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# Glutamatergic transmission in the central nucleus of the amygdala is selectively altered in Marchigian Sardinian alcohol-preferring rats: alcohol and CRF effects

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## Abstract

The CRF system of the central nucleus of the amygdala (CeA) is important for the processing of anxiety, stress, and effects of acute and chronic ethanol. We previously reported that ethanol decreases evoked glutamate transmission in the CeA of Sprague Dawley rats and that ethanol dependence alters glutamate release in the CeA. Here, we examined the effects of ethanol, CRF and a CRF1 receptor antagonist on spontaneous and evoked glutamatergic transmission in CeA neurons from Wistar and Marchigian Sardinian Preferring (msP) rats, a rodent line genetically selected for excessive alcohol drinking and characterized by heightened activity of the CRF1 system. Basal spontaneous and evoked glutamate transmission in CeA neurons from msP rats was increased compared to Wistar rats. Ethanol had divergent effects, either increasing or decreasing spontaneous glutamate release in the CeA of Wistar rats. This bidirectional effect was retained in msP rats, but the magnitude of the ethanol-induced increase in glutamate release was significantly smaller. The inhibitory effect of ethanol on evoked glutamatergic transmission was similar in both strains. CRF also either increased or decreased spontaneous glutamate release in CeA neurons of Wistar rats, however, in msP rats CRF only increased glutamate release. The inhibitory effect of CRF on evoked glutamatergic transmission was also lost in neurons from msP rats. A CRF1 antagonist produced only minor effects on spontaneous glutamate transmission, which were consistent across strains, and no effects on evoked glutamate transmission. These results demonstrate that the genetically altered CRF system of msP rats results in alterations in

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spontaneous and stimulated glutamate signaling in the CeA that may contribute to both the anxiety and drinking behavioral phenotypes.

**Keywords**

amygdala; glutamate; alcohol; CRF; CRF1 antagonist; electrophysiology

## 1. Introduction

The central nucleus of the amygdala (CeA) is the brain region responsible for the emotional processing of internal and external stimuli that contributes to the behavioral manifestations of fear, anxiety, and stress (Koob and Le Moal, 2008). As a major component of the "extended amygdala," the CeA has also been shown to be a major neural substrate for the motivational effects of alcohol and drugs of abuse (Alheid and Heimer, 1988). Primarily composed of GABAergic neurons (Pitkanen and Amaral, 1994; Veinante and Freund-Mercier, 1998), the CeA receives excitatory input from glutamatergic afferent projections from the basolateral amygdala, cortex, thalamus, and brainstem (Pitkanen et al., 2000; Veinante and Freund-Mercier, 1998).

The brain stress peptide corticotropin-releasing factor (CRF) and its G protein-coupled receptors CRF1 and CRF2 are expressed throughout the CeA and exert neuromodulatory effects on excitatory and inhibitory synaptic transmission via intracellular pathways promoting facilitation or depression of neurotransmitter release (Gallagher et al., 2008). CRF plays crucial roles in integrating the body's overall response to stress (Koob et al., 1999) and is critical in behavioral aspects of addiction, including the anxiogenic effects of drug withdrawal (Breese et al., 2005; Heinrichs et al., 1995; Menzaghi et al., 1994). The CRF system is also heavily implicated in negative reinforcement, particularly within the extended amygdala (Koob, 2008; Sinha, 2008). Blockade of CRF in the extended amygdala selectively reduces dependence-induced drinking in rodents (Koob, 2008; Zorrilla et al., 2013). Ethanol dependence has been shown to result in upregulation of the CRF stress system in the CeA (Roberto et al., 2010). Notably, chronic treatment with a systemic CRF1 antagonist blocked development of withdrawal-induced increases in alcohol drinking by dependent rats, and also tempered the moderate increases in alcohol consumption by non-dependent rats over the course of intermittent testing (Roberto et al., 2010).

The actions of CRF in the development of alcohol dependence have recently been expanded to include a role in genetic susceptibility. Gene association studies in humans suggest that the *Crhr1* locus mediates an inherent genetic vulnerability to drinking (Treutlein et al., 2006). It has been theorized that the heightened sensitivity to stress conferred by genetic variation in the CRF1 receptor could also confer a predisposition to develop alcohol dependence, particularly when occurring in conjunction with environmental factors related to stress (Enoch and Goldman, 1999; Pohorecky, 1991; Treutlein et al., 2006). The Marchigian Sardinian Preferring (msP) rat strain is characterized by upregulated CRF1 mRNA in several limbic brain areas, that is linked to two single nucleotide polymorphisms occurring in the promoter region (position −1836 and −2097) of the gene encoding for the

CRF1 receptor (Hansson et al., 2007). The msP rats exhibit an excessive daily ethanol drinking that occurs in binge-like bouts of consumption resulting in blood alcohol levels as high as 100-120 mg/dl (Ciccocioppo et al., 2006). The msP rats display an increased stress response, as well as an increased vulnerability to stress-induced reinstatement (Ayanwuyi et al., 2013; Ciccocioppo et al., 2006; Ciccocioppo et al., 1999b; Cippitelli et al., 2015). There are similarities in drinking behavior of msP rats and control rats that have been made dependent on ethanol, most notably, that the increased ethanol consumption in msP and dependent rats can be reversed by administration of CRF1 antagonists (Gehlert et al., 2007; Hansson et al., 2006). Chronic voluntary drinking has been shown to reverse amygdala CRF1 receptor overexpression and attenuate negative affect in msP rats (Ciccocioppo et al., 1999a; Hansson et al., 2007). Previous studies have implicated changes in the CeA in the ethanol sensitive phenotype of the msP rats (Economidou et al., 2008; Hansson et al., 2007; Hansson et al., 2006). We previously reported changes in the GABA system in the medial CeA of msP rats (Herman et al., 2013) that mimic the changes observed in ethanol dependence (Roberto et al., 2003; Roberto et al., 2004a), but to our knowledge no studies have examined alterations in the glutamatergic system in the msP rats.

Glutamate is the major excitatory neurotransmitter and its modulation by ethanol contributes to ethanol reinforcement, tolerance and dependence (Lovinger and Roberto, 2013). We have previously reported that ethanol decreases evoked glutamatergic transmission in the CeA and that baseline spontaneous glutamatergic transmission is elevated in rats following chronic ethanol treatment (Roberto et al., 2004b), but we did not examine the role of the CRF system in the effects of ethanol on glutamatergic transmission. Previous work shows that CRF increases glutamate release in the CeA and that this effect is increased in fear-conditioned animals (Skorzewska et al., 2009), and altered with cocaine chronic exposure (Liu et al., 2005; Pollandt et al., 2006). CRF also increases the frequency of spontaneous glutamate currents in neurons in the lateral CeA (Silberman and Winder, 2013). Notably, CRF and the CRF-like peptide urocortin I produce opposing effects on glutamatergic transmission in the CeA by presynaptic CRF1 and pre- and postsynaptic CRF2 mechanisms, respectively (Gallagher et al., 2008; Liu et al., 2004; Orozco-Cabal et al., 2006), underscoring the complexity of the CRF system in the regulation of excitatory transmission.

