transmission by superfusing it onto CeA neurons for 15 min. Two-way RM ANOVA indicated a significant interaction between WIN and CIE on sIPSC frequency [F(1,22)=9.44, $p < 0.01$]. *Post-hoc* analysis revealed that WIN significantly ($p < 0.01$) reduced sIPSC frequency in naïve neurons (to 60 $\pm$ 7%; from 0.69 $\pm$ 0.18 to 0.34 $\pm$ 0.05 Hz; n=11), but not CIE neurons (to 87 $\pm$ 7%; from 0.45 $\pm$ 0.06 to 0.39 $\pm$ 0.06 Hz; n=13; Fig. 2A and B). WIN had no significant effects on sIPSC amplitude or rise and decay times in both populations (Fig. 2A and B). As a decrease in sIPSC frequency denotes a decreased probability of GABA release (De Koninck and Mody, 1994; Otis et al., 1994), our data suggest that WIN decreases GABA release and this effect is attenuated after chronic ethanol exposure.

Similar to the sIPSCs, two-way RM ANOVA revealed a significant interaction between WIN and CIE on CeA mIPSC frequency [F(1,16)=11.39, $p < 0.01$]. WIN significantly ($p < 0.01$,

n=8) reduced mIPSC frequency to $58\pm7\%$ (from 0.39 ± 0.08 to 0.21 ± 0.04 Hz) in naïve rats, but had no effect in CIE rats (to $84\pm8\%$; from 0.60 ± 0.14 to 0.50 ± 0.1 Hz; n=10; Fig. 2C and D). WIN did not affect the mIPSC amplitude or rise and decay times in both populations (Fig. 2C and D).

To verify that WIN decreased GABAergic transmission by activating CB₁, we used a CB₁ antagonist (AM251) and a type 2 cannabinoid receptor (CB₂) antagonist (SR144528). In the CeA of naïve rats, WIN significantly ($p<0.05$; n=5; Fig. 2E) decreased sIPSC frequency (to $62\pm9\%$ of control; from 1.02 ± 0.1 to 0.60 ± 0.08 Hz) and subsequent application of 2 μ M AM251 in the continued presence of WIN significantly reverted this inhibition (sIPSC frequency returned to $91\pm9\%$ of control; 0.89 ± 0.08 Hz; n=5; Fig. 2E). In another group of CeA neurons from naïve rats, WIN significantly ($p<0.05$; n=5; Fig. 2F) decreased sIPSC frequency (to $67\pm10\%$ of control; from 0.42 ± 0.08 to 0.27 ± 0.07 Hz) and subsequent 2 μ M SR144528 application in the continued presence of WIN did not alter ($70\pm7\%$; 0.29 ± 0.06 Hz) the inhibition induced by WIN (Fig. 2F). Thus, WIN activates CB₁, but not CB₂, at a presynaptic site to decrease GABA release.

To further confirm the role of CB₁ in the WIN-induced inhibition of spontaneous GABA release, we first applied AM251 and subsequently added WIN. In CeA neurons from both naïve and CIE rats, 2 μ M AM251 significantly ($p<0.05$; Fig. 2G) increased sIPSC frequency [to $131\pm5\%$ (n=6) and $136\pm10\%$ (n=8) of control, respectively] and subsequent application of 2 μ M WIN in the continued presence of AM251 failed to decrease spontaneous GABA release (sIPSC frequency remained at $126\pm7\%$ and $134\pm11\%$ of control, respectively; Fig. 2G). Thus, antagonism of CB₁ blocks the WIN-induced inhibition of spontaneous GABA release, pointing to a clear role for the CB₁ receptor on GABA presynaptic terminals.

