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Article

Alcohol Dependence Induces CRF Sensitivity in Female Central Amygdala GABA Synapses

Larry Rodriguez[1]*, Dean Kirson[1,2]*, Sarah A. Wolfe[1], Reesha R. Patel[1], Florence P. Varodayan[1,3], Angela E. Snyder[1], Pauravi J. Gandhi[1], Sophia Khom[1, 4], Roman Vlkolinsky[1], Michal Bajo[1] and Marisa Roberto[1]

[1] The Scripps Research Institute, Department of Molecular Medicine, La Jolla, CA 92037, USA
[2] The University of Tennessee Health Science Center, Department of Pharmacology, Addiction Science, and Toxicology, Memphis, TN 38163, USA
[3] Binghamton University-SUNY, Department of Psychology, Binghamton, NY, USA
[4] Department of Pharmaceutical Sciences, University of Vienna Josef-Holaubek-Platz 2, A-1090 Vienna, Austria
* These authors contributed equally
Correspondence: Marisa Roberto, e-mail: mroberto@scripps.edu Tel: (858) 784-7262, FAX: (858) 784-7405

Abstract: Alcohol use disorder (AUD) is a chronically relapsing disease characterized by loss of control in seeking and consuming alcohol (ethanol) driven by recruitment of brain stress systems. However, AUD differs among the sexes: men are more likely to develop AUD, but women progress from casual to binge drinking and heavy alcohol use more quickly. The central amygdala (CeA) is a hub of stress and anxiety, with corticotropin releasing factor (CRF)-CRF_1 receptor and GABAergic signaling dysregulation occurring in alcohol dependent male rodents. However, we recently showed that GABAergic synapses in female rats are less sensitive to the acute effects of ethanol. Here, we used patch clamp electrophysiology to examine the effects of alcohol dependence on the CRF-modulation of rat CeA GABAergic transmission of both sexes. We found that GABAergic synapses of naïve female rats were unresponsive to CRF application compared males, although alcohol dependence induced a similar CRF responsivity in both sexes. *In situ* hybridization revealed that females had less CeA neurons containing mRNA for the CRF_1 receptor (*Crhr1)* than males, but in dependence, the percentage of *Crhr1*-expressing neurons in females increased, unlike males. Overall, our data provide evidence for sexually dimorphic CeA CRF system effects on GABAergic synapses in dependence.

Keywords: corticotropin releasing factor (CRF); patch-clamp electrophysiology; sex difference; alcohol use disorder (AUD); Gamma-Aminobutyric Acid (GABA); central amygdala (CeA); spontaneous inhibitory post synaptic currents (sIPSCs)

1. Introduction

Alcohol use disorder (AUD) is a chronically relapsing disorder characterized by a preoccupation with alcohol consumption, a loss of control in limiting intake, and the emergence of negative emotional states during withdrawal (also known as hyperkatifeia, which includes dysphoria, anxiety, irritability) [1, 2]. Excessive alcohol consumption associated with negative emotional states observed in withdrawal occurs via negative reinforcement mechanisms such that alcohol alleviates the symptoms of withdrawal [3-7]. Thus, brain regions, such as the central nucleus of the amygdala (CeA), that are involved in processing stress, anxiety, and other withdrawal-associated states are recruited in the development of AUD. The CeA is a primarily GABAergic nucleus that has been shown to be involved in the excessive alcohol consumption related to dependence and withdrawal, across species [8-11].

The CeA also contains many neuropeptide systems that modulate synaptic activity. The peptide corticotropin releasing factor (CRF), a key mediator of stress responses, is locally produced and released by neurons within multiple brain regions, including the

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hypothalamus, the CeA, and other afferent regions[12]. CRF is a 41-residue polypeptide that binds to the G-protein-coupled CRF type 1 (CRF_1) and CRF type 2 (CRF_2) receptors [13, 14]. While CRF produces its effects by binding to both receptors, it has greater affinity for CRF_1 [12, 15]. The role of the CRF_1 system has been characterized in multiple rodent models of alcohol dependence. For example, ethanol withdrawal increased CRF levels in the amygdala of rats [16]. In alcohol dependent rats, chronic treatment with a CRF_1 antagonist blocked alcohol withdrawal-induced increases in alcohol drinking, and in non-dependent rats the CRF_1 antagonist tempered moderate increases in alcohol consumption [17]. In addition, the escalation of alcohol self-administration and anxiety typically observed during protracted abstinence can be blocked by competitive CRF_1 antagonists [18-21].