In the present study we used an *in vitro* slice preparation to assess the specific electrophysiological differences in spontaneous and evoked glutamatergic transmission in the medial CeA of msP rats compared to Wistar rats, and how ethanol and stimulation or blockade of the CRF system affect glutamatergic transmission in the medial CeA.

## 2. Methods

### 2.1. Animals

In the present study we used 51 adult male (254.3 ± 8.8 g) msP rats maintained at The Scripps Research Institute and obtained from the School of Pharmacy at the University of Camerino (Italy). For the strain comparison, we used 46 adult male (250.9 ± 8.0 g) Wistar rats obtained from Charles River (Raleigh, NC) as this is the strain from which msP rats were originally selected. To control for inter-animal variability, cells were collected from a minimum of 3 rats for each experimental condition. All rats were housed in a temperature-

and humidity-controlled room on a 12-h light/dark cycle (lights on at 6:00 am) with food and water available *ad libitum*. We conducted all care, msP colony breeding and surgical procedures in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Institutional Animal Care and Use Committee (IACUC) policies of The Scripps Research Institute.

***Electrophysiological studies***

## 2.2 Slice preparation

We prepared CeA slices as previously described (Herman et al., 2013) from Wistar rats and genetically-selected Marchigian Sardinian (msP) rats that were anesthetized with isoflurane (3-5%) and rapidly decapitated. We cut coronal slices 300-400 μm thick on a Leica 1000S vibratome (Campden, Lafayette, Indiana), incubated them in an interface configuration for 30-60 min, and then completely submerged and continuously superfused (flow rate of 2-4 ml/min) and equilibrated them with 95% O2/5% CO2 artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl, 130; KCl, 3.5; $NaH_2PO_4$, 1.25; $MgSO_4$•7H2O, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Drugs were added to the aCSF from stock solutions to obtain known concentrations in the superfusate.

## 2.3. Whole-cell patch-clamp recording of glutamate currents

We recorded from 204 neurons in the medial CeA (88 cells from msP rats, 116 cells from Wistar rats) visualized in brain slices (300 μm) using infrared differential interference contrast (IR-DIC) optics and CCD cameras (EXi Aqua and ROLERA-XR, QImaging) (Herman et al., 2013). A w60 or w40 water immersion objective (Olympus) was used for identifying and approaching CeA neurons. Whole-cell voltage-clamp recordings were made with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 2-5 kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a PC using pClamp 10 software (Axon Instruments). All voltage-clamp recordings were performed in a gap-free acquisition mode with a sampling rate per signal of 10 kHz. Patch pipettes (3-6 MΩ) were pulled from borosilicate glass (Warner Instruments and King Precision) and filled with an internal solution composed of (in mM): 145 Kgluconate; 0.5 EGTA; 2 $MgCl_2$; 10 HEPES; 2 Na-ATP; 0.2 Na-GTP. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the presence of 30 μM bicuclline and 1 μM [1-(*S*)-3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*p*-benzylphosphonic acid (CGP 55845A). Miniature EPSCs (mEPSCs) were recorded under identical conditions with the addition of 1 μM tetrodotoxin (TTX). Drugs were constituted in aCSF and applied by bath superfusion. All cells were clamped at −60 mV for the duration of the recording. In all experiments, series resistance (<15 MΩ) was continuously monitored with a 10 mV hyperpolarizing pulse and experiments with >20% change in series resistance were not included in the final analysis.

## 2.4. Intracellular recording of evoked responses

We recorded from 84 CeA neurons (from the medial subdivision of the CeA) with sharp micropipettes filled with 3M KCl using discontinuous current-clamp mode (Cruz et al., 2012; Haubensak et al., 2010; Kallupi et al., 2014; Roberto et al., 2004a). We held most

neurons near their resting membrane potential (RMP). Data were acquired with an Axoclamp-2A preamplifier and stored for later analysis using pClamp software (Axon Instruments, Foster City, CA). We evoked pharmacologically isolated compound excitatory postsynaptic potentials (EPSPs) by stimulating locally within the CeA through a bipolar stimulating electrode and superfusing the slices with the GABA receptor blockers: 30 μM bicuculline (to block $GABA_A$ receptors) and 1 μM CGP 55845A (to block $GABA_B$ receptors) in the aCSF. At the end of the recording we often superfused 30 μM DNQX and 30 μM DL-AP-5 to confirm the glutamatergic nature of the EPSP. To determine the synaptic response parameters for each cell, we performed an input-output protocol (Kallupi et al., 2014; Roberto et al., 2003; Roberto et al., 2004a) consisting of a range of five current stimulations (50-250 mA; 0.125 Hz), 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 entire duration of the experiment.

### 2.5. Drugs

We purchased CGP 55845A, picrotoxin and bicuculline from Sigma (St. Louis, MO), tetrodotoxin from Biotum (Hayward, CA); and ethanol from Remet (La Mirada, CA). r/hCRF was synthesized by Dr. Jean Rivier at the Salk Institute for Biological Studies. R121919 was synthesized by Dr. Kenner Rice at the Drug Design and Synthesis Section, Chemical Biology Research Branch, National Institute on Drug Abuse, National Institutes of Health, Bethesda, MD.

### 2.6. Data analysis and statistics

Frequency, amplitude and kinetics of EPSCs were analyzed and visually confirmed using a semi-automated threshold-based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ). Averages of EPSC characteristics were based on a minimum time interval of 3-5 min and a minimum of 60 events. All detected events were used for event frequency analysis, but superimposed events were eliminated for amplitude and decay kinetic analysis. Experimental groups were divided on a cell-by-cell basis using the functional response (either a clear increase or decrease (> or < than 100%) over normalized baseline values). All data are expressed as mean ± SEM. We quantified the synaptic responses by calculating the EPSP amplitude with Clampfit software (Axon Instruments). We examined paired-pulse facilitation (PPF) in each neuron using paired stimuli at 50 and 100 msec inter-stimulus interval (Kallupi et al., 2014; Roberto et al., 2004a). The stimulus strength was adjusted such that the amplitude of the first EPSP was 50% of maximal, determined from the I-O relationship. We calculated the PPF ratio as the second EPSP amplitude over that of the first EPSP

To analyze data acquired from intracellular and whole cell recordings, we used Clampfit 10.2 (Molecular Devices) and MiniAnalysis 5.1 software (Synaptosoft, Leonia, NJ), respectively. We used GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) for all statistical analysis of results. The EPSC results were analyzed for independent significance using a one-sample t-test and compared using a two-tailed t-test for independent samples, a paired two-tailed t-test for comparisons made within the same recording, and a one-way ANOVA with a Bonferroni *post hoc* analysis for comparisons made between 3 or

more groups. All statistical analysis was performed using Prism 5.02 (GraphPad, San Diego, CA). Data are presented as mean ± standard error. In all cases, $p<0.05$ was the criterion for statistical significance.