Acute ethanol increases spontaneous GABAergic transmission in the CeA of both naïve and chronic ethanol exposed rats

As previously reported (Roberto et al., 2003; Roberto et al., 2004), here we found that application of a maximal dose of ethanol (44mM) increases GABA release in the CeA of both naïve and ethanol exposed rats. Specifically, 44 mM ethanol significantly increased sIPSC frequency to $157\pm17\%$ (from 0.75 ± 0.17 to 1.07 ± 0.18 Hz; $p<0.05$; n=8) and $146\pm11\%$ (from 0.61 ± 0.08 to 0.87 ± 0.12 Hz; $p<0.05$; n=9; Fig. 3A and B) in CeA neurons from naïve and CIE rats, respectively. Acute ethanol did not alter sIPSC amplitudes and kinetics in both populations (Fig. 3A and B). Additionally, as previously demonstrated (Roberto et al., 2010b; Roberto et al., 2003; Roberto et al., 2004), acute ethanol induced a similar and significant ($p<0.05$) increase in the mIPSC frequency in the CeA of naïve ($138\pm3\%$; from 0.56 ± 0.16 to 0.77 ± 0.23 Hz; n=8) and CIE rats ($132\pm8\%$; from 0.38 ± 0.08 to 0.49 ± 0.12 Hz; n=8; Fig. 3C and D). No alterations in mIPSC amplitudes or rise and decay times were observed in both populations (Fig. 3C and D).

CB₁ activation prevented the ethanol-induced increase in spontaneous GABAergic transmission in the CeA

We assessed the influence of CB₁ activation on the ethanol enhancement of sIPSCs by first applying WIN and then applying ethanol in the continued presence of WIN. In 8 CeA neurons from naïve rats, 2 μ M WIN significantly ($p<0.05$) decreased sIPSC frequency to $65\pm8\%$ of control (from 0.51 ± 0.08 to 0.31 ± 0.04 Hz),

and subsequent addition of 44 mM ethanol together with WIN did not alter sIPSC frequency ($63 \pm 9\%$ of control; 0.29 ± 0.04 Hz; Fig. 4A and B). Thus, CB₁ activation prevents ethanol induction of GABA release in naïve animals. Furthermore, in CIE rats, although WIN had no significant effect (to $82 \pm 7\%$ of control; from 0.49 ± 0.08 to 0.40 ± 0.07 Hz; $n=9$) on sIPSC frequency, WIN still blocked the ethanol-induced GABA release (to $87.3 \pm 10\%$ of control; 0.41 ± 0.07 Hz; Fig. 4A and B).

CB₁ activation also blocked the ethanol enhancement of mIPSC frequency in the CeA of naïve and CIE rats. WIN significantly decreased the mIPSC frequency to $54 \pm 6\%$ of control (from 0.43 ± 0.08 to 0.21 ± 0.01 Hz; $n=7$, $p<0.05$), and occluded the ethanol effect (mIPSC frequency remained at $55 \pm 5\%$ of control; 0.21 ± 0.01 Hz; Fig. 4C and D). Additionally, while the WIN inhibitory effect on the mIPSC frequency was significantly attenuated (to $90 \pm 11\%$ of control; from 0.74 ± 0.15 to 0.66 ± 0.14 Hz; $n=7$) in CIE rats compared to naïve rats, acute ethanol failed to increase vesicular GABA release ($102 \pm 12\%$ of control; 0.73 ± 0.14 Hz; Fig. 4C and D).

In another group of naïve rat CeA neurons, we reversed the order of drug application and first applied ethanol and subsequently added WIN in the continued presence of ethanol. Ethanol significantly ($p<0.05$, $n=6$) increased the sIPSC frequency to $145 \pm 14\%$ of control (from 0.41 ± 0.05 to 0.60 ± 0.09 Hz), and the addition of WIN together with ethanol significantly decreased the sIPSC frequency back to the control value (to $101 \pm 6\%$; 0.41 ± 0.04 Hz; Fig. 4E and F), indicating that ethanol and WIN interact presynaptically to modulate GABA release in CeA. Similarly in 8 neurons from CIE rats, acute ethanol application increased the frequency of sIPSCs to $142 \pm 10\%$ of control (from 0.51 ± 0.1 to 0.72 ± 0.1 Hz; Fig 4F) and the addition of WIN in the presence of ethanol significantly decreased it to $97 \pm 6\%$ of control (0.50 ± 0.1 Hz; Fig 4F).