Despite preclinical evidence suggesting that CRF_1 antagonists would be efficacious in the treatment of alcohol dependence, clinical trials of CRF_1 antagonist-based therapies to treat AUD in humans have shown mixed results[22, 23]. Thus, more work is needed to fully understand the neuroadaptations that facilitate and sustain alcohol dependence, and in particular, the impact of sex on the underlying neurobiology of the disease. Potential sex differences are especially important considering the heightened activity of stress- and anxiety-related brain regions in alcohol dependence, and the inherent sex differences in stress responses [24, 25]. Many neuropsychiatric disorders, including anxiety disorders and stress- and trauma-related disorders, differ by sex [26-28], and preclinical studies have shown differences in stress and anxiety processing between males and females [24]. More recently, transgenic reporter mice have been used to study sex differences in the CRF_1 system [29], and while dependence has been shown to sensitize females to the effects of acute alcohol [30], to our knowledge, no one has studied the neuroadaptations in the female CeA CRF system during alcohol dependence.

In this study, we induced alcohol dependence in rats using an established model of chronic intermittent ethanol vapor exposure and used whole-cell patch clamp electrophysiology and *in situ* hybridization (ISH) to identify sex-specific neuroadaptations in the regulatory function of CRF at GABAergic synapses. Within the CeA of naïve and dependent rats of each sex, we determined the effects of exogenous acute application of different concentrations of CRF and assessed tonic CRF_1 receptor activity (using a selective CRF_1 antagonist, R121919) on spontaneous inhibitory GABAergic postsynaptic currents (sIPSCs). We also determined the levels of mRNA co-expression of CRF_1 receptor with GABAergic neuronal markers in the CeA in both sexes.

2. Results

2.1. Sex differences in baseline sIPSC kinetics of CeA GABA synapses

We performed whole-cell patch-clamp recordings of GABAergic sIPSCs (Figure 1A) from neurons (n = 132) in the medial subdivision of the CeA of both male (left) and female (right), naïve (top) and alcohol dependent (bottom) rats. In line with our previous results [30], here we found no main effect of sex or alcohol exposure on baseline sIPSC frequency (Figure 1B), amplitude (Figure 1C), or rise time (Figure 1D). Furthermore, our results revealed a main effect of alcohol exposure on sIPSC decay time (two-way ANOVA; Alcohol Exposure $F_{1,132} = 14.2$, $p < 0.001$) as dependent animal groups had higher decay times on average (10.94 ms) compared to naïve animal groups (8.66 ms) (as in [30]) (Figure 1E). Additionally, there was neither a main effect of Sex nor a Sex x Alcohol Exposure interaction effect. This data suggests that alcohol dependence is altering postsynaptic $GABA_A$ receptor function.