## 3. Results

### 3.1. Baseline spontaneous and evoked CeA glutamatergic transmission is elevated in msP rats compared to Wistar rats

We assessed baseline glutamatergic signaling in adult male Wistar and msP rats using whole-cell voltage clamp recordings of pharmacologically-isolated spontaneous excitatory postsynaptic currents (sEPSCs) in the medial subdivision of the CeA. CeA neurons from msP rats had a significantly higher baseline sEPSC frequency (1.3 ± 0.2 Hz; n = 19) compared to CeA neurons from Wistar rats (0.6 ± 0.1 Hz; n = 26; **Figure 1A and C**; $p<0.05$ by unpaired t-test). There were no differences between msP and Wistar rats in sEPSC amplitude (26.3 ± 1.2 and 25.2 ± 0.8 pA, respectively), rise (1.8 ± 0.1 and 1.8 ± 0.08 ms, respectively) or decay time (1.8 ± 0.2 and 1.5 ± 0.1 ms, respectively; $p>0.05$ by one-way ANOVA). Action potential-independent glutamatergic transmission was examined in recordings of miniature EPSCs (mEPSCs) using whole-cell voltage clamp recordings in the presence of tetrodotoxin (TTX, 1 μM). In contrast to the difference observed in baseline sEPSC frequency, there was no difference in baseline mEPSC frequency between CeA neurons from msP rats (0.5 ± 0.1 Hz; n = 17) and CeA neurons from Wistar rats (0.6 ± 0.1 Hz; n = 18; **Figure 1B and 1D**; $p>0.05$ by unpaired t-test). In addition there were no significant differences between msP and Wistar rats in mEPSC amplitude (26.0 ± 1.8 and 26.8 ± 1.0 pA, respectively), rise (2.1 ± 0.1 and 1.8 ± 0.1 ms, respectively) or decay time (1.9 ± 0.2 and 1.5 ± 0.1 ms, respectively; $p>0.05$ by one-way ANOVA). Generally, changes in the frequency of EPSCs reflect changes in the probability of glutamate release, while changes in EPSC amplitude and kinetics reflect change in postsynaptic glutamatergic receptor function (De Koninck and Mody, 1994). Collectively, these results indicate that msP rats exhibit elevated CeA action potential-dependent, but not vesicular, glutamate release compared to Wistar rats.

We also recorded intracellularly, with sharp pipettes, and evoked pharmacologically isolated compound glutamatergic EPSPs (eEPSPs) by stimulating locally within the medial CeA. We recorded from 84 CeA neurons from either Wistar or msP rats with a mean RMP of −79 ± 1.0 mV and a mean input resistance of 142 ± 7.5 MΩ. The two groups did not show significant differences in these properties, or the voltage–current relationship (not shown). Baseline eEPSP input–output curves generated by equivalent stimulus intensities were significantly ($p<0.05$; by unpaired t-test) higher in CeA neurons from msP rats (n = 36) compared to those from Wistar rats (n = 28) (**Figure 2A**), suggesting a strain difference in evoked glutamatergic transmission. We examined paired-pulse facilitation (PPF) of the eEPSPs at 50 and 100 ms inter-stimulus intervals, a phenomenon whereby a secondary synaptic response is influenced by a preceding primary stimulus of equal intensity (Andreasen and Hablitz, 1994; Manabe et al., 1993). Generally, changes in the PPF ratio (second EPSP/first EPSP) are inversely related to transmitter release such that a reduction of the PPF ratio is associated with an increased probability of transmitter release (Andreasen

and Hablitz, 1994). We found a significantly ($p<0.05$ by unpaired t-test) lower (50 msec: 1.25 ± 0.06; 100 msec, 1.28 ± 0.06; n = 38) basal PPF ratio of EPSPs from neurons of msP rats compared to Wistar rats (50 msec: 1.58 ± 0.08; 100 msec, 1.51 ± 0.09; n = 26) (**Figure 2B**), suggesting augmented baseline evoked network dependent glutamate release in the msP rats. Thus, the elevated eEPSPs, decreased PPF ratio of eEPSPs and the enhanced frequency of sEPSCs taken together point to enhanced action-potential dependent glutamate release in CeA of msP compared to Wistar..

### 3.2. The divergent effects of ethanol on spontaneous CeA glutamatergic transmission are altered in msP as compared to Wistar rats

To determine if ethanol produced strain-specific effects in CeA neurons from msP and Wistar rats, we performed whole-cell recordings of glutamate currents during superfusion of ethanol (EtOH, 44 mM; 10-12 min). When averaged together, the effects of ethanol are found to be nonsignificant (**Figure 3C**), however, a close examination of the data suggests that ethanol produces divergent effects on glutamate transmission. In a subset of neurons (7/17) there was a significant increase in sEPSC frequency (172.7 ± 15.2 % of control; *$p<0.05$ by one-sample t-test), and in the remaining neurons (10/17) there was a significant decrease in sEPSC frequency (58.2 ± 6.9 % of control; *$p<0.05$ by one-sample t-test; **Figure 3A and 3C)**, suggesting increased or decreased action potential-dependent glutamate release, respectively. In contrast, in 9/17 neurons from msP rats, superfusion of ethanol (EtOH, 44 mM) produced an increase in sEPSC frequency that was significantly less than that observed in neurons from Wistar rats (109.7 ± 2.6 % of control *$p<0.05$; #$p<0.05$ by one-way ANOVA Bonferroni *post hoc* analysis). In the remaining 8/17 neurons a significant decrease in sEPSC frequency was still observed (78.2 ± 5.7 % of control; *$p<0.05$ by one-sample t-test; **Figure 3B and 3C**). Ethanol produced no consistent differences in sEPSC amplitude, rise time or decay time between CeA neurons from msP and Wistar rats.