CB₁ antagonism revealed tonic eCB signaling in the CeA—To investigate a possible role of the eCBs in regulating basal CeA GABAergic transmission, we used the CB₁ antagonist AM251. Application of $2 \mu\text{M}$ AM251 for 15 min significantly ($p<0.05$) increased sIPSC frequencies to $135 \pm 9\%$ (from 0.85 ± 0.1 to 1.11 ± 0.1 Hz; $n=10$) and $136 \pm 6\%$ (from 0.54 ± 0.1 to 0.71 ± 0.1 Hz; $n=13$) of control in naïve and CIE rats, respectively (Fig. 5A and B). AM251 did not alter the sIPSC amplitudes or kinetics in both groups (Fig. 5A and B). These data suggest that the CB₁ system tonically regulates inhibitory transmission in the CeA by decreasing GABA release under basal conditions. We also studied the effect of AM251 on CeA mIPSCs in naïve and CIE rats. Two-way RM ANOVA indicated a significant interaction between AM251 and CIE on mIPSC frequency [$F(1,15)=6.19$, $p<0.05$]. *Post-hoc* analysis revealed that AM251 significantly ($p<0.05$) increased the mIPSC frequency to $139 \pm 12\%$ (from 0.42 ± 0.1 to 0.58 ± 0.1 Hz; $n=8$) of control in naïve rats, but had no effect ($111 \pm 3\%$; from 0.40 ± 0.07 to 0.43 ± 0.07 Hz; $n=9$) in CIE rats (Fig. 5C and D). AM251 had no effect on mIPSC amplitudes or kinetics in both groups (Fig. 5C and D).

To investigate the origin of CB₁ activity in the CeA, we filled our patch pipettes with 10 mM BAPTA to buffer the recording cell against the changes in postsynaptic calcium levels that are believed to contribute to the formation and/or mobilization of the eCBs (Hentges et al., 2005; Neu et al., 2007; Roberto et al., 2010a). We observed a significantly higher baseline

sIPSC frequency (1.04 ± 0.1 Hz; $n=10$) in BAPTA loaded neurons compared to the sIPSC frequency in cells recorded with normal internal solution (0.66 ± 0.06 Hz, $n=45$). Furthermore, in BAPTA loaded neurons, $2 \mu\text{M}$ AM251 failed to increase ($106 \pm 7\%$; from 0.83 ± 0.1 to 0.85 ± 0.1 ; $n=7$ and $99 \pm 2\%$; from 0.61 ± 0.1 to 0.61 ± 0.1 Hz; $n=4$) the frequency of sIPSCs in both naive and CIE rats, respectively (Fig. 5E), supporting the finding that eCBs tonically regulate GABA release. We conclude that CeA neurons locally release eCBs that act in a retrograde manner on presynaptic sites to reduce GABA release. Moreover, these results clearly point to basal eCB release that tonically activates CB_1 , rather than constitutive CB_1 activity (Turu and Hunyady, 2010). Additionally, in 4 of the 7 naive rat CeA neurons loaded with BAPTA and exposed to AM251, we were able to subsequently apply ethanol, which significantly ($p < 0.05$) increased (to $140 \pm 9\%$, from 0.9 ± 0.1 to 1.28 ± 0.1 Hz; Fig. 5F) the sIPSC frequency, demonstrating that ethanol acts at a presynaptic site that is independent of eCBs to alter CeA GABA release. The bar graphs of Figure 5F show that the ethanol-induced increases of sIPSC frequency in normal conditions and in high BAPTA loaded neurons are similar.

We also recorded mIPSCs from CeA neurons loaded with BAPTA in both naive and CIE rats. We observed a similar baseline mIPSC frequency in the BAPTA loaded neurons (naive: 0.54 ± 0.1 Hz, $n=9$; and CIE: 0.72 ± 0.1 Hz; $n=7$) compared to those recorded with normal internal solution (naive: 0.44 ± 0.06 Hz, $n=26$; and CIE: 0.49 ± 0.06 Hz, $n=26$) in both groups. Similarly to the sIPSCs, AM251 had no effect on the frequency ($98 \pm 2\%$, $n=9$ and $104 \pm 3\%$, $n=7$) of mIPSCs (Fig. 5G) in both naive and CIE rat CeA neurons loaded with BAPTA, respectively, supporting the finding that eCBs tonically regulate vesicular GABA release. In addition, in naive and CIE rat CeA neurons loaded with BAPTA and exposed to AM251, ethanol significantly ($p < 0.05$) increased (naive: 141 ± 2 , $n=5$; and CIE: $127 \pm 6\%$, $n=5$; Fig. 5H) the mIPSC frequency, demonstrating that ethanol acts independently of eCB to increase vesicular GABA release.

