Published in final edited form as:

Addict Biol. 2018 March ; 23(2): 676–688. doi:10.1111/adb.12525.

CB₁ and Ethanol Effects on Glutamatergic Transmission in the Central Amygdala of male and female msP and Wistar Rats

Dean Kirson¹, Christopher Shaun Oleata¹, Loren Howell Parsons^{1,†}, Roberto Ciccocioppo², and Marisa Roberto¹

¹Department of Neuroscience, The Scripps Research Institute

²School of Pharmacy, Pharmacology Unit, University of Camerino

Abstract

The central amygdala (CeA) is involved in the processing of anxiety and stress, and plays a role in ethanol consumption. Chronic ethanol recruits stress systems in the CeA, leading to aversive withdrawal symptoms. Although primarily GABAergic, CeA contains glutamatergic afferents, and we have reported inhibitory effects of ethanol on locally evoked glutamatergic responses in CeA of Wistar and Marchigian Sardinian alcohol-preferring (msP) rats. Notably, msP rats display enhanced anxiety, stress, and alcohol drinking, simulating the alcohol dependent phenotype. Endocannabinoids (eCB) are also involved in regulation of stress, and we previously demonstrated that cannabinoid receptor type 1 (CB₁) activation decreases CeA GABAergic signaling and blocks ethanol enhancement of GABAergic signaling. Here, we sought to investigate the effects of CB₁ activation (WIN 55,212-2; Win) and antagonism (AM251) with and without acute ethanol on glutamatergic synapses in CeA of female and male Wistar and msP rats. Using intracellular sharp pipette electrophysiology, we examined the effects of CB₁ compounds on locally evoked excitatory postsynaptic potentials (EPSPs) in CeA, and compared effects between strains, gender, and estrous cycle. Acute ethanol decreased EPSP amplitudes in Wistars, and in male but not female msPs. Win decreased EPSP amplitudes in msPs, and in male but not female Wistars. Combined application of Win and ethanol resulted in strain specific effects in female rats. We found no tonic CB₁ signaling at glutamatergic synapses in CeA of any groups, and no interaction with ethanol. Collectively, these observations demonstrate sex-strain-specific differences in ethanol and eCB effects on CeA glutamatergic signaling.

Keywords

CeA; CB₁; eCB; ethanol; glutamate

Correspondence to: Marisa Roberto Ph.D., Professor, Department of Neuroscience, The Scripps Research Institute, SP30-1150, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. mroberto@scripps.edu.

[†]Deceased June 21, 2016.

CONTRIBUTIONS

DK, LHP, RC, and MR were responsible for the study concept and design. DK, CSO, and MR contributed to acquisition of the electrophysiology data. DK, CSO, and MR performed data analysis, and DK, LHP, and MR contributed to the interpretation of findings. DK, CSO, RC, and MR wrote and provided critical revision of the manuscript. All authors (LP passed away prior to final approval) critically reviewed content and approved final version for publication.

INTRODUCTION

The central nucleus of the amygdala (CeA) is a brain region involved in the processing of emotional stimuli and behaviors related to anxiety and stress (Koob and Le Moal, 2008). The CeA serves as the major output of the amygdala, receiving innervation from other amygdala nuclei, prefrontal cortex, hypothalamus, and many other brain regions, as well as local intra-amygdalar connections. The CeA also plays an important role in alcohol addiction, as disruptions in CeA stress system functioning can drive alcohol consumption (Koob and Volkow, 2010). Although the CeA is a primarily GABAergic nucleus, it does receive glutamatergic innervation and we have previously reported effects of ethanol on glutamate transmission in the CeA, having an opposing effect to that seen on GABAergic transmission in the CeA (Herman et al., 2016; Roberto et al., 2004a).

The interaction of stress and alcohol are a vital topic of research considering the high coincidence of alcohol use disorders (AUD) and stress/anxiety disorders like post-traumatic stress disorder (PTSD) (Blanco et al., 2013; Debell et al., 2014; Gilpin and Weiner, 2017; Jacobsen et al., 2001; Shorter et al., 2015; Wood et al., 2016). Interestingly, women are more likely to develop stress/anxiety-related disorders, including PTSD (Gilpin and Weiner, 2017; McLean et al., 2011; Olff et al., 2007), while men are more likely to develop AUD (Erol and Karpyak, 2015; Gilpin and Weiner, 2017). However, in patients with co-morbid PTSD and AUD, women more likely to have PTSD precede development of AUD (Sonne et al., 2003). These studies highlight the complex interactions between gender, stress disorders, and alcoholism. In fact, alcoholism can be considered a stress-related disorder as stress systems are disrupted in the transition from casual alcohol use to alcohol dependence (Galesi et al., 2016; Koob, 2013; Seo and Sinha, 2014). Once in this dependent state, hypersensitive stress systems cause negative affective withdrawal symptoms which drives further drinking to alleviate these symptoms (Koob, 2013). Creating genetic rodent models of these behavioral phenotypes allows study of important systems of interest. In this capacity, the Marchigian Sardinian alcohol-preferring (msP) rat strain (Ciccocioppo et al., 2006) displays elevated stress and anxiety responses as well as excessive ethanol intake that mimics changes observed in ethanol dependent rats (Ayanwuyi et al., 2013; Cippitelli et al., 2015), highlighting the use of the msP as a genetic model of alcohol dependence.

Many neuropeptides and neuromodulators can affect stress and anxiety related signaling in the CeA. The endogenous cannabinoids (endocannabinoids; eCBs) are bioactive lipids that have been implicated in regulatory control of stress signaling in the brain (Riebe and Wotjak, 2011; Ruehle et al., 2012). Additionally, eCB signaling in response to stress occurs in a sex-specific manner in some brain regions (Shonesy et al., 2014; Silva et al., 2016; Tabatadze et al., 2015; Zer-Aviv and Akirav, 2016). The two most prevalent eCBs are N-arachidonoyl ethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG), and these eCBs are synthesized as needed, or “on demand”, to act in a retrograde fashion on presynaptic terminals inhibiting neurotransmitter release. The cannabinoid receptor type 1 (CB₁) is a G-protein coupled receptor and is the primary neuronal eCB receptor in the brain, and CB₁ activation modulates glutamatergic transmission by decreasing glutamate release (Gerdeman and Lovinger, 2001; Ramikie et al., 2014).