The effects of ethanol on mEPSC frequency were similar to those observed with sEPSCs, although with a smaller overall magnitude. In Wistar rats, ethanol produced a small but significant increase in mEPSC frequency (114.7 ± 3.3 % of control; *$p<0.05$ by one-sample t-test) in 7/18 neurons and a significant decrease (68.7 ± 5.1 % of control; *$p<0.05$ by one-sample t-test; **Figure 3D**) in 11/18 neurons. In msP rats, ethanol produced no significant change in mEPSC frequency (112.7 ± 3.7 % of control) in 5/13 neurons and a significant decrease (73.9 ± 5.2 % of control; *$p<0.05$ by one-sample t-test; **Figure 3D**) in 7/13 neurons. Ethanol produced no significant differences in mEPSC amplitude, rise time or decay time between CeA neurons from msP and Wistar rats.

### 3.3. The divergent effects of CRF are lost in action potential-dependent glutamatergic transmission in the CeA of msP as compared to Wistar rats

We then examined the strain-specific effects of CRF on EPSCs in CeA neurons from msP and Wistar rats. Similar to what was observed with ethanol, CRF (100 nM; 12-15 min) produced opposing effects on glutamate transmission in CeA neurons from Wistar rats that, when averaged together, are not significant (**Figure 4C**), but when the effects are separated into two groups based on an increase or decrease from control, divergent effects emerge. In a subset of neurons from Wistar rats, CRF produced a significant increase in sEPSC

frequency (144.8 ± 11.7 % of control; n = 6/12; *p<0.05 by one-sample t-test) and in the remaining neurons there was a significant decrease in sEPSC frequency (61.8 ± 10.1 % of control; n = 6/12; *p<0.05 by one-sample t-test; **Figure 4A and 4C**). In CeA neurons from msP rats, however, the effects of CRF were no longer divergent. CRF produced a significant increase in sEPSC frequency in all neurons examined (156.9 ± 11.3 % of control; n = 10/10; *p<0.05 by one-sample t-test; **Figure 4B and 4C**). We observed no neurons that displayed a decrease or no change in sEPSC frequency with superfusion of CRF in msP rats, suggesting a consistent presynaptic effect of CRF in increasing glutamate release in these animals. In addition, CRF produced no significant change in sEPSC amplitude, rise time or decay time in either strain.

The divergent effects of CRF on mEPSC frequency in CeA neurons from Wistar rats were similar to what was observed with sEPSC frequency, with a subset of CeA neurons displaying a significant increase in mEPSC frequency (143.6 ± 14.7 % of control; n = 9/13; *p<0.05 by one-sample t-test) and a somewhat smaller subset displaying a decrease in mEPSC frequency (76.6 ± 7.5 % of control; n = 4/13; **Figure 4D**). However, as opposed to the uniformly facilitatory effects of CRF on sEPSC frequency in the mSP rats, CRF produced divergent effects on mEPSC frequency with a subset of neurons displaying an increase (157.5 ± 33.5 % of control; n = 5/11) and the remaining neurons displaying a significant decrease (75.5 ± 6.0 % of control; n = 6/11; *p<0.05 by one-sample t-test; **Figure 4D**). CRF produced no significant change in mEPSC amplitude, rise time or decay time.

### 3.4. The effects of CRF receptor-1 blockade on spontaneous CeA glutamatergic transmission are unchanged in msP as compared to Wistar rats

In another set of experiments, we assessed the effects of CRF receptor-1 blockade on EPSCs in CeA neurons from msP and Wistar rats using the CRF1 antagonist R121919 (1 μM; 12-15 min). Similar to what was observed with CRF, R121919 produced opposing effects on glutamate transmission in CeA neurons from Wistar rats, although of a much smaller overall magnitude. When averaged together, these effects are not significant and may simply represent a normal distribution with no overall effect (**Figure 5C**), but when the data are separated into two groups, small divergent effects emerge. R121919 produced an increase in sEPSC frequency in a subset of neurons from Wistar rats (129.5 ± 14.0 % of control; n = 7/15) and a significant decrease in the remaining neurons (73.9 ± 4.0 % of control; n = 8/15; *p<0.05 by one-sample t-test; **Figure 5A and 5C**). R121919 also produced divergent effects on glutamate transmission in CeA neurons from msP rats. In a subset of neurons R121919 produced a significant increase in sEPSC frequency (133.9 ± 10.9 % of control; n = 6/13; *p<0.05 by one-sample t-test) and in the remaining neurons R121919 produced a significant decrease in sEPSC frequency (79.1 ± 6.7 % of control; n =7/13; *p<0.05 by one-sample t-test; **Figure 5B and 5C**).

The effects of R121919 on mEPSC frequency in CeA neurons from Wistar and msP rats were consistent with what was observed with sEPSC frequency. R121919 produced a small but significant increase in mEPSC frequency in a subset of neurons from Wistar rats (112.6 ± 4.0 % of control; n = 7/14; *p<0.05 by one-sample t-test) and produced a significant

decrease in mEPSC frequency in the remaining neurons (66.7 ± 9.2 % of control; n = 7/14; *p<0.05 by one-sample t-test; **Figure 5D**). R121919 also produced divergent effects on glutamate transmission in CeA neurons from msP rats, although the proportions were much less evenly split than what was observed in neurons from Wistar rats. In a small subset of neurons from msP rats R121919 produced an increase in mEPSC frequency (118.8 ± 11.3 % of control; n = 2/18) and in the majority of neurons R121919 produced a significant decrease in mEPSC frequency (69.0 ± 5.3 % of control; n = 16/18; *p<0.05 by one-sample t-test; **Figure 5D**).

### 3.5 The ethanol-induced decrease of evoked glutamatergic transmission in the CeA of msP and Wistar rats is similar

Here we repeated our previous finding (Kallupi et al., 2014; Roberto et al., 2004a) showing that ethanol significantly decreased evoked **compound** glutamatergic responses in 16 CeA neurons (**Figure 6A**) from Wistar rats. Ethanol (44 mM) significantly (p<0.05 by paired t-test) decreased the mean amplitude of evoked EPSPs to 84 ± 3% of control over the three stimulus intensities with recovery on washout **(Figure 6A)**. This ethanol-induced decrease was not associated with a change in the PPF ratios of EPSPs in the CeA of Wistar (control PPF 50 and 100 msec: 1.35 ± 0.12 and 1.35 ± 0.12; ethanol: 1.24 ± 0.09 and 1.47 ± 0.15; respectively, n=13) or msP rats (control PPF 50 and 100 msec: 1.3 ± 0.16 and 1.26 ± 0.12; ethanol: 1.4 ± 0.13 and 1.28 ± 0.15; respectively, n=13) or the I-V relationships (data not shown).

