Published in final edited form as:

Neuropharmacology. 2016 September ; 108: 474–484. doi:10.1016/j.neuropharm.2015.12.005.

Acute and Chronic Ethanol Exposure Differentially Regulate CB1 Receptor Function at Glutamatergic Synapses in the Rat Basolateral Amygdala

Stacey L. Robinson^{1,2}, Nancy J. Alexander¹, Rebecca J. Bluett³, Sachin Patel^{3,4,5,6}, and Brian A. McCool^{1,2}

¹Department of Physiology & Pharmacology, Wake Forest School Medicine, Winston-Salem NC

²Neuroscience and Alcohol Research Training Programs, Wake Forest School Medicine, Winston-Salem NC

³Department of Psychiatry, Vanderbilt University School of Medicine, Nashville, Tennessee

⁴Vanderbilt Brain Institute, Vanderbilt University School of Medicine, Nashville, Tennessee

⁵Vanderbilt-Kennedy Center for Research on Human Development, Vanderbilt University School of Medicine, Nashville, Tennessee

⁶Department Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee

Abstract

The endogenous cannabinoid (eCB) system has been suggested to play a key role in ethanol preference and intake, the acute effects of ethanol, and in the development of withdrawal symptoms following ethanol dependence. Ethanol-dependent alterations in glutamatergic signaling within the lateral/basolateral nucleus of the amygdala (BLA) are critical for the development and expression of withdrawal-induced anxiety. Notably, the eCB system significantly regulates both glutamatergic and GABAergic synaptic activity within the BLA. Chronic ethanol exposure significantly alters eCB system expression within regions critical to the expression of emotionality and anxiety-related behavior, including the BLA. Here, we investigated specific interactions between the BLA eCB system and its functional regulation of synaptic activity during acute and chronic ethanol exposure. In tissue from ethanol naïve-rats, a prolonged acute ethanol exposure caused a dose dependent inhibition of glutamatergic synaptic activity via a presynaptic mechanism that was occluded by CB1 antagonist/inverse agonists SR141716a and AM251. Importantly, this acute ethanol inhibition was attenuated following 10 day chronic intermittent ethanol vapor exposure (CIE). CIE exposure also significantly down-regulated CB1-mediated presynaptic inhibition at glutamatergic afferent terminals but spared CB1-inhibition of GABAergic synapses

Corresponding Author: Brian A. McCool, Ph.D., Professor, Dept. Physiology & Pharmacology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, Ph: 336-716-8608, bmccool@wakehealth.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

arising from local inhibitory-interneurons. CIE also significantly elevated BLA N-arachidonylethanolamine (AEA or anandamide) levels and decreased CB1 receptor protein levels. Collectively, these data suggest a dynamic regulation of the BLA eCB system by acute and chronic ethanol.

Keywords

endogenous cannabinoid; CB1; ethanol; dependence; anandamide; basolateral amygdala

1.0 INTRODUCTION

Dependence-induced anxiety is a significant risk factor for relapse to alcohol use during withdrawal. Ethanol-induced alterations to a network of brain regions involved in the regulation of emotionality likely contribute to this anxiogenic state. The amygdala is critically involved in fear-learning, stress, and anxiety-related behaviors. Importantly, the lateral/basal amygdala (BLA), which serves as the major input nuclei of the amygdaloid complex, undergoes significant alterations during chronic ethanol (for review see [1]). Inhibition of BLA principal neuron activity during withdrawal from ethanol is anxiolytic in rodent dependence models [2], and chronic ethanol-induced alterations to BLA synaptic transmission potentially contribute to the development and expression of withdrawal-related anxiety [2–5]. Specifically, chronic exposure/withdrawal significantly enhances both pre- and postsynaptic components of excitatory transmission coupled with a specific decrease in feedforward inhibitory transmission. Overall, enhanced glutamatergic function and diminished GABAergic function likely drive a net increase in BLA output [2–5]. The impact of chronic ethanol on the neuromodulators controlling glutamatergic and GABAergic transmission is unknown but is poised to dramatically influence these dependence-related outcomes.

The endogenous cannabinoid (eCB) system acts primarily through the modulation of glutamate and GABA synaptic activity and is richly expressed in areas related to the regulation of emotional behavior, including the BLA [6]. Importantly, the eCB system has been implicated in both the acute and chronic effects of ethanol as well as in the development and maintenance of alcohol dependence (For review see [7–9]). In contrast to traditional neurotransmitters which are released by excitation-secretion from presynaptic terminals, eCB ligands are generated ‘on demand’ within postsynaptic compartments and act as retrograde signals that activate presynaptic cannabinoid receptors. The first eCB to be identified, anandamide (N-arachidonylethanolamine or AEA), is generated primarily by the cleavage of the membrane-bound precursor, N-arachidonoyl phosphatidylethanolamine (NAPE), via phospholipase D (NAPE-PLD) (for review see [10]). Following its release into the synapse space, AEA undergoes reuptake and is degraded by fatty acid amide hydrolase (FAAH) [11, 12]. The other most well-studied endogenous ligand, 2-arachidonoylglycerol (2-AG), is synthesized from diacylglycerol by a cellular lipase (DAGL α) and subsequently degraded by monoacylglycerol lipase (MAGL) [13]. These endogenous ligands interact with two main receptors, cannabinoid receptor 1 and 2 (CB1 and CB2, respectively), although CB1 is generally considered the primary cannabinoid receptor in neuronal signaling. CB1 is

a G_{i/o} protein-coupled receptor which inhibits transmitter release at both BLA glutamatergic and GABAergic synapses through numerous downstream intercellular signaling mechanisms [14].

The eCB system serves as a point of particular interest as interactions between ethanol and the eCB system have been observed across a variety of paradigms. Behavioral cross-tolerance between ethanol and CB1 agonists has been observed for decades [15–17] and the eCB system is thought to participate in innate ethanol preference in both rats [18, 19] and mice [20, 21]. Further, both acute and chronic ethanol exposure alter expression levels of the CB1 receptor as well as the endogenous ligands and their respective metabolic and catabolic enzymes [22–34]. Similar alterations have been shown in human alcoholics, indicating ethanol-induced changes in this system may be conserved across species [35, 36]. Importantly, modulation of the eCB system in preclinical models can alleviate withdrawal symptoms, including those associated with altered amygdala function such as anxiety-like behaviors [37]. Critically, the eCB system is also known to play a role in modulating synaptic plasticity within the BLA [38–42]. Ethanol-induced alterations to the relative expression of the endogenous ligands and the function of CB1 receptors are therefore poised to significantly impact the excitatory/inhibitory balance within the BLA.

Within the BLA, CB1 is robustly expressed by CCK+ GABAergic interneurons and is also localized to glutamatergic afferent terminals [43]. Although the majority of BLA CB1 receptors are located on GABAergic terminals, CB1 activation within the BLA decreases regional activity [14], suggesting the modest population of CB1 receptors localized to glutamatergic terminals exerts a privileged influence over BLA-mediated behaviors. We have previously demonstrated that chronic ethanol induces dramatic up-regulation of presynaptic function at glutamatergic projections arriving to the BLA from the internal capsule/stria terminalis [5]. These synapses are potently influenced by the eCB system [40, 41]. Within the current experiments, we evaluated CB1-modulation of glutamatergic transmission at this BLA input during both acute and chronic ethanol exposure. The impact of chronic ethanol exposure on CB1 modulation of GABAergic synapses within the BLA and on the expression levels of the primary eCB ligands and their respective metabolic and catabolic pathways were also investigated.

2.0 METHODS

2.1. Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 100–150g on arrival were group housed for the duration of the experiments (n=138). Animals were given 5–7 days to acclimate to the housing area prior to being placed in experimental groups. Food and water were available ad libitum; and housing conditions were consistent with the NIH Guidelines for the Care and Use of Laboratory Animals (68–74°F, 30–70% relative humidity). All experimental procedures were reviewed and approved by the WFUSM Animal Care and Use Committee.