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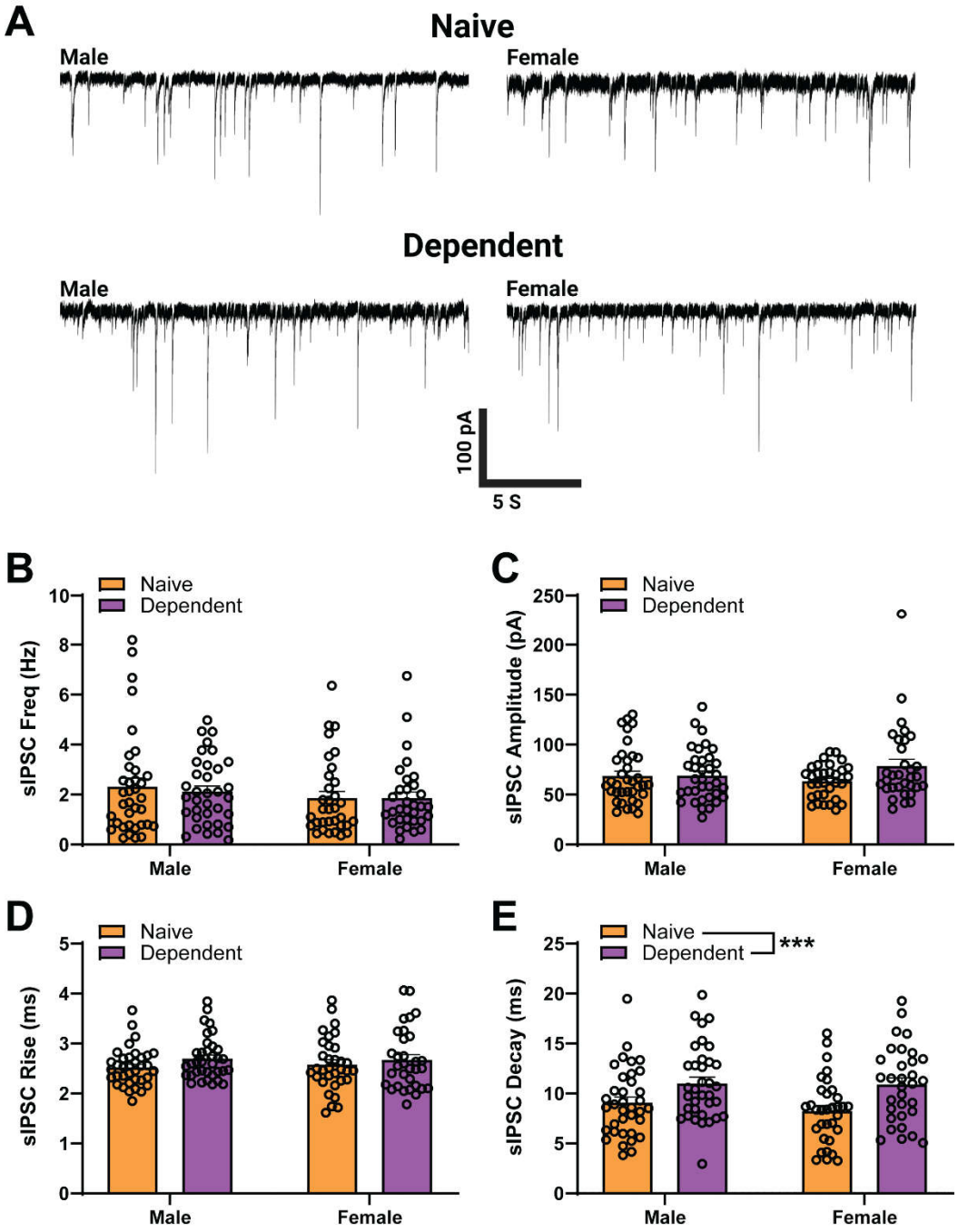

Figure 1. Baseline spontaneous GABAergic transmission in CeA of males and females. (*a-f*) (*a*) Representative GABA$_A$-mediated sIPSCs from CeA neurons of male (left) and female (right) rats, either naïve (upper) or alcohol dependent (lower). There is no effect of Sex or Alcohol Exposure and no interaction effect in CeA baseline sIPSC frequency (*b*), amplitude (*c*), or rise time (*d*). For sIPSC decay time (*e*), there is no main effect of Sex or interaction effect, but there is a main effect of Alcohol Exposure, primarily driven by dependent females having a prolonged decay time (10.88 ± 0.67 ms) compared to naïve females (8.24 ± 0.57 ms). Differences in baseline sIPSC properties are assessed using two-way ANOVA test.; Bars represent *Mean* ± *SEM*; *** denotes $p < 0.001$; n = 31–36 neurons per group; N = 19-25 rats per group.