CB_1 antagonism does not impair the effect of ethanol on spontaneous GABAergic transmission—Blockade of CB_1 by AM251 increases GABA release by occluding the tonic inhibitory control of eCBs on GABA transmission. As AM251 and ethanol display comparable induction of GABA release, we investigated the interactions between CB_1 signaling and ethanol in the CeA.

In naive rats, $2 \mu\text{M}$ AM251 alone increased sIPSC frequency to $137 \pm 11\%$ of control (from 0.72 ± 0.12 to 0.94 ± 0.15 Hz; $n=6$; $p < 0.05$), and application of 44 mM ethanol in the continued presence of AM251 significantly ($p < 0.05$) further increased sIPSC frequency to $174 \pm 22\%$ of control (1.17 ± 0.27 Hz; $n=6$; Fig. 6A and B). Similar additive effects of AM251 and ethanol were observed in CIE rats, where AM251 enhanced sIPSC frequency to $131 \pm 6\%$ of control (from 0.57 ± 0.16 to 0.73 ± 0.21 Hz; $n=10$; $p < 0.05$), and ethanol significantly ($p < 0.05$) further increased sIPSC frequency to $170 \pm 12\%$ of control (0.95 ± 0.26 Hz; $n=10$; Fig. 6A and B). Thus, the effects of CB_1 blockade and ethanol on GABA release in the CeA were additive, suggesting that the two drugs act independently. No changes in amplitudes, rise and decay times were observed upon application of AM251 and ethanol in both groups.

In addition, in the majority of neurons in which we applied both AM251 and WIN (Fig. 2G), we subsequently tested the effects of ethanol. In 5 of the 6 CeA neurons from naïve rats, AM251 significantly ($p<0.05$) increased sIPSC frequency to $132\pm6\%$ of control, application of WIN in the continued presence of AM251 had no effect (the sIPSC frequency remained at $128\pm8\%$) and ethanol then further increased the sIPSC frequency to $167\pm10\%$ (data not shown). Similarly, in 5 of the 8 CeA neurons from CIE rats, the significant AM251-induced increase (at $126\pm7\%$) in sIPSC frequency was not altered by WIN application (remained at $122\pm8\%$), but was additionally increased by ethanol to $164\pm10\%$ (data not shown). These results further support our findings that CB₁ blockade and ethanol effects on GABA release are additive.

Notably, AM251 significantly increased mIPSC frequency to $129\pm7\%$ (from 0.42 ± 0.1 to 0.53 ± 0.1 Hz; $n=7$; $p<0.05$) of control in naïve rats, but had no effect in CIE rats ($111\pm5\%$ of control; from 0.43 ± 0.08 to 0.46 ± 0.09 Hz; $n=7$; $p>0.05$; Fig. 6C and D). Application of ethanol further increased the frequency of mIPSCs to $160\pm14\%$ (0.65 ± 0.1 Hz; $p<0.05$) of control in naïve rats, and to $142\pm12\%$ (0.55 ± 0.08 Hz; $p<0.05$) in CIE rats (Fig. 6C and D). No changes in amplitudes or rise and decay times were observed upon application of AM251 and ethanol in both groups.

DISCUSSION

The eCB system is a key player in the neural basis of addiction (Maldonado et al., 2006; Ramikie and Patel, 2012; Serrano and Parsons, 2011; Sidhpura and Parsons, 2011). Several studies have reported that chronic ethanol treatment significantly altered behavioral and physiological responses to a CB₁ agonist (Pava and Woodward, 2012; Pava, 2014) and reduced CB₁ expression and function in the whole brain (Basavarajappa and Hungund, 1999), hippocampus (Mitrirattanakul et al., 2007), and cortex (Vinod et al., 2006). The CeA GABAergic system plays a critical role in the acute effects of ethanol and the development of ethanol dependence (Roberto et al., 2010b; Roberto et al., 2004). Our previous work focused on the effects of CB₁ activity and acute ethanol on *evoked* GABAergic transmission in the CeA of naïve rats, but *spontaneous* GABAergic transmission and the impact of chronic ethanol exposure on eCB signaling in the CeA remain unstudied. The results presented here indicate that tonic eCB activity at CB₁ inhibits spontaneous GABAergic transmission and that chronic intermittent ethanol exposure significantly downregulates CB₁ function in the CeA. Additionally, CB₁ activation occludes ethanol facilitation of CeA GABAergic transmission, even though ethanol and CB₁ antagonism increase GABA release in the CeA independently.