2.2. Chronic intermittent ethanol exposure

Chronic ethanol vapor exposure was selected to enable consistent, precisely controlled blood ethanol concentrations between subjects throughout experiments (for review see [44]) and was conducted in a manner similar to previous reports [2, 5]. Briefly, animals were placed into air-tight, Plexiglas enclosures (2ft W × 2ft L × 1.3 ft H) in their home cages and intermittently exposed to either ethanol vapor (25–28mg/L) or room air during the light cycle (12 h/day) for 10 consecutive days. Animals were divided into two groups 1) those euthanized while still intoxicated at the end of the last exposure (CIE) and 2) the control (CON) group housed in identical Plexiglas enclosures, exposed only to room air, and euthanized during a time matched period as the CIE group. Trunk blood was collected from the CIE group at euthanasia and analyzed for blood ethanol levels. At the time of brain extraction, blood ethanol levels in the CIE animals were 222.4 ± 9.1 mg/dL as determined by a commercially-available alcohol dehydrogenase assay (Carolina Liquid Chemistries Corp, Brea, CA).

2.3. Electrophysiology methods

2.3.1 Slice preparation—Animals were anesthetized with isoflurane and decapitated in accordance with an approved Wake Forest Baptist Health Institutional ACUC protocol. Brains were quickly removed and incubated in an ice-cold sucrose modified artificial cerebral spinal fluid (aCSF) containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 26 NaHCO_3 , 1.2 NaH_2PO_4 , 0.10 ketamine, and 10 glucose, equilibrated with 95% O_2 and 5% CO_2 . Coronal slices were obtained (400 μm) using a VT1200 S vibrating blade microtome (Leica, Buffalo Grove, IL) and were incubated for at least 1 h in room temperature ($\sim 25^\circ\text{C}$), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 2 MgSO_4 , 2.5 CaCl_2 , 26 NaHCO_3 , and 10 glucose, before initiation of recordings. Sigma-Aldrich (St. Louis, MO) and Tocris (Ellisville, MO) purveyed all chemical reagents.

2.3.2. Whole-cell patch-clamp recording—BLA slices were transferred to a submersion-type recording chamber and perfused with room temperature ($\sim 25^\circ\text{C}$) aCSF (2.0 ml/min) for whole-cell voltage clamp recordings similar to previously published reports [5]. Data were acquired via Axopatch 700B (Molecular Devices, Foster City, CA) and analyzed offline via pClamp software (Molecular Devices). Inclusion criteria for presumptive principal neurons included high membrane capacitance (>100 pF) and low access resistance in the whole-cell configuration (<20 M Ω [45]). Cells in which access resistance changed 20% were excluded from analysis.

Glutamatergic responses were recorded with electrodes filled with an internal solution containing (in mM): 145 K-Gluconate, 5 NaCl, 1 MgCl_2 , 10 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na-GTP, pH 7.25, osmolarity 280–290. In the acute ethanol- excitatory postsynaptic current (EPSC) experiments, a reduced EGTA internal solution was used to facilitate the calcium-dependent production of endocannabinoids during the whole-cell recording. This internal contained (in mM): 121.5 K-Gluconate, 17.5 KCL, 9 NaCl, 1 MgCl_2 , 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.5 Na-GTP, pH 7.25, osmolarity 280–290. Synaptic responses were electrically evoked every 30s using concentric bipolar stimulating electrodes (FHC Inc,

Bowdoin, ME) placed within the internal capsule. Glutamatergic synaptic currents were pharmacologically isolated using 100 μ M picrotoxin (a GABA_A antagonist) to bath aCSF. In the acute ethanol-EPSC experiments, slices were also continuously perfused with 20 μ M SCH50911 (a GABA_B receptor antagonist).

For recordings of inhibitory postsynaptic currents (IPSCs), pipettes contained an internal solution containing (in mM): 100 CsOH, 60 CsCl, 5 NaCl, 1 MgCl₂, 10 EGTA, 10 HEPES, 1 CaCl₂, 4 Mg-ATP, 0.4 Na-GTP, 0.4 QX314, pH 7.25, osmolarity 280-290. Synaptic responses were electrically evoked every 20s using stimulating electrodes placed within close proximity to the patched cell. GABAergic synaptic currents were pharmacologically isolated with 20 μ M 6,7-dinitroquinoxaline-2,3(1*H*,4*H*)-dione (DNQX, an AMPA/kainate receptor antagonist), 50 μ M DL-2-amino-5-phosphono-pentanoic acid (APV, an NMDA receptor antagonist), and 20 μ M SCH50911. Sigma-Aldrich (St. Louis, MO) and Tocris (Ellisville, MO) purveyed all chemical reagents.

As a proxy measure for release probability, paired-electrical stimuli were given to each stimulation site at interpulse intervals of 50 (EPSC) or 100 ms (IPSC). Paired-pulse ratio (PPR) for each stimulation site was calculated as [Peak 2 amplitude/Peak 1 amplitude]. These ratios were expressed as means $\pm$ S.E.M.. The average paired-pulse ratio was determined for a 5–10 minute baseline and was compared to the average ratio of the final five minutes of drug application.

2.3.3 Field Excitatory Post Synaptic Potential (fEPSP) recordings—Slices were placed in the recording chamber and were continuously perfused with aCSF (2 mL/min). All field experiments were recorded in the presence of 100 μ M picrotoxin and 20 μ M SCH50911. Electrodes were filled with an extracellular pipette solution containing aCSF. Ensemble postsynaptic potentials were evoked at 0.1 Hz using concentric bipolar stimulating electrodes (FHC Inc, Bowdoin, ME) placed along the internal capsule input to the BLA. Maximal fEPSP responses were determined in every slice; the stimulus intensity that evoked 60% maximal amplitude was used for each recording. Steady baseline fEPSPs were established and recorded for 5–10 min prior to drug application. Evoked responses were averaged into two minute bins.

2.3.4 Drug Preparation—Stock solutions of SR141716a, WIN 55,212-2, AM251, and URB597 (Cayman Chemicals, Ann Arbor, Michigan) were dissolved in 1ml DMSO (Sigma-Aldrich, St. Louis, MO). Final concentrations of DMSO in the perfusate were equal to or less than 0.05%. To facilitate dissolution, one drop tween 80 (Sigma-Aldrich, St. Louis, MO) was added to all stock solutions save URB597 as per the manufacturer's instructions. Stock solutions were aliquoted and stored at -20°C until use.

2.4 Western Blots

Western blot methods are similar to those previously reported [5]. Each blot contained BLA protein from individual animals (1 per lane) from both the CON and CIE treatment groups (n=6–8 individuals per group). BLA protein was prepared from tissue dissected from coronal slices, disrupted with sonication as previously described [4–5], and quantified in triplicate using the BCA assay (Thermo-Fisher Scientific, Grand Island NY). On any given

blot, the same mass of total protein was loaded for each CON and CIE sample. Several experiments were performed in duplicate with unique cohorts to increase power. Group specific expression was averaged and normalized to the mean CON level and expressed as 'percent CON' to facilitate comparisons across experiments when necessary. The specificity of individual antibodies was indicated by immunoreactive bands at expected molecular weights: CB1 (3.45µg/ml) from Millipore (Billerica, MA) or (1:250) from Cayman Chemicals (Ann Arbor, Michigan), DAGLα (1µg/ml) from (Sigma-Aldrich, St. Louis, MO), FAAH (3µg/ml) from Abcam (Cambridge, Massachusetts), MAGL (1µg/ml), and NAPE-PLD (1:2000) from Cayman Chemicals (Ann Arbor, Michigan). Immuno-reactive bands were detected using standard chemiluminescent detection with an appropriate secondary antibody.