The CB₁ system is also sensitive to the effects of alcohol exposure and withdrawal (Alvarez-Jaimes et al., 2009; Serrano and Parsons, 2011; Serrano et al., 2012). We have previously characterized CB₁ involvement in the effects of acute ethanol on stimulated CeA GABAergic signaling (Roberto et al., 2010). Using an *in vitro* slice preparation, we sought to determine the CB₁ involvement in stimulated CeA glutamatergic signaling in both male and female msP and Wistar (the background strain of msP) rats, using the agonist Win 55,212-2 (Win) and the antagonist AM251, and any possible interactions with acute ethanol application.

MATERIALS AND METHODS

Animals

This study used 15 male and 35 female msP rats and 21 male and 12 female Wistar rats. Average weights were 269.4 ± 11.4 grams for male rats and 141.7 ± 5.7 grams for female rats. Wistar rats were obtained from Charles River (Raleigh, NC, USA); a colony of msP rats is maintained at The Scripps Research Institute and were originally obtained from the School of Pharmacy at the University of Camerino (Italy). For all female rats, estrous cycle was not selected for, and was determined via vaginal cytology from smears obtained under anesthesia the day of recording, to avoid stress axis activation in msP rats. Rats were determined to be within one of three estrous cycle groups, proestrus, estrus, or diestrus (metestrus and diestrus). Additionally, one randomization control group for estrous cycle of female msPs was utilized for some experiments; these rats did not have vaginal cytology determined. All rats were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle (lights off at 1 PM) with food and water available *ad libitum*. All procedures, care, and msP colony breeding were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The Scripps Research Institute.

Electrophysiology

We prepared slices from rats anesthetized with isoflurane (3–5%) followed by rapid decapitation and removal of the brain immediately into an ice-cold high-sucrose cutting solution (sucrose 206 mM; KCl 2.5 mM; CaCl₂ 0.5 mM; MgCl₂ 7 mM; NaH₂PO₄ 1.2 mM; NaHCO₃ 26 mM; glucose 5 mM; HEPES 5 mM; pH 7.4). Coronal slices 400 μ m thick were cut on a Leica 1200S vibratome (Buffalo Grove, IL, USA), incubated in an interface configuration for 17 min, and then completely submerged and continuously superfused (flow rate of 2–4 ml/min) with 95% O₂/5% CO₂ equilibrated room temperature artificial cerebrospinal fluid (aCSF) solution (NaCl 130 mM; KCl 3.5 mM; NaH₂PO₄ 1.25 mM; MgSO₄·7H₂O 1.5 mM; CaCl₂ 2.0 mM; NaHCO₃ 24 mM; glucose 10 mM). All recordings were performed between 1 – 8 hours after slice preparation. We blindly advanced sharp micropipettes filled with 3 M KCl into the medial subdivision of the CeA (Sah et al., 2003), and recorded from all cells in current-clamp mode with neurons held near their resting membrane potential (RMP). Data were acquired with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) and stored for later analysis using pClamp software (Molecular Devices, Sunnyvale, CA, USA). We evoked pharmacologically isolated

compound (AMPA + NMDA) glutamatergic excitatory postsynaptic potentials (EPSPs) by stimulating locally within the CeA through a bipolar stimulating electrode and superfusing the slice with aCSF containing the GABA receptor blockers bicuculline (30 μ M) and CGP 55845A (1 μ M).

In order to determine the synaptic response parameters for each cell, we performed an input–output (I/O) protocol consisting of a range of five current stimulations, starting at the threshold current required to elicit an EPSP, up to the strength required to elicit the maximum amplitude. These stimulus strengths were maintained throughout the duration of the experiment. We examined paired pulse ratio (PPR), whereby two stimuli of the same intensity are applied at inter-stimulus intervals (ISI) of 50, 100 and 200 msec. (Andreasen and Hablitz, 1994; Logrip et al., 2016; Manabe et al., 1993). PPR is calculated as the amplitude of the second EPSP over that of the first EPSP. The stimulus strength was adjusted such that the amplitude of the first EPSP was ~50% of the maximal amplitude determined by the I/O protocol. A drug-induced change in PPR reflects presynaptic effects such that an increase in PPR suggests a decrease in neurotransmitter (glutamate) release. All measures were performed prior to (baseline) and during drug application.

Drugs

We purchased CGP 55845A and bicuculline from Sigma (St. Louis, MO, USA). Ethanol (EtOH) was purchased from Remet (La Mirada, CA, USA). WIN 55,212-2 mesylate (Win; [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone, monomethanesulfonate) and AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1–2,4-dichloro phenyl)-4-methyl-1H-pyrazole-3-carboxamide) were purchased from Tocris (Ellisville, MO, USA). Bicuculline, Win, and AM251 were first dissolved in dimethylsulfoxide before being added to aCSF superfusate (final concentration of 0.05 – 0.1% DMSO). Win and AM251 were applied only once per CeA slice. Drugs were added to the aCSF from stock solutions to obtain known concentrations in the superfusate.

Data Analysis and Statistics

Data were analyzed with Clampfit 10 (Molecular Devices, Sunnyvale, CA, USA), and GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. Sex differences were analyzed in two ways: First, all males and all females of each strain were compared (M vs F), then females were subdivided into estrous cycle groups (Cycle). Because estrous cycle was not selected for, not all groups are represented in each experiment. M vs F analyses include all cells, while Cycle analyses only include groups as stated. EPSPs were analyzed with t-test for individual comparisons or ANOVA for sex and strain comparisons with a Bonferroni *post hoc* test for individual differences as appropriate. In all cases, $p < 0.05$ was the criterion for statistical significance. Data are presented as mean $\pm$ SEM and n refers to the number of cells. Between 1 and 5 cells were recorded per animal, and each drug and gender condition (not estrous cycle groups) contains data from at least 3 animals.

RESULTS

Baseline Sex and Strain Differences in Evoked Glutamatergic Signaling

We recorded locally evoked compound (AMPA + NMDA) glutamatergic EPSPs in medial CeA neurons from both female and male Wistar and msP rats to investigate general strain and sex differences. Average neuronal membrane resistance was 146.8 ± 7.5 and 168.3 ± 8.4 M Ω for Wistar males and females respectively, and 146.1 ± 9.7 and 146.4 ± 6.9 M Ω for msP males and females respectively. RMP was -76.6 ± 0.9 , -80.5 ± 1.2 , -78.8 ± 1.1 , and -79.5 ± 0.7 mV for Wistar males and females and msP males and females respectively. Neither Wistars [$F(1,69) = 0.10$, $n = 37$ cells from 21 males and $n = 34$ cells from 12 females] nor msPs [$F(1,81) = 0.93$, $n = 23$ cells from 15 males and $n = 60$ cells from 35 females] displayed sex differences in baseline I/O curves (Figure 1). Figure 1A shows superimposed EPSP example traces from the five normalized stimulation intensities for both male and female Wistars and msPs. Figure 1B (left panel) shows mean EPSP amplitudes for the five normalized stimulus intensities for neurons from both male and female Wistar rats, with no differences between females and males. Figure 1B (right panel) shows the same relationship between neurons from male and female msP rats.