2.2. Alcohol dependence induces responsivity of female CeA GABAergic synapses to acute CRF application

We next investigated the effects of acute CRF on CeA sIPSCs of both sexes (Figure 2A-B), at a concentration previously determined to have a maximal effect in males (200 nM, [17]). We found a significant main effect of Alcohol Exposure on sIPSC frequency

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(two-way ANOVA; $F_{1,37} = 5.01$, $p < 0.05$; Figure 2C) such that in alcohol dependent groups, acute application of CRF significantly increased the sIPSC frequency (as a percent of baseline) to a larger extent than in naïve groups. While we did not find a significant main effect of sex, there was a significant Sex x Alcohol Exposure interaction effect (two-way ANOVA; $F_{1,37} = 4.37$, $p < 0.05$) driven by naïve females. Specifically, acute application of 200 nM CRF did not significantly increase the sIPSC frequency of naïve females as it did naïve males (Šídák; $t_{9,12} = 3.09$, $p < 0.05$). There were no significant differences in sIPSC amplitude (Figure 2D), rise time (Figure 2E), or decay time (Figure 2F) across groups. These results indicate that acute CRF enhances presynaptic GABA release in all groups except naïve females, and that alcohol dependence induces similar responsivity to acute CRF in males and females.

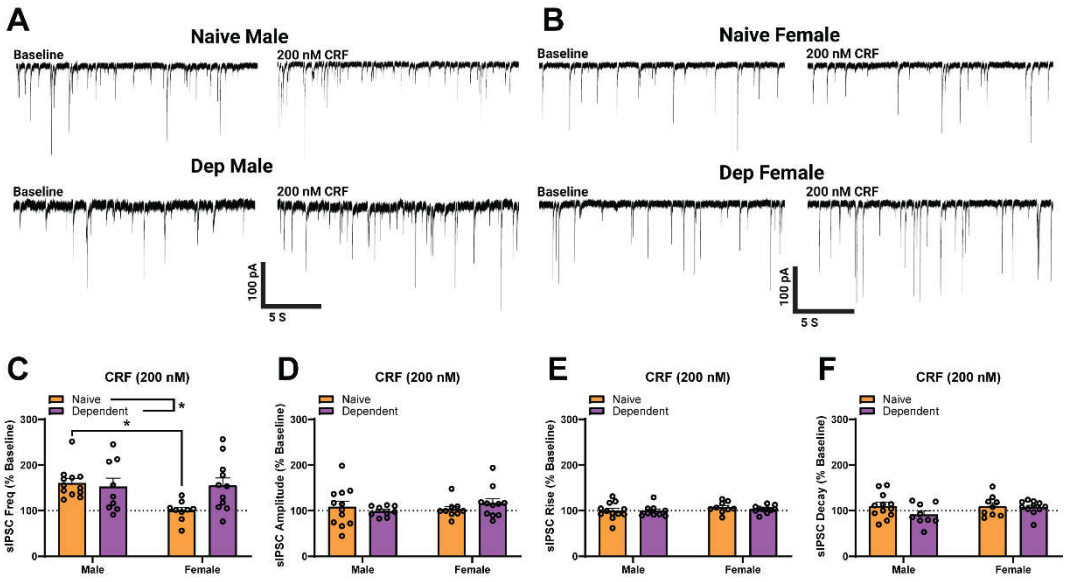

Figure 2. GABAergic transmission in the CeA of naïve females is insensitive to CRF compared to males. (***a***,***b***) Representative $GABA_A$-mediated sIPSCs from CeA neurons of naïve (upper) and dependent (lower) male (***a***) and female (***b***) rats at baseline (left) and during acute application of CRF (200 nM; right). (***c***) Acute CRF increases CeA sIPSC frequency in naïve (160.4 ± 9.8% of baseline) and dependent (157.1 ± 18.2% of baseline) males. Acute CRF has no effect on sIPSC frequency in naïve (100.3 ± 7.3% of baseline) females but increases sIPSC frequency in dependent (155.0 ± 16.9% of baseline) females. For sIPSC frequency, a main effect of Alcohol Exposure was observed, as well as a significant interaction effect between Sex x Alcohol Exposure but no main effect of Sex. There were no significant differences in sIPSC amplitude (***d***), rise time (***e***), or decay time (***f***) during acute CRF application across groups. Differences in sIPSC properties were assessed using two-way ANOVA test with a post hoc Šídák's correction for multiple comparisons where * denotes $p < 0.05$. Bars represent *Mean* ± *SEM*; $n = 9$–12 neurons per group; $N = 5$-8 rats per group.