2.5 Mass spectrometry

Mass spectrometry was performed similar to that described previously [46]. Samples were homogenized in methanol and centrifuged; water was added to the supernatant for a final ratio of 70:30 Methanol:Water. Liquid chromatography/mass spectrometry (LC/MS) was performed as previously described [46]. Briefly, samples were injected into a C-18 column on a gradient from 70:30 to 100:0 Methanol:Water containing 80µM silver acetate and 0.1% glacial acetic acid (v/v). Analytes were detected via selective reaction monitoring (as [M + Ag]⁺ complexes except AA, which is ionized as [(M-H)+2Ag]⁺) in the positive ion mode using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): AA (*m/z* 519(527) to 409(417)); 2-AG (*m/z* 485(493) to 411(419)); and AEA (*m/z* 454(462) to 432(440)).

2.6 Statistics

All values are expressed as mean ± SEM. Primary statistical analyses were conducted using 2-way ANOVA, one-way ANOVA, or *t*-tests (GraphPad, GraphPad Software Inc, La Jolla, CA) depending on the experimental design, with posttests as appropriate. A value of *p* < 0.05 was considered statistically significant.

3.0 RESULTS

3.1 Acute Ethanol Inhibits Internal Capsule BLA Afferents

fEPSPs were stimulated at internal capsule inputs and recorded within the BLA in drug naïve control animals. One-way ANOVA detected a significant effect of 80mM ethanol during the final two minutes of drug application ($F_{3,32} = 6.477$, *p* < .01). Subsequent Dunnett's Multiple Comparison Test was used to directly compare the different EtOH treatment-groups to the aCSF control group. This analysis determined that compared to continuous aCSF perfusion alone (mean percent change: 1.63 ± 2.39 , *n* = 16), at the end of exposure ethanol depressed peak fEPSP amplitude at the 80mM (mean: $-18.77 \pm 3.56\%$, *p* < .001 *n* = 6) and 40mM dose (mean: $-11.56 \pm 3.26\%$, *p* < .01 *n* = 8), but not at 20mM (mean: $1.64 \pm 3.33\%$, *p* > .05 *n* = 6) (Fig. 1A, B). Whole-cell patch clamp recordings were used to evaluate pre- and postsynaptic contributions to this depression (*n* = 18). In 14 out of 18 cells, 25–30 minutes of acute ethanol caused a modest but significant increase in PPR indicative of a decrease in presynaptic release (mean baseline PPR: 1.181 ± 0.091 , post-

ethanol PPR: 1.293 ± 0.102 ; paired t-test $p < .001$, $t(13) = 14.631$) (Fig. 1D, E), but not 10–15 minutes (10–15 minute post-ethanol PPR: 1.173 ± 0.095 ; paired t-test $p > .05$, $t(13) = 0.064$) (see discussion). In 4 cells, ethanol application resulted in no significant change in PPR (mean baseline PPR: 1.029 ± 0.078 , post-ethanol PPR: 0.978 ± 0.084 ; paired t-test $p > .05$, $t(3) = 2.102$) indicating a potential subpopulation of ethanol-sensitive terminals at this input. Collectively, these data suggest that prolonged application of acute ethanol inhibits excitatory transmission at internal capsule afferents to the BLA.

3.2 Ethanol Inhibition of Internal Capsule BLA Afferents is Mediated by CB1 Receptors

Slices were pre-incubated for at least 20 minutes with aCSF or one of two CB1 antagonists ($5\mu\text{M}$ SR141716a or $1\mu\text{M}$ AM251) prior to baseline recordings in drug naïve control rats. In control slices (aCSF only), 20 minutes of 80mM ethanol application resulted in an inhibition of fEPSP peak amplitude (mean = $-17.92 \pm 3.9\%$, $n = 7$) as previously observed. Both CB1 antagonists significantly attenuated this ethanol-inhibition (one-way ANOVA, $F(2,19) = 5.685$; $p < .05$; Dunnett's Multiple Comparison Test: SR141716a $p < .05$ $n = 6$, mean inhibition = $-2.76\% \pm 2.17$; AM251 $p < .05$ $n = 9$, mean inhibition = -1.97 ± 4.04) (Fig. 2A, B, C). We employed Dunnett's post-hoc in this case to directly compare the CB1 antagonists to the control condition. These data suggest acute ethanol inhibition of internal capsule afferents involves activation of CB1 cannabinoid receptors. To explore this, we applied the CB1/2 agonist, WIN 55,212-2 (WIN, $5\mu\text{M}$), 30 minutes after the baseline period in the presence or absence of acute ethanol. Again, consistent with previous experiments, 80mM ethanol significantly decreased fEPSP peak amplitude (t-test $p < .01$, $t(13) = 4.087$). WIN application significantly decreased fEPSP peak amplitude compared to baseline in both aCSF and ethanol pre-treated slices (two-way ANOVA, main effect of time, $F(1,13) = 390.9$, $p < .0001$) but this was not altered by pre-treatment type (aCSF versus ethanol pretreatment, $F(1,13) = 0.05518$, $p > .05$). The total percent inhibition by WIN was likewise not different between ethanol- (mean inhibition: $-58.64 \pm 3.61\%$) and aCSF-treated slices (mean inhibition: $-55.33 \pm 4.87\%$; t-test, $p > .05$, $t(13) = 0.5548$). These data show that ethanol-induced inhibition and WIN inhibition was not additive and may suggest overlapping mechanisms of action.

3.3 Chronic Ethanol Exposure Disrupts CB1-mediated Presynaptic Modulation of Glutamatergic Afferents

As chronic ethanol exposure has been extensively demonstrated to alter eCB system function [7], we evaluated the impact of chronic ethanol on acute ethanol modulation. Two-way ANOVA detected a significant interaction between treatment group (control or CIE) and time ($F(1,19) = 4.800$, $p < .05$). Bonferroni posttest found 80mM acute ethanol decreased fEPSP peak amplitude in drug naïve controls ($p < .001$, $t = 4.408$) but not in BLA slices from CIE exposed animals ($p > .05$, $t = 1.744$) (Fig. 3A, B). Further, the percent inhibition induced by acute ethanol during the final two minutes of treatment was found to be significantly attenuated within CIE exposed animals (mean CON: $13.32 \pm 1.27\%$, $n = 9$; mean CIE: $4.08 \pm 2.82\%$, $n = 12$; t-test $p < .05$, $t(19) = 2.676$) (Fig. 3C). These data suggest CIE exposure disrupts the ability of acute ethanol to modulate glutamatergic transmission at internal capsule BLA afferents.

We next investigated if the attenuation of acute ethanol effects observed within CIE slices were due to alterations in BLA CB1 function following chronic ethanol exposure. Using whole-cell measurements in individual BLA pyramidal neurons from drug-naïve control animals, 5 μ M WIN inhibited the glutamatergic EPSC amplitudes by 51.4 $\pm$ 3.6%. This WIN modulation of EPSC amplitude was significantly reduced in CIE cells relative to controls (8.1 $\pm$ 9.3%; t-test $p < 0.01$; $t(9) = 4.645$). Consistent with previous results, there was no significant difference in baseline (no WIN) PPR between CON and CIE cells (t-test $p > .05$; $t(9) = 0.1843$) [5]. Two-way ANOVA of WIN PPR data from CON and CIE neurons detected a significant interaction between treatment group (CON or CIE exposed) and drug condition (Baseline or WIN, $F(1,8) = 10.19$, $p < .01$). *Bonferroni posttests* determined that WIN significantly increased PPR in control ($p < .01$) but not CIE exposed animals ($p > .05$). Expressing the PPR as percent baseline to underscore WIN modulation, the effect of WIN was significantly less in CIE-treated BLA neurons (5.02 $\pm$ 6.49% effect of WIN on PPR, $n=4$) compared to CON (37.13 $\pm$ 6.15%, $n=6$; t-test $p < .01$, $t(8) = 3.472$) (Fig. 4A, B). These data indicate CIE vapor exposure impairs CB1 modulation of glutamatergic transmission at internal capsule afferents to the BLA.