All following statistics were run on EPSP data from the half-maximal intensity. A two-way ANOVA indicated no significant effect of rat strain [$F(1,150) = 0.94$] or sex [$F(1,150) = 1.22$], and no interaction effect [$F(1,150) = 0.02$] (Figure 1C). We performed further analyses to investigate whether females had different baselines based on estrous cycle that were masked by averaging all females together, thus we also compared CeA EPSP amplitudes from male rats to subdivided groups from the female rats based on estrous cycle. We did not reliably find animals in proestrus, and though we recorded from a couple msP rats in proestrus (none from Wistar females), we did not obtain enough cells in most drug conditions to warrant inclusion of the proestrus data here. Therefore, here (Figure 1D), as in most cases, we examined only diestrus (7.3 ± 0.6 for $n = 20$ cells from 7 Wistars, and 9.1 ± 0.6 mV for $n = 32$ cells from 20 msPs) and estrus (9.4 ± 0.8 for $n = 14$ cells from 5 Wistars, and 8.8 ± 1.0 mV for $n = 16$ cells from 8 msPs). A two-way ANOVA indicated no significant main effect of cycle [$F(2,136) = 1.22$] or strain [$F(1,136) = 0.83$] with no interaction effect [$F(2,136) = 1.30$].

Figure 1E shows representative recordings of paired locally-evoked CeA glutamatergic EPSPs from a diestrus female msP rat at an inter-stimulus interval of 50 ms and an estrus female Wistar rat at inter-stimulus intervals of 50, 100, and 200 ms. PPR for each strain and cycle for the three inter-stimulus intervals can be seen in Figure 1F. By two-way ANOVA, a significant effect of strain was found at the 50 ms inter-stimulus interval [$F(1,127) = 7.085$, $p < 0.01$], with msPs (1.5 ± 0.1 males, 1.4 ± 0.1 females) generally having a higher PPR than Wistars (1.2 ± 0.4 males, 1.2 ± 0.1 females). However, no main effect of sex [$F(1,127) = 0.004$] and no interaction effect [$F(1,127) = 0.05$] was found at the 50 ms inter-stimulus interval. At inter-stimulus intervals of 100 ms (1.3 ± 0.04 and 1.3 ± 0.1 Wistar males and females, 1.3 ± 0.1 and 1.4 ± 0.04 msP males and females, respectively) and 200 ms (1.1 ± 0.04 and 1.1 ± 0.1 Wistar males and females, 1.2 ± 0.1 and 1.1 ± 0.04 msP males and females, respectively), there was no main effect of strain [100 ms $F(1,135) = 2.52$; 200 ms

$F(1,146) = 3.71]$ or cycle [$100\text{ ms } F(1,35) = 0.17$; $200\text{ ms } F(1,146) = 0.78]$, and no interaction effect [$100\text{ ms } F(1,135) = 0.31$; $200\text{ ms } F(1,146) = 0.43]$ by two-way ANOVA.

Acute Ethanol Does Not Decrease Evoked Glutamatergic Signaling in msP Female Rats

We next investigated the effects of acutely applied 44 mM ethanol (Roberto et al., 2004b), in CeA of female Wistar and msP rats and recapitulated a decrease in evoked CeA EPSP amplitudes in male Wistar and msP rats (Figure 2), as we have recently reported (Herman et al., 2016). Figure 2A shows the time course of the effect of ethanol on EPSP amplitudes for Wistar females, with representative traces from before and after drug application for a diestrus Wistar female (inset). After baseline characterization of each cell (Figure 1), application of 44 mM ethanol (10–15 min) significantly decreased EPSP amplitudes to $83.7 \pm 3.6\%$ of baseline (Figure 2B, at the half-maximal intensity) in Wistar males [$t(8) = 6.12$, $p < 0.01$, $n = 9$ cells from 7 rats] and to $84.9 \pm 3.6\%$ of baseline in msP males [$t(6) = 4.22$, $p < 0.01$, $n = 7$ cells from 6 rats]. In Wistar females, ethanol also significantly decreased EPSP amplitudes to $78.8 \pm 6.7\%$ of baseline [$t(8) = 3.14$, $p < 0.05$, $n = 9$ cells from 7 rats]. However, in msP females, ethanol had no significant effect [$t(6) = 1.17$, $n = 7$ cells from 5 rats] on evoked glutamatergic signaling, with EPSP amplitudes essentially unchanged from baseline ($90.9 \pm 7.8\%$). A two-way ANOVA found no main effect of strain [$F(1,28) = 1.34]$ or sex [$F(1,28) = 0.01]$, and no interaction effect [$F(1,28) = 0.89]$.

We again subdivided females by estrous cycle phase for further analysis. In diestrus Wistar females (Figure 2C), ethanol significantly decreased CeA EPSPs to $84.1 \pm 5.2\%$ of baseline [$t(4) = 3.05$, $p < 0.05$, $n = 5$ cells from 3 rats], to a similar extent as Wistar males, while estrus Wistars had a large ($72.2 \pm 14.2\%$) but not significant effect [$t(3) = 1.96$, $n = 4$ cells from 4 rats] of ethanol decreasing EPSP amplitudes. In diestrus msP females (Figure 2D), ethanol had no effect [$t(4) = 0.20$, $n = 5$ cells from 4 rats] on EPSPs ($101.0 \pm 5.2\%$ of baseline). We recorded only 2 cells from estrus msPs, which displayed a non-significant [$t(1) = 3.52$, $n = 2$ cells from 1 rat] decrease ($65.5 \pm 9.8\%$) upon ethanol application (data not shown). A two-way ANOVA found a significant main effect of cycle [$F(2,26) = 4.95$, $p < 0.05]$, but no effect of strain [$F(1,26) = 0.47]$ and no interaction effect [$F(2,26) = 1.50]$. A Bonferroni *post hoc* revealed a significant difference between diestrus and estrus females [$t(26) = 3.11$, $p < 0.05]$.