Within the central nervous system, calcium-dependent postsynaptic eCB synthesis promotes retrograde inhibition of afferent neurotransmission (Alger and Tang, 2012; Kano et al., 2009; Lovinger, 2008; Ramikie and Patel, 2012). Specifically, eCBs bind to CB₁ localized to the axon terminals of GABAergic and glutamatergic neurons, activating intracellular G_{i/o} protein signaling cascades to suppress vesicular neurotransmitter release in several brain regions (e.g., cerebellum, hippocampus, striatum, amygdala, and neocortex) (Castillo et al., 2012; Chevaleyre et al., 2006; Lupica and Riegel, 2005; Melis and Pistis, 2012). Here, we report that the CB agonist WIN decreased spontaneous (both action potential-dependent and

-independent) GABA release in the CeA of naïve rats. The WIN effect was reverted by the CB₁ antagonist AM251, but not the CB₂ antagonist SR144528, and WIN did not inhibit GABA release in the presence of AM251, indicating that the WIN effect was mediated exclusively by CB₁ (Roberto et al., 2010a). In contrast, Ramikie et al. recently reported that evoked GABAergic transmission in the lateral CeA was relatively unaffected by CB₁ agonism (Ramikie et al., 2014), pointing to differential sensitivity across the amygdala circuitry.

We have previously reported profound neuroadaptations in the CeA after ethanol dependence was induced by prolonged chronic intermittent ethanol exposure (5–7 weeks) (Roberto et al., 2010b; Roberto et al., 2004). We hypothesized that CeA eCB signaling may contribute to the development of ethanol dependence and be compromised after a relatively short chronic ethanol exposure. Thus, in contrast to our prior studies (Cruz et al., 2012; Roberto et al., 2006; Roberto et al., 2010b; Roberto et al., 2004), the present experiments employed a short-term ethanol exposure (2–3 weeks) to investigate the effects of eCB signaling on GABAergic transmission in the CeA. In the CIE rats used in the present study, we did not observe the elevated GABA release typical of ethanol-dependent rats (Roberto et al., 2004). Instead we found no significant difference (and perhaps a slight reduction) in spontaneous GABA release and a reduction in postsynaptic GABA_A receptor function in the CeA of CIE rats compared to naïve rats. Interestingly, the CIE rats showed attenuation of the WIN-induced decrease in spontaneous GABA release observed in the naïve rats, suggesting decreased CB₁ function. Recent evidence that the gene expression of eCB metabolic enzymes is disrupted by comparable levels of ethanol exposure (Serrano et al., 2012) provides additional evidence of ethanol-induced dysregulation of the eCB system that may contribute to disrupted GABAergic transmission in the CeA. We speculate that dysregulation of other systems (e.g. adenosine) may also contribute to and/or compensate for the decrease in CB₁ function, resulting in no significant changes in baseline GABA transmission after the 2–3 week ethanol exposure.

In contrast to CB₁ activation, we showed that acute ethanol increased spontaneous GABA release. CB₁ activation prevents and abolishes this ethanol effect, indicating that CB₁ activation is preemptive over the action of ethanol at these synapses. These findings are consistent with our previous work (Roberto et al., 2010a) on evoked CeA GABAergic transmission and are similar to the effects reported in the cerebellum (Kelm et al., 2008) and nucleus accumbens (NAc) (Perra et al., 2005). Interestingly, despite the diminished CB₁ function in the CIE rats, CB₁ agonism continues to occlude ethanol's actions on CeA GABAergic transmission.