### 3.6 The CRF-induced decrease of evoked glutamatergic transmission in the CeA of Wistar rats is lost in msP rats

To determine whether CRF regulates baseline evoked **compound** glutamatergic transmission in the CeA, we applied CRF (100 nM) for a period of 12-15 min during eEPSP recordings. A two-way ANOVA revealed a significant difference in the strain responsivity to CRF (100 nM) across the intensity strengths tested [$F(1,81) = 12.73$; $p<0.001$]. Application of CRF significantly (p<0.05) decreased eEPSPs in 12 CeA neurons from Wistar rats (**Fig. 6B**). In contrast, CRF overall did not alter eEPSPs in 17 msP CeA neurons. However, the eEPSP amplitude was increased by CRF in 7/17 neurons, while it was decreased in 9/17 neurons (one neuron showed no effect). CRF did not alter the PPF ratios of EPSPs in the CeA of either Wistar (control PPF 50 and 100 msec: 1.72 ± 0.18 and 1.45 ± 0.19; CRF: 1.6 ± 0.15 and 1.52 ± 0.11; n=11) or msP rats (control PPF 50 and 100 msec: 1.36 ± 0.09 and 1.49 ± 0.12; CRF: 1.42 ± 0.13 and 1.58 ± 0.17 ; n=17).

### 3.7 CRF1 blockade does not affect evoked glutamatergic transmission in CeA of Wistar and msP rats

To determine whether CRF1 receptors regulate baseline evoked glutamatergic transmission in the CeA, we then tested R121919 (1 μM) for a period of 12-15 min. We found no significant effect of the CRF1 antagonist on the evoked glutamate response in CeA neurons from either Wistar or msP rats (**Figure 6C**). R121919 did not alter the PPF ratios of EPSPs (Wistar control PPF 50 and 100 msec: 1.50 ± 0.1 and 1.34 ± 0.05; R121919: 1.59 ± 0.08 and

1.41 ± 0.08; respectively, n=7; msP control PPF 50 and 100 msec: 1.29 ± 0.07 and 1.28 ± 0.15; R121919: 1.35 ± 0.12 and 1.41 ± 0.18 respectively, n = 9) in either group.

## 4. Discussion

The results of this study indicate that msP rats have a dysregulated CeA glutamate system in both the basal and stimulated state. Within the CeA, msP rats have an increased level of basal spontaneous excitatory glutamatergic activity as compared to Wistar controls, but there was no difference in action-potential independent glutamate transmission between the two strains. In addition, the effects of ethanol and CRF on spontaneous glutamate transmission were altered in CeA neurons from msP rats as compared to Wistar controls, suggesting that the response of the CRF system to either acute ethanol or CRF is dysfunctional in msP rats. Conversely, the effects of CRF1 blockade were unchanged in msP rats as compared to Wistar controls. This suggests that the baseline activity of the CRF1 receptor is not significantly different in msP rats, although the overall sensitivity of the system is altered. Given the significance of the CeA in the behavioral aspects of alcohol dependence and the behavioral phenotype of the msP rats, it is likely that the heightened glutamatergic state and dysregulated glutamate signaling in the CeA contribute to the increased sensitivity to stress, increased anxiety, and increased ethanol consumption observed in msP rats.

The majority of the significant differences in glutamatergic transmission that we observed in CeA neurons from msP rats occurred in spontaneous, action potential-dependent synaptic transmission. In general, there were little to no changes in miniature excitatory postsynaptic currents (mEPSCs) between msP and Wistar control rats. There was little to no difference between the baseline frequency of action potential-dependent (sEPSC) and action potential-independent (mEPSC) glutamate transmission in CeA neurons from Wistar rats. This suggests that in Wistar rats, the majority of baseline glutamatergic signaling in the CeA is driven by vesicular glutamate release and not overall network activity. In msP rats, baseline action potential-dependent glutamate transmission (both spontaneous and evoked) was significantly higher in the CeA than that observed in neurons from Wistar rats, suggesting that the dysregulation associated with the msP phenotype is due mainly to changes in the CRF system at the network level. This idea is consistent with the diffuse expression of CRF throughout the brain, including at several key sites that form networks with the CeA (Gallagher et al., 2008), as well as the heterogeneous expression of CRF1 and CRF2 receptors at both pre- and postsynaptic sites (Gallagher et al., 2008; Liu et al., 2004; Orozco-Cabal et al., 2006). The data demonstrating changes in vesicular glutamate release with application of ethanol, CRF or the CRF1 antagonist R121919 suggest that these drugs may act on distinct sites located at or near the presynaptic terminals. However, the main differences observed in spontaneous excitatory postsynaptic currents (sEPSCs) in msP rats point to clear network-dependent presynaptic effects of these drugs, possibly within local CeA circuitry or from upstream afferents. However, we cannot rule out the possibility of postsynaptic effects of either ethanol or the CRF system across the synaptic network. It has previously been shown that both CRF and a CRF1 antagonist increase spontaneous glutamate release onto lateral CeA neurons, however CRF did not alter selective optical stimulation of glutamatergic afferents from the cortex and hippocampus (Silberman and

Winder, 2013), suggesting that the effects of CRF are specific to the input pathway or to the state of the neuron (spontaneous versus stimulated). The increases in glutamate release observed with CRF or a CRF1 antagonist in lateral CeA neurons are consistent with what we observed in the medial CeA, suggesting some commonalities between lateral and medial CeA. However, the decrease in glutamate release we observed with CRF or a CRF1 antagonist was not reported in the lateral CeA, suggesting that the medial CeA may possess more complex cell populations and/or connectivity.

The effects of ethanol, CRF and the CRF1 antagonist R121919 on glutamatergic transmission in the CeA are reported here as divergent and the data are described in two separate groups. However, in each case the data are also illustrated with each data point as part of a single continuous group to allow for comparisons with the grouped data. In some instances the divergent effects are more clear (i.e., with ethanol) while in others the effects are smaller in magnitude (R121919), raising the possibility that the effects are not in fact divergent, but simply represent an even distribution with a net result of no significant change. In all cases, the divergent effects are more pronounced in action potential-dependent glutamatergic transmission, which is consistent with a significant role of overall network activity in these effects. Given the heterogeneous cell population of the CeA, we hypothesize that the divergent effects of ethanol and CRF on spontaneous excitatory transmission are the result of differential receptor expression and/or connectivity of distinct CeA cell populations. However, as current methods to classify neuronal populations in the CeA are lacking, we cannot go into further detail on what specific populations are preferentially excited and/or inhibited at this time. Work to clarify the cellular makeup of the CeA is ongoing and outside the scope of the present study.