Acute ethanol treatment did not significantly change PPR from baseline at inter-stimulus intervals of 50 ms ($128.8 \pm 18.3\%$ and $114.8 \pm 9.7\%$ Wistar males and females, $119.3 \pm 14.3\%$ and $93.0 \pm 14.7\%$ msP males and females, respectively), 100 ms ($105.8 \pm 9.1\%$ and $91.4 \pm 8.8\%$ Wistar males and females, $112.9 \pm 19.4\%$ and $96.3\% \pm 6.7\%$ msP males and females, respectively), or 200 ms ($109.2 \pm 13.3\%$ and $93.0 \pm 9.4\%$ Wistar males and females, $111.0 \pm 17.5\%$ and $119.3 \pm 15.3\%$ msP males and females, respectively) for any of the groups tested (data not shown).

CB₁ Agonism Does Not Decrease Evoked Glutamatergic Signaling in Wistar Female Rats

Because of the afore mentioned involvement of amygdalar eCB signaling in stress and anxiety, we next sought to test the effects of CB₁ activation, using the agonist Win, on glutamatergic signaling in the CeA of male and female msP and Wistar rats. After stable

baseline characterization of each cell as above, we applied 2 μ M Win to the superfusate for 15 min. Figure 3A shows representative evoked EPSPs at baseline and during 2 μ M Win in neurons from both a male and a diestrus female Wistar rat. The time course of the effect of Win on EPSP amplitudes in msP females is shown in Figure 3B. In Wistar males, Win induced a significant yet modest decrease in EPSP amplitudes to $88.5 \pm 3.7\%$ of baseline [$t(11) = 3.14$, $p < 0.01$, $n = 12$ cells from 9 rats], though Win had no effect [$t(8) = 0.04$, $n = 9$ cells from 5 rats] on EPSPs from Wistar females ($99.6 \pm 8.9\%$ of baseline). Win caused a significant decrease in EPSPs for both sexes of msPs, with males at $81.1 \pm 6.7\%$ of baseline [$t(7) = 2.84$, $p < 0.05$, $n = 8$ cells from 5 rats] and females at $74.6 \pm 3.2\%$ of baseline [$t(23) = 7.94$, $p < 0.001$, $n = 24$ cells from 17 rats]. A two-way ANOVA found a significant main effect of strain [$F(1,49) = 9.13$, $p < 0.01$] with msPs having a greater Win effect (Figure 3C), but no main effect of sex [$F(1,49) = 0.18$] and no interaction effect [$F(1,49) = 2.70$].

Figures 3D and 3E show the effects of Win on EPSPs when grouped by estrous cycle for Wistars and msPs respectively. Similar to the grouped female data, compared to baseline, Win had no significant effect on either diestrus ($110.4 \pm 13.3\%$ of baseline) [$t(4) = 0.78$, $n = 5$ cells from 3 rats] or estrus ($86.1 \pm 8.6\%$ of baseline) [$t(3) = 1.62$, $n = 4$ cells from 3 rats] Wistar females. Again similar to the grouped female data, Win caused a significant decrease in CeA EPSPs to $77.1 \pm 3.5\%$ of baseline for diestrus [$t(9) = 6.61$, $p < 0.001$, $n = 10$ cells from 9 rats] and $66.5 \pm 9.0\%$ of baseline for estrus [$t(4) = 3.71$, $p < 0.05$, $n = 5$ cells from 3 rats] msP females. In addition, in a control group of msP females that did not have estrous cycle determined, Win significantly decreased [$t(8) = 4.00$, $p < 0.01$, $n = 9$ cells from 5 rats] EPSP amplitudes to $76.2 \pm 5.8\%$ of baseline. A two-way ANOVA of strain and cycle (cycle control excluded) again found a significant main effect of strain [$F(1,38) = 12.69$, $p = 0.001$], but no main effect of cycle [$F(2,38) = 2.77$] and no interaction effect [$F(2,38) = 2.21$].

As with ethanol, Win application did not significantly alter PPR at inter-stimulus intervals of 50 ms ($122.2 \pm 9.9\%$ and $113.5 \pm 20.6\%$ Wistar males and females, $110.7 \pm 5.9\%$ and $100.5 \pm 6.3\%$ msP males and females, respectively) or 200 ms ($108.4 \pm 9.8\%$ and $100.7 \pm 9.3\%$ Wistar males and females, $99.3 \pm 9.0\%$ and $94.9 \pm 4.5\%$ msP males and females, respectively) for any of the groups tested (data not shown). However, at the 100 ms inter-stimulus interval, Win significantly increased PPR to $127.2 \pm 8.7\%$ of baseline in msP males [$t(6) = 3.13$, $p < 0.05$], indicating a presynaptic effect of Win to decrease evoked glutamate release. Win did not significantly alter PPR in Wistars ($99.8 \pm 9.8\%$ and $113.7 \pm 7.2\%$ males and females, respectively) or msP females ($98.6\% \pm 6.5\%$) at the 100 ms inter-stimulus interval (data not shown). A two-way ANOVA of strain [$F(1,40) = 0.49$] and sex [$F(1,40) = 0.70$] found a significant interaction effect [$F(1,40) = 5.8$, $p < 0.05$] at 100 ms inter-stimulus interval; however, a Bonferroni *post hoc* test indicated no significant differences between individual groups.

Win and Ethanol Interact in a Strain Specific Manner in Female Rats

Ethanol has been shown to interact with the eCB system in many different ways, including altering endocannabinoid levels (Alvarez-Jaimes et al., 2009) and altering synaptic transmission (Roberto et al., 2010; Varodayan et al., 2016a; Varodayan et al., 2016b).

Therefore, in a subset (5 of 12) of the cells superfused with Win (Figure 3), we subsequently co-applied 44 mM EtOH in the continued presence of Win (10 – 15 min) to test possible interaction effects of Win and EtOH (Figure 4). In Wistar males, this subset of cells retained the modest decreasing effect of Win but is no longer significantly different from baseline [$t(4) = 1.22$, $n = 5$ cells from 5 rats]. In msP males, all cells tested with Win received subsequent co-application with ethanol. In both Wistar and msP males, the acute ethanol-induced inhibition of glutamate signaling remains in the presence of Win (Figure 4B). Ethanol co-applied with Win significantly decreased EPSPs from the effects of Win alone to $64.0 \pm 6.7\%$ of baseline for Wistars [$t(4) = 9.45$, $p < 0.001$] and $53.9 \pm 4.0\%$ of baseline for msPs [$t(7) = 11.60$, $p < 0.001$]. However, in Wistar females (Figure 4A,B), Win blocks ethanol's effects to where EPSPs in the presence of both drugs were not significantly different [$t(7) = 1.44$, $n = 8$ cells from 5 rats] from baseline at $87.4 \pm 8.7\%$ of baseline. Interestingly, in female msPs (Figure 4A,B), Win induces a significant [$t(14) = 4.94$, $p < 0.001$, $n = 15$ cells from 13 rats] decreasing effect of ethanol on CeA glutamatergic EPSP amplitudes ($54.8 \pm 4.4\%$ of baseline) that was not seen with ethanol alone (Figure 2).