As CB1 is likewise known to significantly modulate BLA local interneuron GABAergic synapses, we also measured WIN modulation of GABAergic synapses following CIE exposure. In agreement with extant literature in the BLA, 5 μ M WIN inhibited IPSC amplitudes from CON neurons by 54.1 $\pm$ 8.1% ($n=6$); and this effect was not significantly different in CIE neurons (48.3 $\pm$ 5.4%; t-test $p > 0.05$, $t(11) = 0.515$). As previously reported [3], no difference in GABA PPR was observed between CON and CIE animals (t-test $p > .05$; $t(11) = 1.134$). Importantly, CIE vapor exposure did not alter WIN modulation of GABA PPR, with paired two-way ANOVA detecting a significant impact of WIN ($F(1,11) = 17.63$, $p < .01$) but no significant interaction ($F(1,11) = 0.8376$, $p > 0.05$) or main effect treatment group ($F(1,11) = 4.224$, $p > .05$). Consistent with this, no significant difference was found in the percent change of PPR induced by WIN in control and CIE cells (mean control: 31.6 $\pm$ 6.2%, $n = 6$; mean CIE: 34.4 $\pm$ 17.2%, $n = 7$; t-test $p > .05$, $t(11) = 0.1443$) (Fig. 4C, D). These findings suggest that CIE dysregulation of CB1 presynaptic modulation is specific to glutamatergic synapses.

3.4 Chronic Ethanol Exposure Specifically Alters CB1 Expression Levels

To evaluate potential sources for the observed reductions in CB1 function on glutamatergic afferents to the BLA, expression levels for CB1 receptor protein and specific components of eCB synthesis and degradation were assessed in control and CIE exposed animals. Total protein levels of BLA CB1 was modestly but significantly decreased in CIE vapor exposed animals compared to control (t-test, $p < .05$, $t(20) = 2.325$ CON $n = 11$, CIE $n = 11$) (Fig. 5A). However, total protein expression levels for primary metabolic enzymes of AEA, NAPE-PLD (t-test, $p > .05$, $t(6) = 0.4981$; CON $n = 4$, CIE $n = 4$) (Fig. 5B) and FAAH (t-test, $p > .05$, $t(22) = 0.3108$; CON $n = 12$, CIE $n = 12$) (Fig. 5C) were unperturbed by chronic ethanol. Similarly, no change was seen in the total expression levels of DAGL- α (t-test, $p > .05$, $t(14) = 1.133$; CON $n = 8$, CIE $n = 8$) (Fig. 5D) or MAGL (t-test, $p > .05$, $t(14) = 0.2859$; CON $n = 8$, CIE $n = 8$) (Fig. 5E), the primary metabolic enzymes associated with

2-AG. Overall, these data suggest CIE vapor exposure specifically down-regulates CB1 receptor expression.

3.5 Chronic Ethanol Exposure Selectively Elevates BLA AEA Content

To further explore CIE impact on the eCB system, the relative levels of the primary endogenous ligands were assessed at baseline and following CIE exposure using mass spectrometry across individual animals. Relative to control, CIE did not alter the levels of the eCB precursor arachidonic acid (t-test $p > .05$, $t(21) = 0.1429$; CON $n = 11$, CIE $n = 12$) or the eCB agonist, 2-AG (t-test $p > .05$, $t(20) = 0.5467$; CON $n = 10$, CIE $n = 12$) (Fig. 6A). AEA was significantly increased in BLA tissue from CIE animals compared to control (t-test $p < .05$, $t(20) = 2.215$; CON $n = 9$, CIE $n = 9$) (Fig. 6B). These findings suggest that ethanol specifically alters the synthesis or degradation of this eCB.

To distinguish these possibilities, we pre-incubated treatment naïve slices with the selective FAAH-inhibitor URB597 (20 μ M) for at least 30 minutes. URB597 was constantly perfused throughout recordings. Although we did not directly assess the effect of URB597 itself on fEPSP peak amplitude, there was no significant difference in baseline fEPSP size between URB- and aCSF-treated slices (t-test $p > .05$, $t(5) = 0.1425$) prior to perfusion of the slices with acute ethanol. Notably, acute ethanol produced a significant effect of time (two-way ANOVA, $F(1,12) = 22.41$, $p < .001$) but there was no significant effect of URB597 ($F(1,12) = 0.0076$, $p > .05$) (Fig. 7A, B) suggesting FAAH inhibition did not significantly alter acute ethanol effects. In support of this, the percent inhibition induced by ethanol was not significantly different between control and URB597-treated samples during ethanol incubation (mean ethanol inhibition: $18.73 \pm 2.44\%$, $n = 7$; mean URB+ethanol: $18.92 \pm 7.52\%$, $n = 7$; t-test $p > .05$, $t(12) = 0.0245$) (Fig. 7C). These data suggests that the ethanol inhibition of internal capsule BLA afferents is not dependent upon modulation of AEA catabolism via FAAH.

3.0 DISCUSSION

Ethanol significantly alters the expression and function of endogenous cannabinoid system components during both acute and chronic ethanol exposure across numerous brain regions (for review see [7] and [9]). The present study suggests glutamatergic inputs to the BLA arriving along the internal capsule number among these sites of interaction. Pharmacologically relevant doses of acute ethanol inhibited fEPSPs produced by stimulation of these terminals. Notably, this inhibition was not additive to that induced by the full CB1/2 agonist WIN 55,212-2. Given a ceiling effect following the use of a full CB1-agonist may have also contribute to this result, CB1 involvement was further assessed through pre-application of two different CB1 antagonists.—Consistent with the proposed CB1 involvement, pre-incubation with SR141716a or AM251 occluded ethanol-induced inhibition of fEPSP peak amplitude. Together, these data strongly suggest acute ethanol increases endocannabinoid levels in the BLA, potentially by modulating ligand synthesis/degradation, and that these endogenous ligands interact with presynaptic CB1 receptors found on glutamatergic synapses. Consistent with previous findings in the amygdala and associated limbic regions, chronic ethanol significantly increased AEA concentration and

reduced CB1 receptor expression [26, 27, 37]. These alterations occurred without concurrent changes in 2-AG or arachidonic acid concentration. Notably, the modest decrease in CB1 receptor expression was paralleled by a significant and selective attenuation of acute ethanol inhibition of evoked excitatory glutamatergic transmission measured with both ensemble EPSP responses and whole-cell synaptic responses. Specifically, CIE exposure selectively reduced CB1-modulation of these glutamatergic terminals with no concurrent change in apparent CB1 modulation of evoked release at local interneuron GABAergic synapses. Overall, these data suggest a dynamic regulation of eCB in the BLA by both acute and chronic ethanol exposure within this model that may contribute to ethanol modulation of anxiety-like behaviors. The relatively prolonged period of acute ethanol application (20–30 minute) required to consistently observe significant inhibition of excitatory transmission at the internal capsule input suggests of a lack of direct interaction between ethanol and the CB1 receptor or the immediate, downstream targets of CB1 activation. We and others have shown that acute ethanol can facilitate BLA GABA release within 10 minutes of the initial application [3, 47, 48]. Given the more prolonged time course, acute ethanol likely produces an indirect activation of CB1 through increasing the synaptic eCB concentration. However, this interpretation must be taken with some caution. Numerous variables, including receptor desensitization/internalization or insertion, may impact the timecourse and relative size of treatment effects observed during drug superfusion within *ex vivo* electrophysiological recordings. Further experiments are needed to more extensively evaluate this hypothesis. With these limitations in mind, a number of observations support the proposed indirect activation of CB1 by ethanol. First, acute ethanol can facilitate eCB levels both *in vitro* [34] and *in vivo* [22, 23]. Notably, acute ethanol also inhibits BLA glutamatergic projections to the NAcc and cortico-striatal excitatory transmission via a similarly proposed mechanism [49, 50]. The time course required to observe acute ethanol inhibition of excitatory synapses in our study is also similar to that needed to inhibit presynaptic glutamate release by postsynaptic loading with AEA or application of a FAAH inhibitor during room temperature *ex vivo* recordings [38, 51]. Given the selective impact of chronic ethanol exposure on AEA levels reported here, we initially hypothesized that acute ethanol might suppress excitatory transmission by inhibiting AEA degradation. Contrary to these expectations, a FAAH-selective inhibitor did not alter the modulation by acute ethanol. This may indicate acute ethanol specifically increases 2-AG synthesis/release at these terminals similar to selective effects of ethanol on this ligand observed by *in vivo* microdialysis [23]. Alternatively, similar to observations made in the hippocampus or cerebellum, acute ethanol may increase BLA AEA concentration by increasing release and/or suppressing reuptake/degradation [33, 34]. However, the numerous pathways for AEA synthesis and paucity of selective ligands occluded our ability to directly test the effects of inhibiting AEA production within field recordings. It is noteworthy that our recordings were performed at room temperature since these conditions would tend to produce modest responses by synthetic pathways in general. Further studies will be required to fully elucidate the relative contributions of AEA and 2-AG synthesis, release, and degradation in response acute ethanol.