CB₁ antagonism revealed that eCBs exert tonic inhibitory control over spontaneous GABA release in the CeA of naïve rats. Similar basal CB₁ activity is not present in the NAc, bed nucleus of the stria terminalis (BNST) (Hoffman and Lupica, 2001; Massi et al., 2008), or other nuclei of the amygdala complex (Azad et al., 2003; Marsicano et al., 2002). To identify the origin of this CB₁ activity, we used BAPTA to prevent calcium-dependent formation/mobilization of eCBs in the postsynaptic recording cell. Intracellular BAPTA prevented the AM251-induced increase in spontaneous and vesicular GABAergic transmission, supporting the notion that this effect is driven by enhanced eCB tone, rather than the inverse-agonist

properties of AM251 or CB₁ constitutive activity (Turu and Hunyady, 2010). Thus, CeA neurons locally release eCBs that act in a retrograde manner on presynaptic sites to tonically decrease GABA release.

Interestingly, CB₁ antagonism reverses ethanol effects in the NAc, VTA and basolateral amygdala (Perra et al., 2008; Perra et al., 2005), but not in the CeA, where we found that CB₁ blockade increased GABA release in an additive manner to ethanol. In addition, superfusion of ethanol in the presence of AM251 and WIN still enhances spontaneous GABA release in both naive and CIE rats, indicating that the ethanol-induced facilitation of GABA does not involve CB₁ receptor activation. Thus, CB₁ does not mediate the effects of ethanol in the CeA and ethanol does not act by relieving eCB tone. Furthermore, postsynaptic BAPTA did not alter the ethanol enhancement of both spontaneous and vesicular GABA release, indicating that this effect is not reliant on CeA eCB signaling. In the cerebellum, CB₁ activation decreases and ethanol increases spontaneous GABA release via protein kinase A (PKA)-dependent mechanisms (Kelm et al., 2008). We hypothesize a similar scenario at CeA GABAergic synapses, with ethanol and eCBs acting on a common signaling pathway to regulate GABA release. In fact, we have reported that the adenylylate cyclase/PKA pathway mediates the ethanol effect in the CeA (Cruz et al., 2011; Cruz et al., 2012). Thus, this pathway may be targeted in opposing directions by eCBs and ethanol to regulate CeA GABA release. Other potential candidates, such as protein kinase C epsilon (PKC ϵ) (Bajo et al., 2008) and the combined crosstalk between PKA and PKC ϵ , may also regulate these opposing effects of ethanol and eCBs on GABA release (Kelm et al., 2008).

In both the naive and CIE rats, CB₁ antagonism increased action potential-dependent GABA release, suggesting that the final output of the eCB system is not altered after chronic ethanol exposure. However, action potential-independent vesicular GABA release was no longer augmented by AM251 in CIE animals. As discussed above (3rd paragraph), given the present evidence of a tonic CB₁ inhibitory control of basal GABA transmission and impairment of CB₁ function with chronic ethanol exposure, it is unclear what mechanisms sustain normal levels of action potential-dependent GABA release following chronic ethanol exposure. It is possible that the diminished CB₁ activity is compensated by augmented eCB production across the synaptic network and/or that alterations in non-cannabinoid mechanisms impinge on baseline GABA release. It is important to note that the present recordings were made during the initial hours of abstinence from a moderate period of alcohol exposure. The profile of eCB function following long-term alcohol exposure and during more protracted abstinence remains uncharacterized.

The CeA is critical for mediating the negative affective states associated with stress and alcohol dependence (Gilpin and Roberto, 2012; Koob and Volkow, 2010), as it is the major output nucleus of the amygdala (Cassell et al., 1999; Sah and Lopez De Armentia, 2003) and belongs to the conceptual macrostructure called the extended amygdala (including BNST and NAc shell) (Koob and Volkow, 2010). Thus, the CeA is uniquely situated to function as an integrative hub that converts emotionally relevant sensory information about the external environment and internal milieu into appropriate behavioral and physiological responses. A substantial body of evidence indicates that the eCB system participates in a negative feedback system that limits the expression of stress responses and anxiety under stressful