When analyzing the female data grouped by estrous cycle, both diestrus ($89.1 \pm 14.0\%$ of baseline) [$t(4) = 2.12$, $n = 5$ cells from 3 rats] and estrus ($84.7 \pm 7.3\%$ of baseline) [$t(2) = 0.63$, $n = 3$ cells from 2 rats] Wistar females showed the effect of Win blocking the ethanol induced decrease in EPSP amplitudes (Figure 4C). Notably, in the presence of Win, ethanol induced a significant [$t(6) = 3.66$, $p < 0.05$, $n = 7$ cells from 7 rats] reduction in EPSPs in the diestrus ($54.5 \pm 7.2\%$ of baseline) msP females (Figure 4D). In contrast, in msP estrus females, ethanol induced only a modest reduction in the presence of Win, from $66.5 \pm 9.0\%$ to $53.3 \pm 8.0\%$ of baseline [$t(4) = 1.88$, $n = 5$ cells from 3 rats]. The co-application of ethanol and Win only affected PPR for one group, Wistar males at the 200 ms inter-stimulus interval. A one-way RM ANOVA found a significant effect of drug on PPR [$F(2,8) = 5.322$, $p < 0.05$]; however, a Bonferroni *post hoc* test indicated no significant differences between individual groups (data not shown).

Tonic CB₁ Signaling is Lacking at CeA Evoked Glutamatergic Synapses

We previously reported tonic CB₁ signaling at GABAergic synapses in the CeA of male rats susceptible to blockade by the CB₁ specific antagonist AM251 (Varodayan et al., 2016a; Varodayan et al., 2016b). Therefore, we applied AM251 (2 μ M) to slices for 15 min to investigate whether tonic CB₁ signaling occurs at glutamatergic synapses in the CeA of male and female Wistar and msP rats. (Figure 5). Figure 5A shows the time course of the effects of AM251 on EPSP amplitudes in msP females, with representative traces from before and after drug application for a diestrus msP female (inset). We found that in all groups, AM251 had no significant effect on EPSP amplitudes in Wistars (94.3 ± 3.1 and $94.0 \pm 5.5\%$ of baseline for males [$t(8) = 1.85$, $n = 9$ cells from 6 rats] and females [$t(11) = 1.10$, $n = 12$ cells from 5 rats] respectively) or msPs (82.7 ± 7.4 and $99.0 \pm 4.0\%$ of baseline for males [$t(6) = 2.35$, $n = 7$ cells from 3 rats] and females [$t(18) = 0.24$, $n = 19$ cells from 15 rats] respectively), suggesting no tonic CB₁ signaling at glutamatergic synapses in the CeA (Figure 5B). Further analysis of females into sub-groups by estrous cycle (including cycle control females) did not reveal any significant effects of AM251 [Wistar: diestrus $t(5) = 0.83$, $n = 6$ cells from 2 rats; estrus $t(5) = 0.68$, $n = 6$ cells from 3 rats; msP: diestrus $t(12) =$

0.39, $n = 13$ cells from 8 rats; estrus $t(2) = 1.95$, $n = 3$ cells from 3 rats; cycle control $t(2) = 0.03$, $n = 3$ cells from 3 rats] (Figures 5C,D).

Additionally, AM251 treatment resulted in no significant changes in PPR from baseline at inter-stimulus intervals of 50 ms ($141.7 \pm 23.0\%$ and $109.8 \pm 6.0\%$ Wistar males and females, $100.4 \pm 10.4\%$ and $100.0 \pm 8.0\%$ msP males and females, respectively) and 200 ms ($95.7 \pm 9.0\%$ and $113.2 \pm 11.3\%$ Wistar males and females, $103.5 \pm 12.2\%$ and $122.1 \pm 12.3\%$ msP males and females, respectively). A two-way ANOVA of strain and sex found a significant main effect of sex [$F(1,34) = 5.5$, $p < 0.05$] at the 100 ms ($99.2 \pm 9.6\%$ and $118.2 \pm 7.7\%$ Wistar males and females, $93.6 \pm 6.3\%$ and $109.6 \pm 5.2\%$ msP males and females, respectively) inter-stimulus interval with no effect of strain [$F(1,34) = 0.91$] and no interaction effect [$F(1,34) = 0.04$]; however, a Bonferroni *post hoc* test indicated no significant differences between individual groups.

CB1 Antagonism Does Not Alter Acute Ethanol Effects

Finally, we examined whether blockade of CB₁ receptors via AM251 interfered with the effects of acute ethanol. Therefore, in most of the cells where we applied AM251, we subsequently co-applied AM251 and 44 mM ethanol (Figure 6). In all groups tested, combined application of ethanol and AM251 (subsequent to AM251 alone) did not alter the effects of acute ethanol alone seen for each group (Figure 2A,B). Subdividing female data into cycles (Figure 6C,D) did not reveal a change in the lack of interaction between AM251 and ethanol. The co-application of ethanol and AM251 did not alter PPR from baseline at any of the inter-stimulus intervals tested (data not shown).

DISCUSSION

In this study, we have characterized CeA stimulated glutamatergic transmission in a rat genetic model of high stress and anxiety (mimicking the alcohol dependent phenotype) and its background strain, investigated modulation of this signaling by ethanol and cannabinoids, and found both sexually dimorphic and strain specific effects of these drugs in modulating glutamatergic signaling. Because the CeA is heavily involved in both stress responses and the effects of alcohol drinking (Koob and Le Moal, 2008; Koob and Volkow, 2010), understanding neuronal functioning in this area is key to understanding the interplay of stress and alcohol. As previous research has shown sexual dimorphism in the responses to both stress and alcohol (Chester et al., 2008; Logrip et al., 2016; Marco et al., 2017; Varlinskaya et al., 2017), understanding differential CeA glutamatergic signaling and modulation between sexes is integral to developing treatments for these highly comorbid disorders.