In good keeping with increased eCB concentrations found following multiple different chronic ethanol exposure paradigms [26–33], CIE exposure selectively elevated AEA within the BLA without altering levels of the primary anabolic/catabolic enzymes. However, AEA

levels do not strictly correlate with NAPE-PLD protein levels. For example, AEA levels are similar between wildtype and NAPE-PLD knockout mice [52]. Thus, we cannot exclude the possibility CIE exposure may facilitate one or more of the established AEA synthetic pathways, including phospholipase A2 (PLA2) [29]. It is likewise possible that significant reductions in BLA FAAH enzymatic activity could occur independently from changes in total FAAH protein levels [53]. Thus, CIE exposure may increase AEA concentration through dysregulation of production, re-uptake, degradation, or a combination of all these mechanisms [33].

The CB1 receptor plays a critical role in modulating the balance between excitatory and inhibitory transmission [14]. The selective functional impairment of CB1 located on glutamatergic terminals following CIE exposure in our study may therefore have profound implications for the regulation of BLA activity during excessive ethanol use. Differential alterations to distinct CB1 populations have also been observed in the hippocampus and striatum [54, 55]; but the underlying mechanisms involved in these selective impairments remain an area of intense investigation and are potentially region-dependent. One contributing factor may be a preferential down-regulation of 'CB1 receptor populations more sensitive to low levels of agonist following prolonged activation. In support of this, a recent evaluation of mutant mouse lines which possess CB1 conditional knock-out at either glutamatergic or GABAergic synapses found that the anxiolytic properties of a low-dose, exogenous CB1 agonist were specifically mediated by activation of glutamatergic-CB1 receptors while the anxiogenic effects apparent at higher agonist concentrations occurred via CB1 localized on GABAergic synapses [56]. These results suggest these distinct CB1 populations may be differentially sensitive to prolonged agonist exposure. Of further note, enhancing 2-AG concentration in hippocampal principal neurons was recently shown to specifically alter GABAergic signaling at perisomatic interneuron synapses while elevation of AEA concentration through FAAH inhibition had no CB1-mediated effect at these same synapses [57]. Similar CB1-mediated tonic inhibition of GABAergic transmission has been previously demonstrated within the amygdala, however any potential differences in CB1-tonic regulation of GABA and glutamate transmission were not directly evaluated in this present work [58]. Together, these data suggest that differential sensitivity of distinct synaptic CB1 receptor populations, specifically to CIE-induced increases in AEA, may have significantly contributed to the preferential down-regulation of CB1 receptor function expressed by glutamatergic BLA afferents. Alternatively, the relative distribution and unique efficacy of CB1 receptors localized to glutamatergic and GABAergic synapses could also confer a unique sensitivity to chronic ethanol. CB1 receptors on glutamatergic synapses compose approximately 25% of total CB1 expression in most cortical regions, yet these receptors control almost 50% of the total cannabinoid-dependent GTPgammaS binding [59]. Thus, relatively small reductions in CB1 expression at glutamatergic terminals might have a much more dramatic impact on receptor function relative to GABAergic terminals which express relatively high levels of the receptor. A more prolonged CIE exposure may be potentially required before reductions in GABAergic CB1 expression sufficient to impair CB1 function of this terminal population occur [60].

It is important to note WIN 55,212-2 acts as a non-specific CB1/2 agonist and a potential influence of CB2 function was not directly evaluated within this work. Traditionally, CB2

signaling within the CNS is primarily associated with immune function (for review see [61]), but recent work suggests CB2 may participate in some ethanol-related behaviors. For instance, CB2 influences ethanol preference and consumption, but is not involved in cannabinoid modulation of ischemia-induced hippocampal glutamate release following chronic ethanol exposure [62–64]. In this present work, the occlusion of acute ethanol-induced effects on fEPSP peak amplitude by the highly selective-CB1 antagonist AM251 suggests a predominate role of CB1. Further, WIN 55, 212-2 inhibition of amygdalar GABAergic transmission is attributed entirely to CB1-mediated effects [58]. However, potential chronic ethanol-induced alterations CB2 in addition to CB1 receptor function at glutamatergic synapses cannot be excluded. The potential role of CB2 in the acute and chronic effects of ethanol serves as an important area of future investigation.

Work from our lab has previously demonstrated that CIE dramatically enhances presynaptic function at the internal capsule-BLA input [2, 5], producing a presynaptically-expressed LTP-like state. Functional impairments in homeostatic mechanisms regulating glutamate release at these synapses, like the CB1 receptor, may critically contribute to the induction of this ethanol-induced plasticity [42]. This hypothesis is consistent with work showing that manipulation of eCB signaling at excitatory synapses in the hippocampus dramatically modulates LTP induction [39]. Recent studies also demonstrate that knockout of CB1 specifically localized to glutamatergic terminals facilitates LTP induction at excitatory synapses while deletion of GABAergic CB1 serves to impair induction of this LTP [42]. This if note as the internal capsule BLA CB1 receptor population specifically restricts LTP induction during associative plasticity following coincident activation of both internal and external capsule inputs into the BLA [41]. Thus, the functional impairment of CB1 receptors on BLA glutamatergic terminals by chronic ethanol may ultimately help facilitate the development of ethanol-induced plasticity at these synapses and so contribute to the development of withdrawal-induced angiogenesis [2, 5].

In summary, we have demonstrated that within a rodent model ethanol and the eCB system interact at internal capsule input terminals of the BLA during both acute and chronic ethanol exposure. These interactions may have implications for the development and expression of withdrawal-induced increases in anxiety-like behavior. Of note, previous work has demonstrated CIE-induced alterations to the eCB system persist into protracted withdrawal in both rodent and human models [35, 65]. The eCB system may therefore be poised to serve as an important target for further investigation.

Acknowledgments

This work is funded by National Institutes of Health grants T32 AA007565 (BAM & SLR), F31 MH106192 (RJB), R21 MH103515 (SP), R01 MH100096 (SP), and R01 AA014445 (BAM).