Given that the sex difference in CRF response was driven by naïve females, we tested lower (100 nM) and higher (400 nM) concentrations of CRF in naïve rats of each sex to compare their responsivity (Figure 3A-B). As shown in Figure 3C, we found a main effect of Sex (two-way ANOVA; $F_{1,53} = 14.34$, $p < 0.001$), but no main effect of CRF concentration on sIPSC frequency. However, we do report a Sex x CRF Concentration interaction effect ($F_{1,53} = 3.73$, $p < 0.05$) which was driven by the difference between male and female naïve responses to 200 nM CRF (Sidak; $t_{12,9} = 4.45$, $p < 0.001$). In addition, naïve males responded to low (one sample t-test, $t_9 = 3.06$, $p < 0.05$) and high (one sample t-test, $t_{11} = 2.69$, $p < 0.05$) concentrations of CRF, with a maximally effective concentration of 200 nM (one sample t-test, $t_{12} = 6.14$, $p < 0.0001$), which recapitulates our previous work. In contrast, we observed no concentration of CRF that produced an effective response in females (one sample t-test, $p > 0.05$). Collectively, these results suggest that acute CRF produces concentration-dependent responses in naïve males but not in females. There were no effects of Sex or CRF

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Concentration on sIPSC amplitude (Figure 3D), rise time (Figure 3E), or decay time (Figure 3F).