We analyzed sex differences by first comparing males to females, then grouping females according to estrous cycle for further comparison. Because we did not select for cycle, the majority of female neurons we recorded from were from rats in diestrus or estrus at the time of sacrifice. The proestrus phase of the estrous cycle is only approximately 12 hours long, and we found few animals in this phase. As we were not able to record from enough CeA neurons in proestrus animals to represent each experimental condition, we could not include the proestrus data in this study. Since diestrus is the phase consistent with the lowest levels

of cycling hormones, we hypothesized that glutamatergic synaptic responses from diestrus animals would be most comparable to those from males, with synaptic responses from estrus animals more likely to differ. However, we found no differences in baseline CeA glutamatergic transmission between males and females of either msP or Wistar rats, regardless of estrous cycle. We found baseline strain differences, regardless of sex, in PPR at 50 ms interstimulus interval, with PPR generally higher in msPs compared to Wistars, which suggests one of a few possible changes in presynaptic calcium functioning in msP terminals at the second stimulation. One is that the calcium influx from the first stimulation is high enough to saturate calcium buffer proteins, resulting in higher levels of calcium during the second stimulation. Another is that calcium influx is greater for the second stimulation due to modulation of calcium channels. The last is that there are separate release mechanisms that respond to the initial high level of calcium influx or to the sustained residual calcium after the first stimulation. Interestingly, synaptotagmin 7 has recently been implicated as a low affinity calcium sensor responsible for short term facilitation at glutamatergic synapses (Jackman et al., 2016). Which of these mechanisms might be responsible for the increased facilitation seen in the msPs warrants further investigation.

Here we recapitulated the ethanol-induced decrease in evoked CeA EPSP amplitudes as previously reported in msP and Wistar males, with no change in PPR, indicating a lack of presynaptic site for the actions of ethanol (Herman et al., 2016). However, we find a strain specific sex difference in the effects of ethanol as Wistar female EPSPs have the same effect as the males, while msP female EPSPs are unaffected by ethanol. Considering estrous cycle for the females, the diestrus msPs are unaffected by ethanol, while the effects of ethanol on estrus Wistar and msP females are less straightforward. In general, estrus females showed a greater (though non-significant) effect of ethanol than diestrus females, suggesting an interaction with some component of estrogen signaling. Estrogen receptor immunoreactivity is present in the amygdala (Mitra et al., 2003), and many amygdala neurons have coexpression of estrogen receptors and NMDA receptors (Kia et al., 2002), suggesting glutamatergic signaling in the CeA may be susceptible to estrous cycle dependent reorganization. This also suggests the lack of estrogen signaling in diestrus msP females likely interacts with heightened stress system signaling seen in the msPs to block ethanol's effects, though it is not clear how this occurs, but further investigation of these interactions is warranted.

CB₁ activation has been shown to decrease glutamate release in many brain areas, including the central amygdala, of male rodents (Gerdeman and Lovinger, 2001; Ramikie et al., 2014). However, to our knowledge, this is the first study to investigate CB₁ receptor function on CeA glutamatergic signaling in both female and male rodents. Generally, CB₁ agonists are thought to decrease anxiety like behavior, while disruptions in CB₁ signaling are anxiogenic. A disruption in CB₁ signaling in the msPs might then explain their high stress and anxiety phenotype via decreased receptor expression or function, or decreased eCB signaling. However, here we find that Win decreased EPSP amplitudes in both male and female msPs, suggesting expression of functional CB₁ receptors in CeA. Additionally, we find no effects of the CB₁ specific antagonist AM251, suggesting no tonic CB₁ signaling at glutamatergic synapses of msPs or Wistars. Thus, it is possible that the msPs express greater numbers of CB₁ receptors in CeA, leading to the enhanced effect of Win in these animals. Interestingly,

in Wistars, Win had sexually dimorphic effects, modestly decreasing EPSP amplitudes in males but having no effect in females. This lack of effect with Win was consistent in both diestrus and estrus animals, indicating this irregular result is not likely dependent on estrogen signaling.

The enhanced Win effect seen in msPs may in fact be directly connected with enhanced glutamatergic activity. CeA glutamate neurons in Wistars may be under tighter regulatory control of glutamate release, thus diminishing further inhibition by Win. Metabotropic glutamate receptors from the group 2 family (mGluR₂) have previously been shown to block effects of CB₁ signaling by competing at downstream activators (Xi et al., 2006), in this case already acting through G_i/G_o proteins to inhibit neurotransmitter release via inhibition of cAMP-dependent signaling. Wistar rats from the Charles River line fully express mGluR₂, and although expression levels of mGluR₂ is unknown for msP rats, msPs were bred from Sardinian-preferring (sP) rats that have been found to have lower expression of mGluR₂ (Wood et al., 2016). Thus, it is possible that lowered expression of mGluR₂ in msPs results in increased cAMP-dependent PKA signaling, which downstream leads to increased calcium causing enhanced glutamatergic signaling. This system would be very susceptible to the effects of Win in activating G_i/G_o proteins to inhibit cAMP production. Thus, future experiments will need to examine possible interactions between the CB₁ and mGluR₂/PKA systems in the msP rats.

We find that for msP and Wistar males, the combined effects of Win and ethanol have a somewhat additive effect. Despite the diminished effects of Win in the subset of cells that also received ethanol for Wistar males, in both Wistar and msP males, the added ethanol further inhibited EPSPs beyond the magnitude seen with ethanol or Win alone, indicating that the method of inhibition on glutamatergic signaling is distinct for each drug. However, for females we find specific effects of Win and ethanol dependent on strain. In Wistar females, Win blocks the inhibitory effects of ethanol, while in msP females, Win induces an inhibitory effect of ethanol that was not present for ethanol alone. For Wistar females, this suggests ethanol acts through a shared mechanism on a coincident downstream target, so the effect is occluded by the pre-applied Win. For msP females, these data suggest that CB₁ receptor activation of a downstream signaling cascade may be priming the effects of ethanol. Although unlikely, in both cases we cannot rule out the possibility that ethanol directly acts on CB₁ receptors. Notably, these mechanisms would then be sexually dimorphic and strain specific. Examination of estrous cycle in these effects is not more informative. In Wistars, both diestrus and estrus females exhibit the block of ethanol effects. In msPs, diestrus females exhibit the induced ethanol effect, while estrus females have a diminished ethanol effect on top of the Win. This would suggest that estrogens may prevent the Win induced ethanol effect in CeA of msP females. Further investigation of these interactions is warranted.

In summary, we have found sex and strain specific effects of ethanol, sex and strain specific effects of the CB₁ agonist Win, sex and strain specific combined effects of Win and ethanol, and no interaction between CB₁ antagonism via AM251 and the effects of acute ethanol in CeA. The msP rat line is suggestive of effects likely to be seen in chronic ethanol exposed animals, and because we found specific effects in both this line and the background naïve

Wistar strain, it is likely that the transition to alcohol dependence occurs in a sexually dimorphic manner. In fact, many effects of ethanol and stress occur in a sexually dimorphic manner (Bekhhbat et al., 2016; Logrip et al., 2016; Morales et al., 2015; Retson et al., 2015), and these differential effects must be considered in the pursuit of treatments for alcohol and stress disorders, like alcohol dependence.