References

1. McCool BA, Christian DT, Diaz MR, Läck AK. Glutamate plasticity in the drunken amygdala: the making of an anxious synapse. *Int Rev Neurobiol.* 2010; 91:205–33. [PubMed: 20813244]
2. Läck AK, Diaz MR, Chappell A, DuBois DW, McCool BA. Chronic ethanol and withdrawal differentially modulate pre- and postsynaptic function at glutamatergic synapses in rat basolateral amygdala. *J Neurophysiol.* Dec; 2007 98(6):3185–96. [PubMed: 17898152]

3. Diaz MR, Christian DT, Anderson NJ, McCool BA. Chronic ethanol and withdrawal differentially modulate lateral/basolateral amygdala paracapsular and local GABAergic synapses. *J Pharmacol Exp Ther.* Apr; 2011 337(1):162–70. [PubMed: 21209156]
4. Christian DT, Alexander NJ, Diaz MR, Robinson S, McCool BA. Chronic intermittent ethanol and withdrawal differentially modulate basolateral amygdala AMPA-type glutamate receptor function and trafficking. *Neuropharmacology.* Jun; 2012 62(7):2430–9. [PubMed: 22387532]
5. Christian DT, Alexander NJ, Diaz MR, McCool BA. Thalamic glutamatergic afferents into the rat basolateral amygdala exhibit increased presynaptic glutamate function following withdrawal from chronic intermittent ethanol. *Neuropharmacology.* Feb.2013 65:134–42. [PubMed: 22982568]
6. Häring M, Guggenhuber S, Lutz B. Neuronal populations mediating the effects of endocannabinoids on stress and emotionality. *Neuroscience.* Mar.2012 204:145–58. [PubMed: 22233782]
7. Pava MJ, Woodward JJ. A review of the interactions between alcohol and the endocannabinoid system: implications for alcohol dependence and future directions for research. *Alcohol.* May; 2012 46(3):185–204. [PubMed: 22459871]
8. Vinod KY, Hungund BL. Endocannabinoid lipids and mediated system: implications for alcoholism and neuropsychiatric disorders. *Life Sci.* Aug; 2005 77(14):1569–83. [PubMed: 16005471]
9. Basavarajappa BS. The endocannabinoid signaling system: a potential target for next-generation therapeutics for alcoholism. *Mini Rev Med Chem.* Aug; 2007 7(8):769–79. [PubMed: 17692039]
10. Okamoto Y, Wang J, Morishita J, Ueda N. Biosynthetic pathways of the endocannabinoid anandamide. *Chem Biodivers.* Aug; 2007 4(8):1842–57. [PubMed: 17712822]
11. Egertová M, Cravatt BF, Elphick MR. Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. *Neuroscience.* 2003; 119(2): 481–96. [PubMed: 12770562]
12. Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF. Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci.* Jul; 2004 20(2):441–58. [PubMed: 15233753]
13. Ueda N, Tsuboi K, Uyama T, Ohnishi T. Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. *Biofactors.* Jan-Feb;2011 37(1):1–7. [PubMed: 21328621]
14. Azad SC, Eder M, Marsicano G, Lutz B, Zieglgänsberger W, Rammes G. Activation of the cannabinoid receptor type 1 decreases glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. *Learn Mem.* Mar-Apr;2003 10(2):116–28. [PubMed: 12663750]
15. Newman LM, Lutz MP, Gould MH, Domino EF. 9-Tetrahydrocannabinol and ethyl alcohol: evidence for cross-tolerance in the rat. *Science.* Mar; 1972 175(4025):1022–3. [PubMed: 5061865]
16. Sprague GL, Craigmill AL. Ethanol and delta-9-tetrahydrocannabinol: mechanism for cross-tolerance in mice. *Pharmacol Biochem Behav.* Oct; 1976 5(4):409–15. [PubMed: 1005489]
17. Pava MJ, Blake EM, Green ST, Mizroch BJ, Mulholland PJ, Woodward JJ. Tolerance to cannabinoid-induced behaviors in mice treated chronically with ethanol. *Psychopharmacology (Berl).* Jan; 2012 219(1):137–47. [PubMed: 21701813]
18. Ortiz S, Oliva JM, Pérez-Rial S, Palomo T, Manzanares J. Differences in basal cannabinoid CB1 receptor function in selective brain areas and vulnerability to voluntary alcohol consumption in Fawn Hooded and Wistar rats. *Alcohol Alcohol.* Jul-Aug;2004 39(4):297–302. [PubMed: 15208160]
19. Vinod KY, Maccioni P, Garcia-Gutierrez MS, Femenia T, Xie S, Carai MA, Manzanares J, Cooper TB, Hungund BL, Colombo G. Innate difference in the endocannabinoid signaling and its modulation by alcohol consumption in alcohol-preferring sP rats. *Addict Biol.* Jan; 2012 17(1):62–75. [PubMed: 21309960]
20. Vinod KY, Yalamanchili R, Thanos PK, Vadasz C, Cooper TB, Volkow ND, Hungund BL. Genetic and pharmacological manipulations of the CB(1) receptor alter ethanol preference and dependence in ethanol preferring and nonpreferring mice. *Synapse.* Aug; 2008 62(8):574–81. [PubMed: 18509854]

21. Hungund BL, Basavarajappa BS. Distinct differences in the cannabinoid receptor binding in the brain of C57BL/6 and DBA/2 mice, selected for their differences in voluntary ethanol consumption. *J Neurosci Res.* Apr; 2000 60(1):122–8. [PubMed: 10723075]
22. Ceccarini J, Casteels C, Koole M, Bormans G, Van Laere K. Transient changes in the endocannabinoid system after acute and chronic ethanol exposure and abstinence in the rat: a combined PET and microdialysis study. *Eur J Nucl Med Mol Imaging.* Oct; 2013 40(10):1582–94. [PubMed: 23740372]
23. Caillé S, Alvarez-Jaimes L, Polis I, Stouffer DG, Parsons LH. Specific alterations of extracellular endocannabinoid levels in the nucleus accumbens by ethanol, heroin, and cocaine self-administration. *J Neurosci.* Apr; 2007 27(14):3695–702. [PubMed: 17409233]
24. Ferrer B, Bermúdez-Silva FJ, Bilbao A, Alvarez-Jaimes L, Sanchez-Vera I, Giuffrida A, Serrano A, Baixeras E, Khaturia S, Navarro M, Parsons LH, Piomelli D, Rodriguez de Fonseca F. Regulation of brain anandamide by acute administration of ethanol. *Biochem J.* May; 2007 404(1):97–104. [PubMed: 17302558]
25. Rubio M, McHugh D, Fernández-Ruiz J, Bradshaw H, Walker JM. Short-term exposure to alcohol in rats affects brain levels of anandamide, other N-acyl ethanolamines and 2-arachidonoyl-glycerol. *Neurosci Lett.* Jun; 2007 421(3):270–4. [PubMed: 17574742]
26. González S, Valenti M, de Miguel R, Fezza F, Fernández-Ruiz J, Di Marzo V, Ramos JA. Changes in endocannabinoid contents in reward-related brain regions of alcohol-exposed rats, and their possible relevance to alcohol relapse. *Br J Pharmacol.* Oct; 2004 143(4):455–64. [PubMed: 15371286]
27. Vinod KY, Yalamanchili R, Xie S, Cooper TB, Hungund BL. Effect of chronic ethanol exposure and its withdrawal on the endocannabinoid system. *Neurochem Int.* Nov; 2006 49(6):619–25. [PubMed: 16822589]
28. Basavarajappa BS, Cooper TB, Hungund BL. Chronic ethanol administration down-regulates cannabinoid receptors in mouse brain synaptic plasma membrane. *Brain Res.* May; 1998 793(1–2):212–8. [PubMed: 9630633]
29. Basavarajappa BS, Cooper TB, Hungund BL. Effect of chronic ethanol exposure on mouse brain arachidonic acid specific phospholipase A2. *Biochem Pharmacol.* Feb; 1998 55(4):515–21. [PubMed: 9514087]
30. Basavarajappa BS, Hungund BL. Chronic ethanol increases the cannabinoid receptor agonist anandamide and its precursor N-arachidonoylphosphatidylethanolamine in SK-N-SH cells. *J Neurochem.* Feb; 1999 72(2):522–8. [PubMed: 9930723]
31. Basavarajappa BS, Hungund BL. Down-regulation of cannabinoid receptor agonist-stimulated [35S]GTP gamma S binding in synaptic plasma membrane from chronic ethanol exposed mouse. *Brain Res.* Jan; 1999 815(1):89–97. [PubMed: 9974126]
32. Basavarajappa BS, Saito M, Cooper TB, Hungund BL. Stimulation of cannabinoid receptor agonist 2-arachidonoylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. *Biochim Biophys Acta.* Dec; 2000 1535(1):78–86. [PubMed: 11113634]
33. Basavarajappa BS, Saito M, Cooper TB, Hungund BL. Chronic ethanol inhibits the anandamide transport and increases extracellular anandamide levels in cerebellar granule neurons. *Eur J Pharmacol.* Apr; 2003 466(1–2):73–83. [PubMed: 12679143]
34. Basavarajappa BS, Ninan I, Arancio O. Acute ethanol suppresses glutamatergic neurotransmission through endocannabinoids in hippocampal neurons. *J Neurochem.* Nov; 2008 107(4):1001–13. [PubMed: 18796007]
35. Ceccarini J, Hompes T, Verhaeghen A, Casteels C, Peuskens H, Bormans G, Claes S, Van Laere K. Changes in cerebral CB1 receptor availability after acute and chronic alcohol abuse and monitored abstinence. *J Neurosci.* Feb; 2014 34(8):2822–31. [PubMed: 24553924]
36. Vinod KY, Kassir SA, Hungund BL, Cooper TB, Mann JJ, Arango V. Selective alterations of the CB1 receptors and the fatty acid amide hydrolase in the ventral striatum of alcoholics and suicides. *J Psychiatr Res.* Jul; 2010 44(9):591–7. [PubMed: 20015515]
37. Rubio M, Fernández-Ruiz J, de Miguel R, Maestro B, Michael Walker J, Ramos JA. CB1 receptor blockade reduces the anxiogenic-like response and ameliorates the neurochemical imbalances