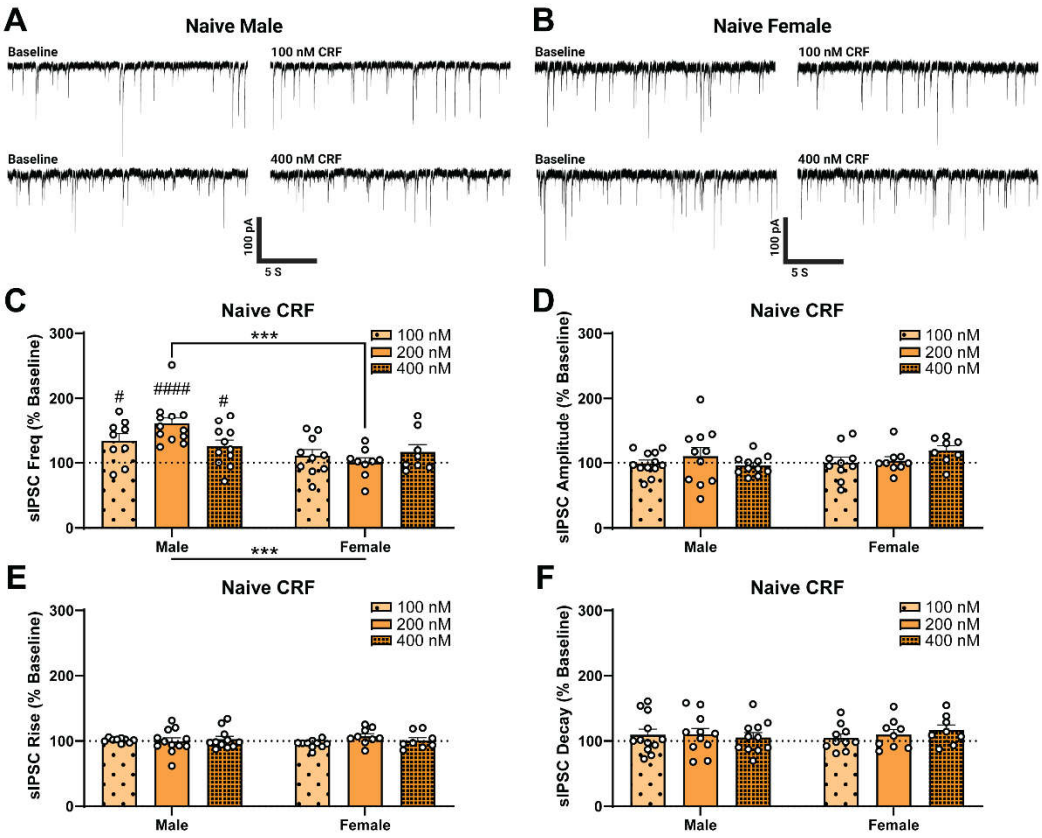

Figure 3. Spontaneous GABAergic transmission in the CeA of naïve females is insensitive to CRF. (***a-f***) Male and female sIPSC responses to varied concentrations of CRF. Representative $GABA_A$-mediated sIPSCs from CeA neurons of naïve male (***a***) and female (***b***) rats at baseline (left) and during acute application of 100 nM CRF (right, upper) or 400 nM CRF (right, lower). (***c***) While CRF significantly increases sIPSC frequency above baseline in males, CRF has no significant effect on naïve females. In addition to a main effect of Sex, we observed a Sex x CRF Concentration interaction effect, but no main effect of CRF Concentration. There were no main effects of Sex or CRF Concentration and no interaction effect on sIPSC amplitude (***d***), rise time (***e***), or decay time (***f***). Changes from baseline sIPSC properties were assessed using one sample t-tests where # denotes $p < 0.05$ and #### denotes $p < 0.0001$. Differences between naïve male and female sIPSC properties in response to CRF Concentrations were assessed using two-way ANOVA test with a post hoc Šídák's correction for multiple comparisons where * denotes $p < 0.05$ and *** denotes $p < 0.001$. Bars represent *Mean* ± *SEM*; 200 nM responses are the same as in Figure 2; n = 8–12 neurons per group; N =4-7 rats per group.

We then assessed the CRF sensitivity of male and female dependent rats (Figure 4A-B). Consistent with previous findings [17] high (400 nM) concentrations of CRF increased sIPSC frequency of dependent males (one sample t-test, $t_9 = 2.52$, $p < 0.05$) while a low concentration of CRF (100 nM; one sample t-test, $p > 0.05$) did not. In addition, high (400 nM) but not low (100 nM) concentrations of CRF also increased sIPSC frequency of dependent females (one sample t-test, $t_{11} = 5.99$, $p < 0.001$), similar to what we observed in the dependent males (Figure 4C). In contrast to the naïves, group analysis of dependent rats revealed a significant main effect of CRF Concentration (two-way ANOVA test, $F_{2,51} = 6.19$, $p < 0.01$) on sIPSC frequency, but no main effect of Sex and no interaction effect. Additionally, there was no main effect of Sex, no main effect of CRF Concentration, and no interaction effect on sIPSC amplitude (Figure 4D), rise time (Figure 4E), or decay time (Figure 4F). These results indicate that in alcohol dependence, the female CeA becomes

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responsive to acute CRF, and the CRF concentration responsivity is similar to dependent males.