Acknowledgments

This is manuscript number 29450 from The Scripps Research Institute. This work was supported by NIH grants R01 AA017447, AA015566, AA06420, U01 AA013498, F32 AA025262, R01 AA021491, and T32 AA007456. We thank Hui Sun (NIH/NIAAA) for genotyping of the msP rats. We dedicate this manuscript in loving memory to our departed friend and colleague, Dr. Loren (Larry) Parsons, whose studies of endocannabinoids and drug addiction greatly enriched the field and formed a foundation for future scientific growth.

References

- Alvarez-Jaimes L, Stouffer DG, Parsons LH. Chronic ethanol treatment potentiates ethanol-induced increases in interstitial nucleus accumbens endocannabinoid levels in rats. *J Neurochem.* 2009; 111:37–48. [PubMed: 19650871]
- Andreasen M, Hablitz JJ. Paired-pulse facilitation in the dentate gyrus: a patch-clamp study in rat hippocampus in vitro. *J Neurophysiol.* 1994; 72:326–336. [PubMed: 7965017]
- Ayanwuyi LO, Carvajal F, Lerma-Cabrera JM, Domi E, Bjork K, Ubaldi M, Heilig M, Roberto M, Ciccocioppo R, Cippitelli A. Role of a genetic polymorphism in the corticotropin-releasing factor receptor 1 gene in alcohol drinking and seeking behaviors of marchigian sardinian alcohol-preferring rats. *Front Psychiatry.* 2013; 4:23. [PubMed: 23630503]
- Bekhhbat M, Merrill L, Kelly SD, Lee VK, Neigh GN. Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: Implications for tissue collection methods. *Behav Brain Res.* 2016; 305:122–125. [PubMed: 26946276]
- Blanco C, Xu Y, Brady K, Perez-Fuentes G, Okuda M, Wang S. Comorbidity of posttraumatic stress disorder with alcohol dependence among US adults: results from National Epidemiological Survey on Alcohol and Related Conditions. *Drug Alcohol Depend.* 2013; 132:630–638. [PubMed: 23702490]
- Chester JA, Barrenha GD, Hughes ML, Keuneke KJ. Age- and sex-dependent effects of footshock stress on subsequent alcohol drinking and acoustic startle behavior in mice selectively bred for high-alcohol preference. *Alcohol Clin Exp Res.* 2008; 32:1782–1794. [PubMed: 18652593]
- Ciccocioppo R, Economidou D, Cippitelli A, Cucculelli M, Ubaldi M, Soverchia L, Lourdusamy A, Massi M. Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats: an animal model to study the neurobiology of alcoholism. *Addict Biol.* 2006; 11:339–355. [PubMed: 16961763]
- Cippitelli A, Ayanwuyi LO, Barbier E, Domi E, Lerma-Cabrera JM, Carvajal F, Scuppa G, Li H, Ubaldi M, Heilig M, Roberto M, Ciccocioppo R. Polymorphism in the corticotropin-releasing factor receptor 1 (CRF1-R) gene plays a role in shaping the high anxious phenotype of Marchigian Sardinian alcohol-preferring (msP) rats. *Psychopharmacology (Berl).* 2015; 232:1083–1093. [PubMed: 25260340]
- Debell F, Fear NT, Head M, Batt-Rawden S, Greenberg N, Wessely S, Goodwin L. A systematic review of the comorbidity between PTSD and alcohol misuse. *Social psychiatry and psychiatric epidemiology.* 2014; 49:1401–1425. [PubMed: 24643298]
- Erol A, Karpyak VM. Sex and gender-related differences in alcohol use and its consequences: Contemporary knowledge and future research considerations. *Drug Alcohol Depend.* 2015; 156:1–13. [PubMed: 26371405]
- Galesi FL, Ayanwuyi LO, Mijares MG, Cippitelli A, Cannella N, Ciccocioppo R, Ubaldi M. Role of Hypothalamic-Pituitary-Adrenal axis and corticotropin-releasing factor stress system on cue-induced relapse to alcohol seeking. *Eur J Pharmacol.* 2016; 788:84–89. [PubMed: 27316790]
- Gerdeman G, Lovinger DM. CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. *J Neurophysiol.* 2001; 85:468–471. [PubMed: 11152748]