- associated with alcohol withdrawal in rats. *Neuropharmacology*. May; 2008 54(6):976–88. [PubMed: 18371990]
38. Gunduz-Cinar O, MacPherson KP, Cinar R, Gamble-George J, Sugden K, Williams B, Godlewski G, Ramikie TS, Gorka AX, Alapafuja SO, Nikas SP, Makriyannis A, Poulton R, Patel S, Hariri AR, Caspi A, Moffitt TE, Kunos G, Holmes A. Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. *Mol Psychiatry*. Jul; 2013 18(7):813–23. [PubMed: 22688188]
 39. Basavarajappa BS, Nagre NN, Xie S, Subbanna S. Elevation of endogenous anandamide impairs LTP, learning, and memory through CB1 receptor signaling in mice. *Hippocampus*. Jul; 2014 24(7):808–18. [PubMed: 24648181]
 40. Shin RM, Tully K, Li Y, Cho JH, Higuchi M, Suhara T, Bolshakov VY. Hierarchical order of coexisting pre- and postsynaptic forms of long-term potentiation at synapses in amygdala. *Proc Natl Acad Sci U S A*. Nov; 2010 107(44):19073–8. [PubMed: 20956319]
 41. Fonseca R. Asymmetrical synaptic cooperation between cortical and thalamic inputs to the amygdala. *Neuropsychopharmacology*. Dec; 2013 38(13):2675–87. [PubMed: 23884343]
 42. Monory K, Polack M, Remus A, Lutz B, Korte M. Cannabinoid CB1 receptor calibrates excitatory synaptic balance in the mouse hippocampus. *J Neurosci*. Mar; 2015 35(9):3842–50. [PubMed: 25740514]
 43. Marsicano G, Lutz B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci*. Dec; 1999 11(12):4213–25. [PubMed: 10594647]
 44. Vendruscolo LF, Roberts AJ. Operant alcohol self-administration in dependent rats: focus on the vapor model. *Alcohol*. May; 2014 48(3):277–86. [PubMed: 24290310]
 45. Washburn MS, Moises HC. Electrophysiological and morphological properties of rat basolateral amygdaloid neurons in vitro. *J Neurosci*. Oct; 1992 12(10):4066–79. [PubMed: 1403101]
 46. Shonesy BC, Bluett RJ, Ramikie TS, Báldi R, Hermanson DJ, Kingsley PJ, Marnett LJ, Winder DG, Colbran RJ, Patel S. Genetic disruption of 2-arachidonoylglycerol synthesis reveals a key role for endocannabinoid signaling in anxiety modulation. *Cell Rep*. Dec; 2014 9(5):1644–53. [PubMed: 25466252]
 47. Silberman Y, Ariwodola OJ, Weiner JL. β 1-adrenoceptor activation is required for ethanol enhancement of lateral paracapsular GABAergic synapses in the rat basolateral amygdala. *J Pharmacol Exp Ther*. Nov; 2012 343(2):451–9. [PubMed: 22904357]
 48. Weiner JL, Valenzuela CF. Ethanol modulation of GABAergic transmission: the view from the slice. *Pharmacol Ther*. Sep; 2006 111(3):533–54. [PubMed: 16427127]
 49. Perra S, Pillolla G, Luchicchi A, Pistis M. Alcohol inhibits spontaneous activity of basolateral amygdala projection neurons in the rat: involvement of the endocannabinoid system. *Alcohol Clin Exp Res*. Mar; 2008 32(3):443–9. [PubMed: 18215217]
 50. Cho HS, Jeun SH, Li QZ, Kim KJ, Choi SJ, Sung KW. Involvement of the endocannabinoid system in ethanol-induced corticostriatal synaptic depression. *J Pharmacol Sci*. 2012; 120(1):45–9. [PubMed: 22971846]
 51. Adermark L, Lovinger DM. Retrograde endocannabinoid signaling at striatal synapses requires a regulated postsynaptic release step. *Proc Natl Acad Sci U S A*. Dec; 2007 104(51):20564–9. [PubMed: 18077376]
 52. Leung D, Saghatelian A, Simon GM, Cravatt BF. Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry*. Apr; 2006 45(15):4720–6. [PubMed: 16605240]
 53. Gray JM, Vecchiarelli HA, Morena M, Lee TT, Hermanson DJ, Kim AB, McLaughlin RJ, Hassan KI, Kühne C, Wotjak CT, Deussing JM, Patel S, Hill MN. Corticotropin-releasing hormone drives anandamide hydrolysis in the amygdala to promote anxiety. *J Neurosci*. Mar; 2015 35(9):3879–92. [PubMed: 25740517]
 54. Adermark L, Jonsson S, Ericson M, Söderpalm B. Intermittent ethanol consumption depresses endocannabinoid-signaling in the dorsolateral striatum of rat. *Neuropharmacology*. Dec; 2011 61(7):1160–5. [PubMed: 21251919]