We have previously reported that ethanol decreases evoked EPSP amplitudes in CeA neurons of naïve control Sprague Dawley rats, an effect that was not associated with significant changes in the PPF ratio, indicating a lack of a significant presynaptic site of action for ethanol on evoked glutamatergic transmission (Kallupi et al., 2014; Roberto et al., 2006; Roberto et al., 2004b). These results were confirmed in the present study in which we demonstrated that ethanol-induced decreases of evoked glutamatergic responses in the CeA of Wistar rats are equivalent to that of msP rats. CRF, like ethanol, has similar inhibitory effects on evoked CeA glutamatergic transmission, without affecting PPF in Wistar rats. This observation of the CRF-induced inhibition of locally evoked EPSPs in Wistar rats is in agreement with previous work showing that CRF inhibits the BLA to CeA synapses (Liu et al., 2004). Notably, in our study, the CRF-induced inhibition of evoked glutamate responses is lost in the CeA of msP rats. Finally, application of the CRF1 receptor antagonist did not affect evoked glutamatergic responses in either Wistar or msP rats, suggesting the absence of tonic CRF1 activity on evoked glutamate release in the CeA. Overall, the differences we observe in the effects of ethanol and CRF on spontaneous and evoked glutamatergic transmission (bidirectional effects vs. inhibition) are likely due to differential presynaptic release mechanisms (different synaptic vesicle pool, release machinery etc..) (Atasoy et al., 2008).

We recently reported an increase in basal action potential-independent, but not evoked, GABA release in the CeA of msP rats as compared to Wistar rats (Herman et al., 2013). We

found no difference in the sensitivity of CeA GABAergic synapses to acute ethanol, CRF and a CRF1 antagonist compared to Wistar rats. The present study acts as a complement to our earlier work examining alterations in the GABA system in msP rats. As a balance between excitatory and inhibitory tone is essential to normal network function, it is possible that the increased basal GABA release we observed in our previous results is an attempt to counter the increased excitatory tone we report in the present study. Notably, the significant baseline difference in GABA release observed between msP and Wistar rats was action potential-independent pointing to the presynaptic terminal as the critical site for dysregulation of the msP GABAergic synapses (Herman et al., 2013). Despite the increased baseline GABA release in msP rats, there was no difference in the response of this transmission to exogenously applied ethanol, CRF or a CRF1 antagonist (Herman et al., 2013). Interestingly, our present findings show that the sensitivity of glutamatergic synapses to ethanol and CRF is altered in msP rats compared to Wistar rats and this change occurs at the network level. Based on our previous and present results, we hypothesize that the innate propensity to excessive drinking in the msP rat line may be an attempt to compensate for the negative affect-like effects (i.e., anxiety and depression) linked to hyperactivity of the extrahypothalamic CRF system that affects both GABAergic and glutamatergic transmission in CeA.

Collectively, the results of this study emphasize the critical role of the CRF system in normal synaptic functioning of the CeA, and the potential implications for the contribution of CeA dysfunction to the behavioral manifestations of stress, anxiety and alcohol dependence, although these are complex disorders that almost certainly involve dysfunction in a number of brain areas and systems beyond the CeA. The commonalities of the msP rats with ethanol dependent rats on a cellular (both glutamatergic and GABAergic systems) and behavioral level suggest that upregulation of the CRF system plays a dominant role in the CeA abnormalities associated with alcoholism (Sommer et al., 2008). Given that acamprosate (Campral), which may act by antagonizing glutamatergic receptors, is one of the few effective therapeutic options for alcoholism (De Witte et al., 2005; Koob et al., 2002; Mann et al., 2008), the dysregulation of the glutamate system observed in msP rats may provide some insight into the role of dysregulated CeA glutamate in alcohol dependence. Thus, the CRF system may be an important target, either for the identification of individuals with an inherent genetic vulnerability to alcohol dependence or in the development of therapeutics to treat alcohol dependence in vulnerable individuals.

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## Highlights

- CeA glutamate release is elevated in msP rats compared to Wistar rats
- EtOH produces divergent effects on glutamate release in msP and Wistar rats
- The increase in CeA glutamate release with EtOH is less in msP than in Wistar rats
- CRF produces divergent effects on glutamate release in Wistar rats
- In CeA neurons of msP rats CRF only increases glutamate release

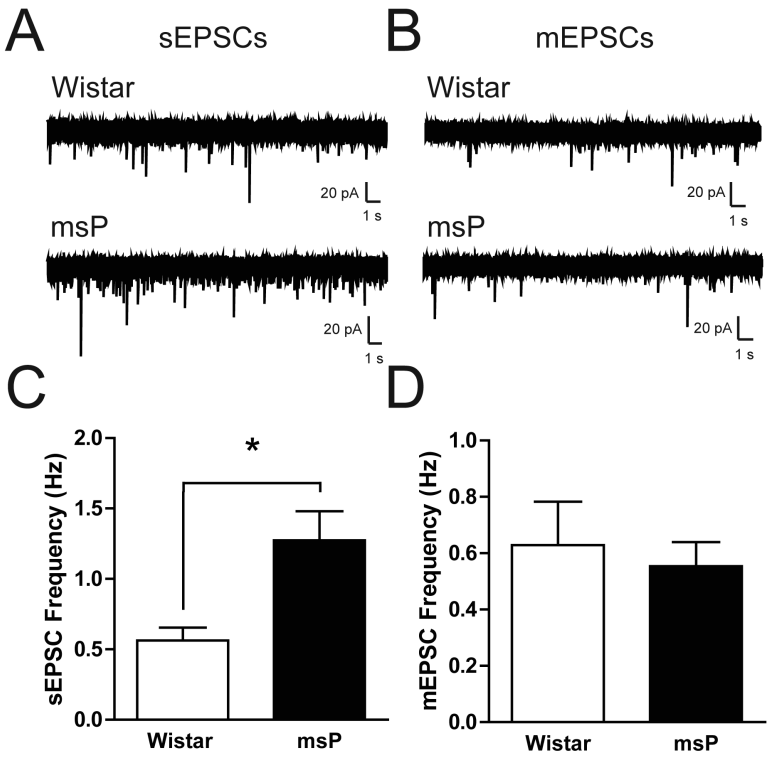


**Figure 1.**

Baseline spontaneous glutamate transmission is elevated in msP rats as compared to Wistar rats. **A**) Representative sEPSCs in CeA neurons from a Wistar rat (top trace) and an msP rat (bottom trace). **B**) Representative mEPSCs in CeA neurons from a Wistar rat (top trace) and an msP rat (bottom trace). **C**) Summary (mean ± SEM) of baseline sEPSC frequency in CeA neurons from Wistar (n = 26) and msP (n = 19) rats (*p<0.05 by unpaired t-test). **D**) Summary of baseline mEPSC frequency in CeA neurons from Wistar (n = 18) and msP (n = 17) rats.