circumstances, a function that in part involves the CeA (Riebe and Wotjak, 2011; Ruehle et al., 2012; Serrano and Parsons, 2011). Accordingly, dysregulation of CeA eCB function may contribute to abnormal anxiety-like responses to stress and reduced capacity to recover from stressful experiences. The CeA is 95% GABAergic, with local GABA interneurons and GABAergic projection neurons that may inhibit each other via axon collaterals (Herman et al., 2013; Herman and Roberto, 2014; Pape and Pare, 2010). CB₁ signaling constrains local inhibitory transmission in CeA (present results and (Roberto et al., 2010a)), thereby allowing increased activity of CeA projection neurons that inhibit downstream brain regions to mediate emotional stress responses. Accordingly, the present finding that this influence is attenuated following chronic intermittent ethanol exposure implicates eCB/CB₁ signaling in the dysregulation of CeA GABAergic transmission that contributes to aberrant emotional responses to stress and excessive alcohol consumption that are characteristic of alcohol dependence.

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Figure 1.

Baseline properties of s/mIPSCs in the CeA of naive and CIE rats **A: Left panel:** Representative sIPSC recordings from CeA neurons in naive and CIE rats. **Right Panel:** sIPSC frequency in naive rats is similar to that of CIE rats. **B:** Mean sIPSC amplitude in naive rats is similar to that of CIE rats. **C:** sIPSC rise and decay times in naive rats are similar to that of CIE rats. **D: Left panel:** Representative mIPSC recordings from CeA neurons in naive rats and CIE rats. **Right Panel:** Mean mIPSC frequency in naive rats is similar to that of CIE rats. **E:** mIPSC amplitudes in CIE rats are significantly (* $p < 0.05$,

unpaired t-test) decreased compared to naïve rats. **F**: mIPSC rise and decay times in naïve rats are similar to that of CIE rats.

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Figure 2.

CB₁ agonism decreases CeA GABA release and chronic ethanol exposure attenuates this effect. **A:** Representative sIPSCs in control conditions and during WIN 55,212-2 (WIN; 2 μ M) application in CeA neurons from naive (top) and CIE (bottom) rats. **B:** WIN significantly ($p < 0.01$) decreased the mean sIPSC frequency in CeA neurons of naive rats, but not CIE rats. WIN did not alter the amplitudes, and rise and decay times of sIPSCs in either group. # indicates significant ($p < 0.01$) differences between naive and CIE rats by two-way RM ANOVA. **C:** Representative mIPSCs in CeA neurons from naive (top) and CIE

(bottom) rats. **D:** WIN significantly ($*p<0.01$) decreased the mean mIPSC frequency in CeA neurons of naive rats, but not CIE rats. WIN did not alter the amplitudes, and rise and decay times of mIPSCs in either group. #indicates significant ($p<0.01$) differences between naive and CIE rats by two-way RM ANOVA. **E:** In naive rats, WIN significantly ($*p<0.05$) decreased the frequency of sIPSCs. This WIN-induced inhibition of sIPSC frequency is significantly ($#p<0.05$) reverted (by one way ANOVA) by co-application of the CB₁ antagonist, AM251 (2 μ M). **F:** The WIN-induced significant ($*p<0.05$) inhibition of sIPSC frequency is not altered by co-application of the CB₂ antagonist, SR144528 (2 μ M). **G:** In both naive and CIE rats, antagonism of CB₁ with AM251 significantly ($*p<0.05$) increased the sIPSC frequency and blocked the WIN-induced decrease of sIPSC frequency.

Figure 3.

Acute ethanol application increases action potential-dependent and -independent GABA release in both naive and CIE rats. **A:** Representative sIPSCs from CeA neurons of naive (top) and CIE (bottom) rats in control conditions and during ethanol (EtOH; 44 mM) application. **B:** Ethanol significantly ($*p < 0.05$) increased the mean sIPSC frequency in CeA neurons of both naive and CIE rats. Ethanol did not alter the amplitudes, and rise and decay times of sIPSCs in either group. **C:** Representative mIPSCs from CeA neurons of naive (top) and CIE (bottom) rats in control conditions and during EtOH application. **D:** Ethanol

significantly ($*p<0.05$) increased the mean mIPSC frequency in CeA neurons of both naive and CIE rats, without altering the amplitudes, and rise and decay times in either group.

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Figure 4.