55. Reich CG, Mihalik GR, Iskander AN, Seckler JC, Weiss MS. Adolescent chronic mild stress alters hippocampal CB1 receptor-mediated excitatory neurotransmission and plasticity. *Neuroscience*. Dec.2013 253:444–54. [PubMed: 24035826]
56. Rey AA, Purrio M, Viveros MP, Lutz B. Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA(B) receptors in the balance of GABAergic and glutamatergic neurotransmission. *Neuropsychopharmacology*. Nov; 2012 37(12):2624–34. [PubMed: 22850737]
57. Lee SH, Ledri M, Tóth B, Marchionni I, Henstridge CM, Dudok B, Kenesei K, Barna L, Szabó SI, Renkecz T, Oberoi M, Watanabe M, Limoli CL, Horvai G, Soltesz I, Katona I. Multiple Forms of Endocannabinoid and Endovanilloid Signaling Regulate the Tonic Control of GABA Release. *J Neurosci*. Jul; 2015 35(27):10039–57. [PubMed: 26157003]
58. Zhu PJ, Lovinger DM. Retrograde endocannabinoid signaling in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala. *J Neurosci*. Jun; 2005 25(26):6199–207. [PubMed: 15987949]
59. Steindel F, Lerner R, Häring M, Ruehle S, Marsicano G, Lutz B, Monory K. Neuron-type specific cannabinoid-mediated G protein signalling in mouse hippocampus. *J Neurochem*. Mar; 2013 124(6):795–807. [PubMed: 23289830]
60. Varodayan FP, Soni N, Bajo M, Luu G, Madamba SG, Schweitzer P, Parsons LH, Roberto M. Chronic ethanol exposure decreases CB1 receptor function at GABAergic synapses in the rat central amygdala. *Addict Biol*. May.2015
61. Cabral GA, Raborn ES, Griffin L, Dennis J, Marciano-Cabral F. CB2 receptors in the brain: role in central immune function. *Br J Pharmacol*. Jan; 2008 153(2):240–51. [PubMed: 18037916]
62. Ishiguro H, Iwasaki S, Teasent L, Higuchi S, Horiuchi Y, Saito T, Arinami T, Onaivi ES. Involvement of cannabinoid CB2 receptor in alcohol preference in mice and alcoholism in humans. *Pharmacogenomics J*. Dec; 2007 7(6):380–5. [PubMed: 17189959]
63. Ortega-Álvarez A, Ternianov A, Aracil-Fernández A, Navarrete F, García-Gutiérrez MS, Manzanares J. Role of cannabinoid CB2 receptor in the reinforcing actions of ethanol. *Addict Biol*. Jan; 2015 20(1):43–55. [PubMed: 23855434]
64. Zheng L, Wu X, Dong X, Ding X, Song C. Effects of Chronic Alcohol Exposure on the Modulation of Ischemia-Induced Glutamate Release via Cannabinoid Receptors in the Dorsal Hippocampus. *Alcohol Clin Exp Res*. Oct; 2015 39(10):1908–16. [PubMed: 26343919]
65. Mitrirattanakul S, López-Valdés HE, Liang J, Matsuka Y, Mackie K, Faull KF, Spigelman I. Bidirectional alterations of hippocampal cannabinoid 1 receptors and their endogenous ligands in a rat model of alcohol withdrawal and dependence. *Alcohol Clin Exp Res*. May; 2007 31(5):855–67. [PubMed: 17386072]

Highlights

- Acute ethanol inhibits amygdala extracellular synaptic responses (fEPSPs)
- Acute ethanol inhibition of fEPSPs is blocked by CB1 receptor antagonists
- Chronic ethanol disrupts WIN 515,212-2 modulation of whole-cell EPSCs but not IPSCs
- Chronic ethanol up-regulates anandamide levels and down-regulates CB1 protein levels

Figure 1.

Acute ethanol dose-dependently inhibits EPSP amplitude in response to excitatory internal capsule afferent activation in the rat basolateral amygdala. A) Time course of the impact of continuous aCSF (n = 16), 20mM (n = 5), 40mM (n = 9), and 80mM (n = 6) ethanol application on fEPSP peak amplitude in drug naïve animals. B) Traces are averages of 10 minute baseline and final two minutes of treatment and illustrate significant ethanol inhibition at 40mM and 80mM doses, with no impact of continued aCSF or 20mM application. C) Dose response curve representation of percent inhibition induced by each treatment. D) 80mM ethanol increases PPR during the final five minutes of application

compared to baseline, indicating a presynaptic mechanism of inhibiting excitatory transmission ($n = 14$). Individual points represent PPR from single cells at baseline and following acute ethanol (connected by lines). E) Traces are averages of 5 minutes baseline and the final five minutes of drug application recorded from BLA pyramidal neurons. * $p < 0.05$, ** $p < 0.01$, Dunnett's Multiple Comparison Test; ### $p < 0.001$, paired t-test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

Acute ethanol inhibits excitatory internal capsule afferent terminals to rat basolateral amygdala principal neurons via the CB1 cannabinoid receptor. A) Time course of 80mM ethanol impact on fEPSP peak amplitude applied alone ($n = 7$) or with CB1 antagonist SR14176a ($5\mu\text{M}$, $n = 6$) or AM251 ($1\mu\text{M}$, $n = 9$). B) Traces are averages of 10 minute baseline and final two minutes of treatment. C) Bar graph representation of the percent inhibition in fEPSP peak amplitude during the final two minutes of 80mM ethanol treatment following aCSF or CB1 antagonist co-application. * $p < 0.05$, Dunnett's Multiple Comparison Test.

Figure 3.

Acute ethanol inhibition of excitatory internal capsule afferent terminals to rat basolateral amygdala is attenuated following chronic ethanol exposure. A) Time course of 80mM ethanol impact on fEPSP peak amplitude applied to drug naive control (n = 9) or chronic intermittent ethanol vapor exposed slices (n = 12). B) Bar graph representation of the percent inhibition in fEPSP peak amplitude during the final two minutes of ethanol treatment illustrating a difference in inhibition induced by 80mM ethanol between groups. C) Traces are averages of 10 minute baseline and final two minutes of treatment. # $p < 0.05$, t-test

Figure 4.

Chronic ethanol exposure selectively impairs CB1 function on glutamatergic terminals in rat basolateral amygdala. A) Bar graph representation that PPR relative to baseline at glutamatergic internal capsule inputs during the final 5 minutes of WIN application is increased in CON, but not CIE cells. B) INSET: Average PPR traces for glutamatergic EPSCs at baseline (black lines) and after WIN application (gray lines) in CON and CIE cells. See text. Calibration bars are provided to denote the relative amplitude size (pA) and response duration (ms). Larger traces represent these same traces normalized for the peak amplitude of second EPSC (dashed lines) to illustrate WIN modulation of PPR. In these traces, vertical lines denote normalization effect on the relative response amplitude after scaling and are the magnitude as the inset. C) Bar graph representation that WIN increases PPR from synapses formed with GABAergic interneurons relative to baseline equally in both CON and CIE cells during the final five minutes of drug application. D) INSET: Average PPR traces for GABAergic IPSCs at baseline (black lines) and following WIN application (gray) in CON and CIE cells. Normalized traces are scaled as illustrated in B to emphasize WIN modulation of PPR. ## $p < 0.01$, t-test.

Figure 5.

Chronic ethanol exposure decreases CB1 protein expression in rat basolateral amygdala. Bar graphs represent relative protein expression levels in control (CON; inset bands on left) and following chronic ethanol exposure (CIE: inset bands on right). Boxes represent approximate regions of the gel analyzed for protein expression level. See methods. A) CB1 protein expression is significantly decreased. In contrast, B) NAPE-PLD, C) FAAH, D) DAGL α , and E) MAGL expression were not significantly affected by CIE. # $p < 0.05$, t-test.

Figure 6.

Chronic ethanol exposure selectively elevates tissue content of the endogenous cannabinoid AEA in rat basolateral amygdala. Bar graph representation that following chronic ethanol exposure. A) 2-AG concentrations within in the basolateral amygdala remain unchanged following chronic ethanol (CIE) relative to control (CON). In contrast, B) AEA concentration is significantly increased following chronic ethanol. # $p < 0.05$, t-test.

Figure 7.

Acute ethanol inhibition of internal capsule afferent terminals to rat basolateral amygdala is not altered by FAAH inhibition. A) Time course of 80mM ethanol impact on fEPSP peak amplitude applied alone ($n = 7$) or following pre-incubation with the selective FAAH inhibitor URB597 ($n = 7$). B) Bar graph representation of the percent inhibition in fEPSP peak amplitude during the final two minutes of treatment illustrating no difference in inhibition induced by 80mM ethanol in the presence or absence of URB597. C) Traces are averages of 10 minute baseline and final two minutes of treatment.