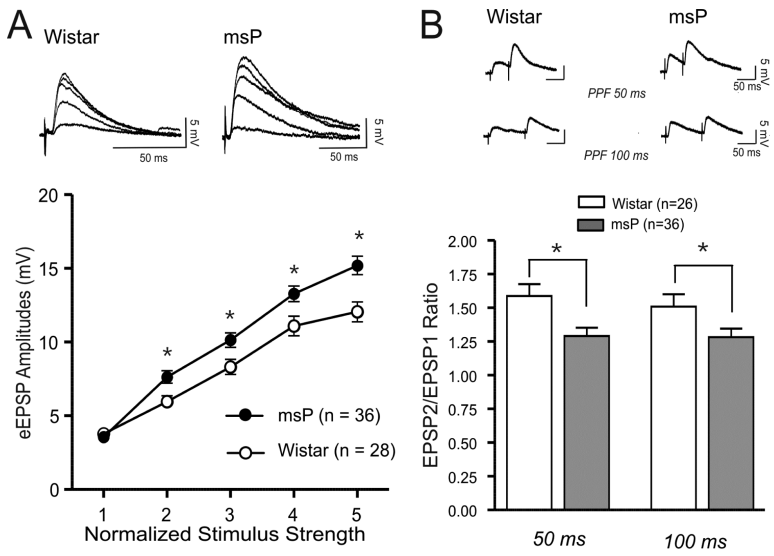


**Figure 2.**

Baseline evoked EPSP amplitudes are elevated in CeA neurons of msP rats compared to Wistar rats. **A**) *Top panel*: Representative evoked glutamatergic-EPSPs in CeA neurons from Wistar and msP rats. *Bottom Panel*: The input-output curves of mean baseline eEPSPs are enhanced in CeA neurons from msP (n = 38) compared to Wistar rats (n = 28). **B**) *Top Panel*: Representative recordings of evoked 50 and 100 msec PPF of eEPSPs in CeA neurons from msP and Wistar rats. *Bottom Panel*: Histograms (mean ± SEM) plotting the baseline PPF ratio of eEPSPs in CeA neurons from msP and Wistar rats. In the msP (n = 38) baseline PPF ratios are significantly (*$p < 0.05$) lower than Wistar (n = 26) neurons.

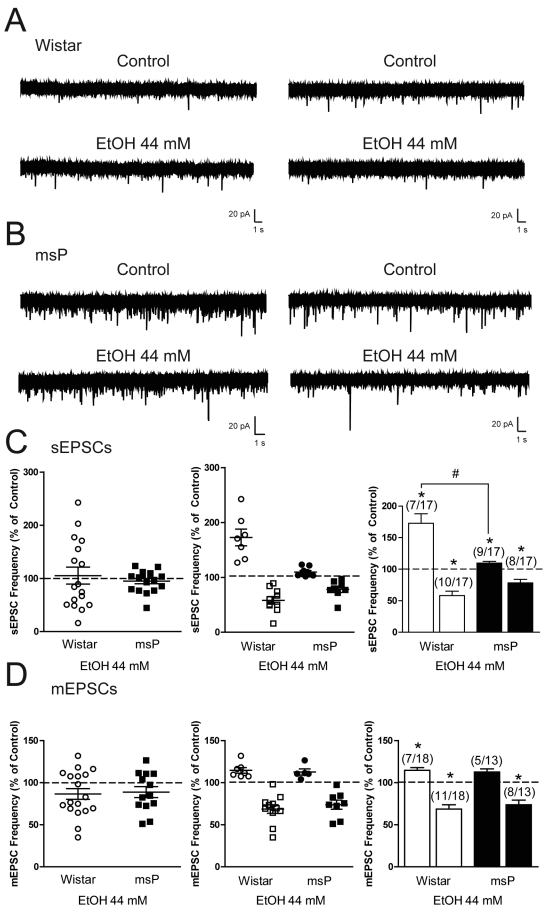


**Figure 3.**

The divergent effects of ethanol on CeA glutamatergic transmission are altered in msP rats as compared to Wistar rats. **A**) Representative sEPSCs in CeA neurons from a Wistar rat showing an increase (left) and decrease (right) in sEPSC frequency following ethanol (EtOH 44 mM) superfusion. **B**) Representative sEPSCs in a CeA neuron from a msP rat showing a small increase in sEPSC frequency (left) and a decrease in sEPSC frequency following ethanol (EtOH 44mM) superfusion. **C) Left panel:** Scatterplot displaying the unbinned individual changes in sEPSC frequency following ethanol application in each cell. **Center panel:** Scatterplot displaying the individual changes in sEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in sEPSC frequency (% of control) following ethanol superfusion in CeA neurons from Wistar (n = 17) and msP (n = 17) rats (*p<0.05 by one sample t-test; #p<0.05 by one-way ANOVA with Bonferroni *post hoc* comparison). **D**) **Left panel:** Scatterplot displaying the unbinned individual changes in mEPSC frequency following ethanol application in each cell. **Center panel:** Scatterplot displaying the individual changes in mEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in mEPSC frequency (% of control) following ethanol superfusion in CeA neurons from Wistar (n = 18) and msP (n = 13) rats.