- Gilpin NW, Weiner JL. Neurobiology of comorbid post-traumatic stress disorder and alcohol-use disorder. *Genes Brain Behav.* 2017; 16:15–43. [PubMed: 27749004]
- Herman MA, Varodayan FP, Oleata CS, Luu G, Kirson D, Heilig M, Ciccocioppo R, Roberto M. Glutamatergic transmission in the central nucleus of the amygdala is selectively altered in Marchigian Sardinian alcohol-preferring rats: Alcohol and CRF effects. *Neuropharmacology.* 2016; 102:21–31. [PubMed: 26519902]
- Jackman SL, Turecek J, Belinsky JE, Regehr WG. The calcium sensor synaptotagmin 7 is required for synaptic facilitation. *Nature.* 2016; 529:88–91. [PubMed: 26738595]
- Jacobsen LK, Southwick SM, Kosten TR. Substance use disorders in patients with posttraumatic stress disorder: a review of the literature. *Am J Psychiatry.* 2001; 158:1184–1190. [PubMed: 11481147]
- Kia HK, Yen G, Krebs CJ, Pfaff DW. Colocalization of estrogen receptor alpha and NMDA-2D mRNAs in amygdaloid and hypothalamic nuclei of the mouse brain. *Brain Res Mol Brain Res.* 2002; 104:47–54. [PubMed: 12117550]
- Koob GF. Addiction is a Reward Deficit and Stress Surfeit Disorder. *Front Psychiatry.* 2013; 4:72. [PubMed: 23914176]
- Koob GF, Le Moal M. Addiction and the brain antireward system. *Annu Rev Psychol.* 2008; 59:29–53. [PubMed: 18154498]
- Koob GF, Volkow ND. Neurocircuitry of addiction. *Neuropsychopharmacology.* 2010; 35:217–238. [PubMed: 19710631]
- Logrip ML, Oleata C, Roberto M. Sex differences in responses of the basolateral-central amygdala circuit to alcohol, corticosterone and their interaction. *Neuropharmacology.* 2016; 114:123–134. [PubMed: 27899281]
- Manabe T, Wyllie D, Perkel DJ, Nicoll RA. Modulation of synaptic transmission and long-term potentiation in the CA1 region of the hippocampus. *J Neurophysiol.* 1993; 70:1451–1459. [PubMed: 7904300]
- Marco EM, Ballesta JA, Irala C, Hernandez MD, Serrano ME, Mela V, Lopez-Gallardo M, Viveros MP. Sex-dependent influence of chronic mild stress (CMS) on voluntary alcohol consumption; study of neurobiological consequences. *Pharmacol Biochem Behav.* 2017; 152:68–80. [PubMed: 27894930]
- McLean CP, Asnaani A, Litz BT, Hofmann SG. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. *Journal of psychiatric research.* 2011; 45:1027–1035. [PubMed: 21439576]
- Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology.* 2003; 144:2055–2067. [PubMed: 12697714]
- Morales M, McGinnis MM, McCool BA. Chronic ethanol exposure increases voluntary home cage intake in adult male, but not female, Long-Evans rats. *Pharmacol Biochem Behav.* 2015; 139:67–76. [PubMed: 26515190]
- Olf M, Langeland W, Draijer N, Gersons BP. Gender differences in posttraumatic stress disorder. *Psychological bulletin.* 2007; 133:183–204. [PubMed: 17338596]
- Ramkise TS, Nyilas R, Bluett RJ, Gamble-George JC, Hartley ND, Mackie K, Watanabe M, Katona I, Patel S. Multiple mechanistically distinct modes of endocannabinoid mobilization at central amygdala glutamatergic synapses. *Neuron.* 2014; 81:1111–1125. [PubMed: 24607231]
- Retson TA, Hoek JB, Sterling RC, Van Bockstaele EJ. Amygdalar neuronal plasticity and the interactions of alcohol, sex, and stress. *Brain structure & function.* 2015; 220:3211–3232. [PubMed: 25081549]
- Riebe CJ, Wotjak CT. Endocannabinoids and stress. *Stress (Amsterdam, Netherlands).* 2011; 14:384–397.
- Roberto M, Cruz M, Bajo M, Siggins GR, Parsons LH, Schweitzer P. The endocannabinoid system tonically regulates inhibitory transmission and depresses the effect of ethanol in central amygdala. *Neuropsychopharmacology.* 2010; 35:1962–1972. [PubMed: 20463657]

- Roberto M, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Increased GABA release in the central amygdala of ethanol-dependent rats. *J Neurosci*. 2004a; 24:10159–10166. [PubMed: 15537886]
- Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis. *J Neurosci*. 2004b; 24:1594–1603. [PubMed: 14973247]
- Ruehle S, Rey AA, Remmers F, Lutz B. The endocannabinoid system in anxiety, fear memory and habituation. *J Psychopharmacol*. 2012; 26:23–39. [PubMed: 21768162]
- Sah P, Faber ES, Lopez De Armentia M, Power J. The amygdaloid complex: anatomy and physiology. *Physiol Rev*. 2003; 83:803–834. [PubMed: 12843409]
- Seo D, Sinha R. The neurobiology of alcohol craving and relapse. *Handbook of clinical neurology*. 2014; 125:355–368. [PubMed: 25307585]
- Serrano A, Parsons LH. Endocannabinoid influence in drug reinforcement, dependence and addiction-related behaviors. *Pharmacol Ther*. 2011; 132:215–241. [PubMed: 21798285]
- Serrano A, Rivera P, Pavon FJ, Decara J, Suarez J, Rodriguez de Fonseca F, Parsons LH. Differential effects of single versus repeated alcohol withdrawal on the expression of endocannabinoid system-related genes in the rat amygdala. *Alcohol Clin Exp Res*. 2012; 36:984–994. [PubMed: 22141465]
- Shonesy BC, Bluett RJ, Ramikie TS, Baldi 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 reports*. 2014; 9:1644–1653. [PubMed: 25466252]
- Shorter D, Hsieh J, Kosten TR. Pharmacologic management of comorbid post-traumatic stress disorder and addictions. *Am J Addict*. 2015; 24:705–712. [PubMed: 26587796]
- Silva L, Black R, Michaelides M, Hurd YL, Dow-Edwards D. Sex and age specific effects of delta-9-tetrahydrocannabinol during the periadolescent period in the rat: The unique susceptibility of the prepubescent animal. *Neurotoxicology and teratology*. 2016; 58:88–100. [PubMed: 26898326]
- Sonne SC, Back SE, Diaz Zuniga C, Randall CL, Brady KT. Gender differences in individuals with comorbid alcohol dependence and post-traumatic stress disorder. *Am J Addict*. 2003; 12:412–423. [PubMed: 14660155]
- Tabatadze N, Huang G, May RM, Jain A, Woolley CS. Sex Differences in Molecular Signaling at Inhibitory Synapses in the Hippocampus. *J Neurosci*. 2015; 35:11252–11265. [PubMed: 26269634]
- Varlinskaya EI, Kim EU, Spear LP. Chronic intermittent ethanol exposure during adolescence: Effects on stress-induced social alterations and social drinking in adulthood. *Brain Res*. 2017; 1654:145–156. [PubMed: 27048754]
- Varodayan FP, Bajo M, Soni N, Luu G, Madamba SG, Schweitzer P, Roberto M. Chronic alcohol exposure disrupts CB1 regulation of GABAergic transmission in the rat basolateral amygdala. *Addict Biol*. 2016a
- 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*. 2016b; 21:788–801. [PubMed: 25940135]
- Wood CM, Nicolas CS, Choi SL, Roman E, Nylander I, Fernandez-Teruel A, Kiianmaa K, Bienkowski P, de Jong TR, Colombo G, Chastagnier D, Wafford KA, Collingridge GL, Wildt SJ, Conway-Campbell BL, Robinson ES, Lodge D. Prevalence and influence of cys407* Grm2 mutation in Hannover-derived Wistar rats: mGlu2 receptor loss links to alcohol intake, risk taking and emotional behaviour. *Neuropharmacology*. 2016
- Xi ZX, Gilbert JG, Peng XQ, Pak AC, Li X, Gardner EL. Cannabinoid CB1 receptor antagonist AM251 inhibits cocaine-primed relapse in rats: role of glutamate in the nucleus accumbens. *J Neurosci*. 2006; 26:8531–8536. [PubMed: 16914679]
- Zer-Aviv TM, Akirav I. Sex differences in hippocampal response to endocannabinoids after exposure to severe stress. *Hippocampus*. 2016; 26:947–957. [PubMed: 26928784]

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.