CB₁ activation prevented the ethanol-induced increase in GABAergic transmission in the CeA. **A:** Representative sIPSCs in control conditions, during WIN (2 μ M) application and co-application of WIN and EtOH (44 mM) in CeA neurons from naive (left) and CIE (right) rats. **B:** WIN significantly ($*p < 0.05$) decreased the mean sIPSC frequency in CeA neurons of naïve rats, but not CIE rats, and blocked the ethanol-induced increase in both groups. **C:** Representative mIPSCs in control conditions, during WIN application and co-application of WIN and EtOH in CeA neurons from naive (left) and CIE (right) rats. **D:** WIN significantly

(* $p < 0.05$) decreased the mean mIPSC frequency in CeA neurons of naïve rats, but not CIE rats, and prevented the ethanol-induced increase in both groups. **E:** Representative sIPSCs in control conditions, during EtOH application and co-application of EtOH and WIN, in a CeA neuron from a naïve rat. **F:** Acute ethanol significantly (* $p < 0.05$) increased the mean sIPSC frequency in the CeA of both naïve and CIE rats, and the addition of WIN significantly (# $p < 0.05$) decreased it to baseline levels.

Figure 5.

Blockade of CB₁ reveals a tonic eCB activity in the CeA and postsynaptic calcium buffering with BAPTA eliminates this activity. **A:** Representative sIPSCs in control conditions, and during AM251 (2 μ M) application in CeA neurons from naive (left) and CIE (right) rats. **B:** AM251 significantly ($p < 0.05$) increased the mean sIPSC frequency in CeA neurons of both naive and CIE rats. AM251 did not alter the amplitude, and rise and decay times of sIPSCs in either group. **C:** Representative mIPSCs from CeA neurons of naive (left) and CIE (right) rats in control conditions and during AM251 application. **D:** AM251 significantly ($p < 0.05$) increased the mean mIPSC frequency in CeA neurons of naive, but not CIE, rats without altering the amplitude, and rise and decay times in either group. # indicates significant ($p < 0.05$) differences between naive and CIE rats by two-way RM ANOVA. **E:** Bar graphs plotting the AM251-induced changes in the mean sIPSC frequency in normal conditions (same neurons of panel **B**) or with BAPTA in the recording pipette. Note that BAPTA abolished the AM251-induced increase in the sIPSC frequency in CeA neurons of both naive and CIE rats. # indicates significant ($p < 0.01$) differences between the AM251 alone and BAPTA treatment. **F:** Bar graphs plotting the ethanol-induced enhancement of sIPSC

frequency in normal conditions (same neurons of Figure 3B) and in BAPTA loaded neurons. EtOH (44 mM) similarly and significantly ($p<0.05$) increases the mean sIPSC frequency in both conditions. **G:** Bar graphs plotting the AM251-induced changes in the mean mIPSC frequency in normal conditions (same neurons of panel **D**) or with high BAPTA in the recording pipette. BAPTA abolished the AM251-induced enhancement of the mIPSC frequency in naive neurons and had no effect in CIE neurons (where there was no *per se* AM251 effect). # indicates significant ($p<0.01$) differences between the AM251 alone and BAPTA treatment. **H:** Bar graphs plotting the ethanol-induced enhancement of mIPSC frequency in BAPTA loaded neurons exposed to AM251 from naive and CIE rats. EtOH similarly and significantly ($p<0.05$) increases the mean mIPSC frequency in both conditions.

Figure 6.

CB₁ antagonism does not impair the effect of ethanol on spontaneous GABAergic transmission. **A:** Representative sIPSCs in control conditions, during AM251 (2 μ M) application and AM251 and EtOH (44 mM) in CeA neurons from naive (left) and CIE (right) rats. **B:** AM251 significantly ($*p < 0.05$) increased the mean sIPSC frequency and ethanol co-applied with AM251 increased it further, in CeA neurons of both naive and CIE rats. **C:** Representative mIPSCs in control conditions, during AM251 application and AM251 and EtOH in CeA neurons from naive (left) and CIE (right) rats. **D:** AM251 significantly ($*p < 0.05$) increased the mean mIPSC frequency in CeA neurons of naive rats, but not CIE rats. Co-application of AM251 and EtOH significantly ($*p < 0.05$) increased the mean mIPSC frequency of CeA neurons in both naive and CIE rats.