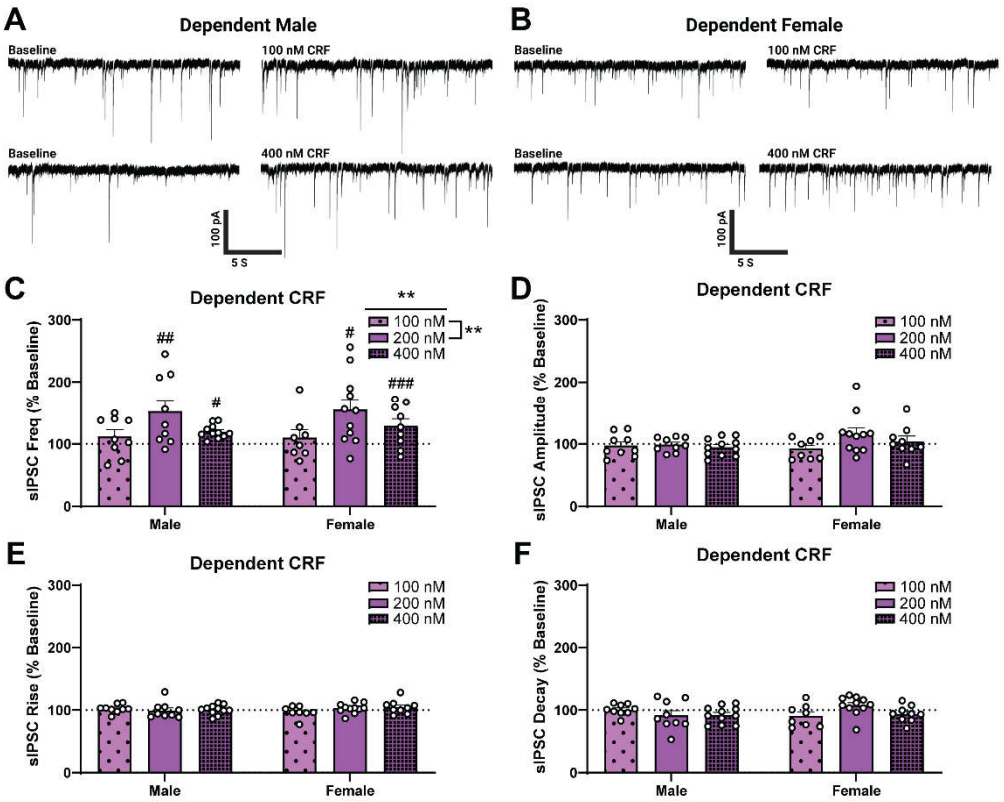

Figure 4. Alcohol dependence induces CRF responsivity of GABAergic synapses in female CeA. (***a***, ***b***) Representative $GABA_A$-mediated sIPSCs from CeA neurons of dependent male (***a***) and female (***b***) rats at baseline (left) and during acute application of 100 nM CRF (right, upper) or 400 nM CRF (right, lower). (***c-f***) Alcohol dependent male and female sIPSC responses to varied CRF Concentrations. There is a main effect of CRF Concentration on sIPSC frequency, but no main effect of Sex and no interaction effect. There was no main effect of Sex or CRF Concentration and no interaction effect on sIPSC amplitude (**d**), rise time (**e**), or decay time (**f**). Changes from baseline sIPSC properties were assessed using one sample t-tests where # denotes $p < 0.05$ and ### denotes $p < 0.001$. Differences between male and female sIPSC properties in response to CRF Concentrations were assessed using two-way ANOVA test with a post hoc Šídák's correction for multiple comparisons where ** denotes $p < 0.01$. Bars represent *Mean* ± *SEM*; 200 nM responses are the same as in Figure 2; $n = 8–11$ neurons per group; $N = 3$-8 rats per group.

2.3. Alcohol dependence alters CRF_1 receptor expression in females.

CRF_1 receptors mediate most of the effects of CRF on GABA signaling within the CeA [17, 31, 32]. Given that CRF responses of GABAergic synapses varied by sex, we investigated whether these differences reflected changes in CRF_1 receptor expressing in GABAergic neurons in the CeA. To address this, we utilized *in situ* hybridization (RNAscope) to identify the percent of nuclei expressing CRF_1 (*Crhr1*+), and GAD2 (*Gad2*+) mRNA in CeA sections of naïve (Figure 5A) and alcohol dependent (Figure 5B) female and male rats. First, we analyzed the basal CeA expression patterns of *Crhr1* in naïve rats of each sex and found that females have significantly less *Crhr1*+ cells relative to males (Figure 5C; $p < 0.05$). We then determined the expression pattern of *Crhr1* in dependent rats. We found that in dependent males, the amount of *Crhr1*+ cells did not significantly differ from naïve males (Figure 5D); however, female dependent rats had significantly more *Crhr1*+ cells than naïve females (Figure 5E; $p < 0.05$). Both female and male rats displayed a high co-expression of *Gad2*+ in the *Crhr1*+ cell population (Figure 5F-G). These data suggest that the CeA of naïve female rats have a lower percentage of *Crhr1*+ cells than naïve males, but alcohol dependence increases the percentage of *Crhr1*+ cells in females.