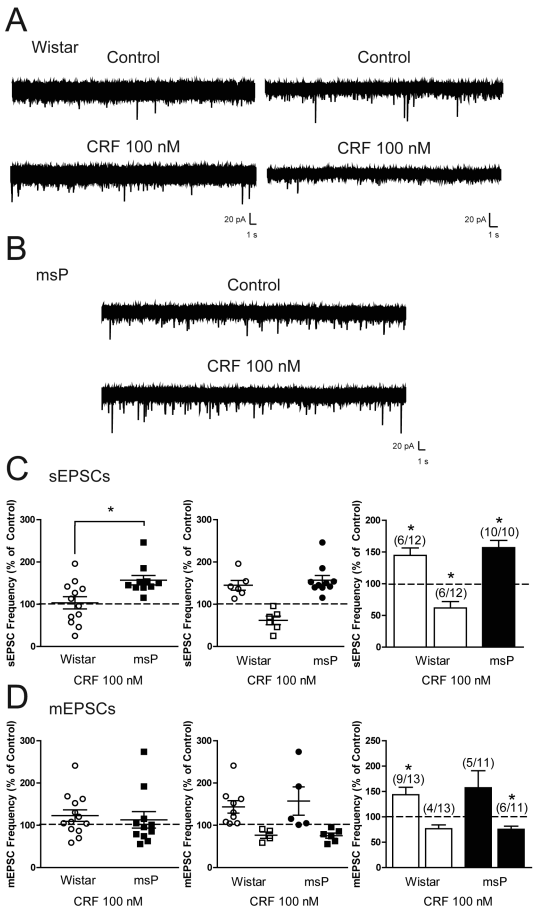


**Figure 4.**

The divergent effects of CRF on CeA glutamatergic transmission are lost in msP rats as compared to Wistar rats. **A**) Representative sEPSCs in CeA neurons from a Wistar rat showing an increase (left) and decrease (right) in sEPSC frequency following CRF (100 nM) superfusion. **B**) Representative sEPSCs in a CeA neuron from an msP rat showing an increase in sEPSC frequency following CRF superfusion. **C**) **Left panel:** Scatterplot displaying the unbinned individual changes in sEPSC frequency following CRF application in each cell. **Center panel:** Scatterplot displaying the individual changes in sEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in sEPSC frequency (% of control) following CRF superfusion in CeA neurons from Wistar (n = 12) and msP (n = 10) rats (*p<0.05 by one sample t-test). **D**) **Left panel:** Scatterplot displaying the unbinned individual changes in mEPSC frequency following CRF application in each cell. **Center panel:** Scatterplot displaying the individual changes in mEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in mEPSC frequency (% of control) following CRF superfusion in CeA neurons from Wistar (n = 13) and msP (n = 11) rats.

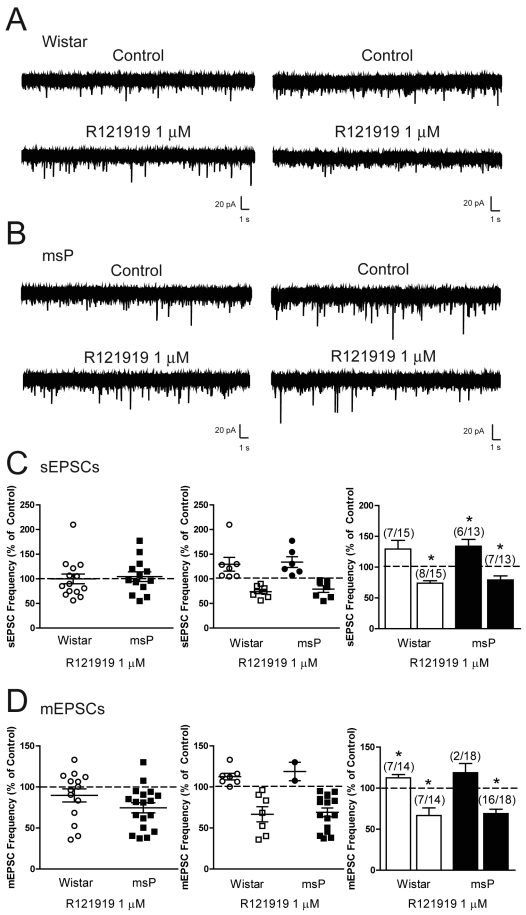


**Figure 5.**

The divergent effects of CRF1 receptor blockade on CeA glutamatergic transmission are retained in msP rats as compared to Wistar rats. **A)** Representative sEPSCs in CeA neurons from a Wistar rat showing an increase (left) and decrease (right) in sEPSC frequency following R121919 (1 μM) superfusion. **B**) Representative sEPSCs in CeA neurons from msP rats showing an increase (left) and a decrease in sEPSC frequency (right) following R121919 (1 μM) superfusion. **C**) **Left panel:** Scatterplot displaying the unbinned individual changes in sEPSC frequency following R121919 application in each cell. **Center panel:** Scatterplot displaying the individual changes in sEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in sEPSC frequency (% of control) following R121919 superfusion in CeA neurons from Wistar (n = 15) and msP (n = 13) rats (*p<0.05 by one sample t-test). **D**) **Left panel:** Scatterplot displaying the unbinned individual changes in mEPSC frequency following R121919 application in each cell. **Center panel:** Scatterplot displaying the individual changes in mEPSC frequency in each cell grouped by net change from control levels. **Right panel:** Summary (mean ± SEM) of the change in mEPSC frequency (% of control) following R121919 superfusion in CeA neurons from Wistar (n = 14) and msP (n = 18) rats.

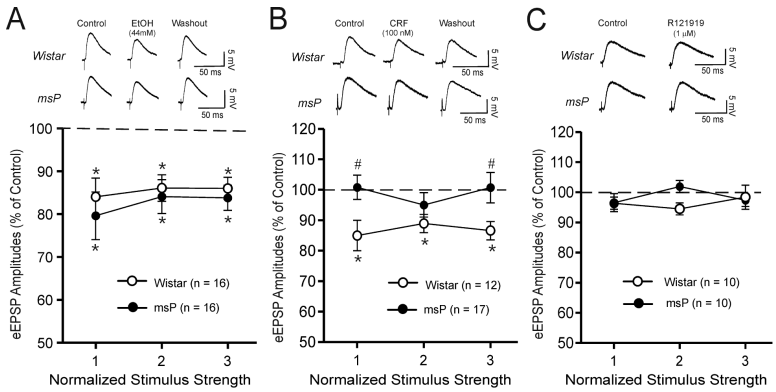


**Figure 6.**

Effects of ethanol, CRF and R121919 on evoked EPSP amplitude in CeA neurons of Wistar and msP rats. **A)** Ethanol significantly and reversibly decreases EPSP amplitude. *Top:* Representative eEPSPs in CeA neurons of Wistar and msP rats during baseline, ethanol (44 mM) and washout. *Bottom:* Ethanol significantly (*p<0.05 by paired t-test) decreases the mean amplitudes of eEPSP over the middle three stimulus strength intensities tested in both Wistar and msP rats. **B)** CRF significantly (*p<0.05) decreases eEPSP amplitudes in the CeA of Wistar rats, but had no effect in msP rats (#p<0.05 as compared to Wistar by two-way ANOVA). *Top:* Representative CeA eEPSP recordings from Wistar and msP rats during baseline, CRF (100 nM), and washout. *Bottom:* CRF decreases the mean amplitude of eEPSPs in CeA neurons of Wistar, but not msP, rats. **C)** The CRF1 antagonist, R121919, does not alter eEPSP amplitudes in either strain. *Top:* Representative eEPSPs in CeA neurons of Wistar and msP rats during baseline, R121919 (1μM) and washout. *Bottom:* R121919 does not affect the mean amplitudes of eEPSPs over the middle three stimulus strength intensities in either Wistar or msP rats.