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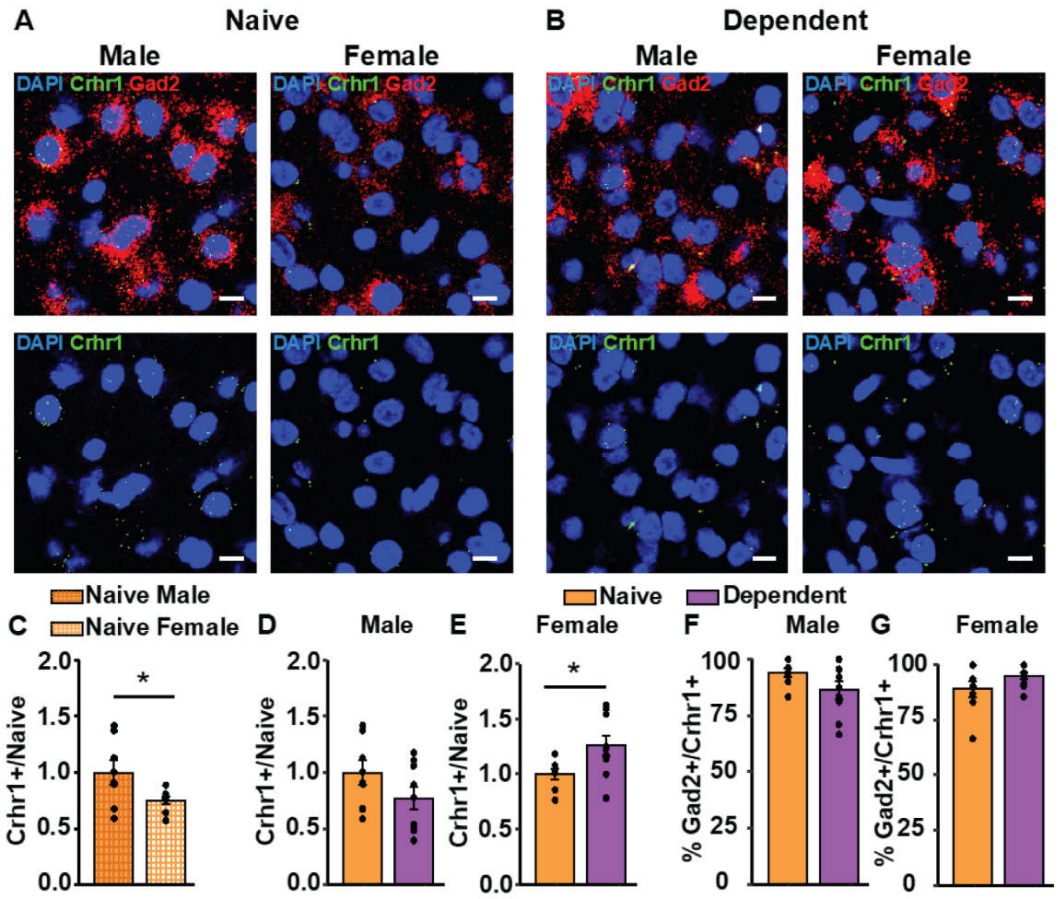

Figure 5. Alcohol dependence alters *Crhr1*+ cells in the CeA in females. Representative images of *Crhr1* (green), *Gad2* (red), and DAPI (blue) for (***a***) naïve and (***b***) alcohol dependent male (*left*) and female (*right*) rats in the CeA. Scale bar = 10 μm. (***c***-***e***) Summary bar graphs indicating relative proportion of nuclei expressing *Crhr1* (Crhr1+) between (***c***) naïve male and female rats, (***d***) naïve male and dependent male rats, and (***e***) naïve female and dependent female rats. (***f***-***g***) Summary bar graphs indicating the percentage of CeA nuclei co-expressing *Gad2* in the *Crhr1*+ population (*Gad2*+/*Crhr1*+) in naïve and dependent male (***f***) and female (***g***) rats. $n = 7$-9 images from 3 rats/group; bars represent *Mean* ± *SEM*; * denotes $p < 0.05$.

2.4. Alcohol dependence induces tonic activation of CRF₁ at CeA GABAergic synapses in males only.

Lastly, to compare the basal function of CRF₁ on CeA GABA transmission in each sex, we tested the effects of CRF₁ selective antagonism on sIPSCs via acute application of 1 μM R121919 (Figure 6A) [17, 33]. There was no significant main effect of Sex or Alcohol Exposure and no interaction effect on sIPSC frequency (Figure 6B) amplitude (Figure 6C), rise time (Figure 6D), or decay time (Figure 6E). While selective antagonism of CRF₁ did not alter CeA GABAergic transmission in naïve males, it significantly decreased GABA release from baseline in dependent male rats (Figure 6B; one sample t-test, $t_9 = 2.971$, $p < 0.05$). In contrast to the males, R121919 did not alter sIPSC properties in either naïve or dependent female rats, suggesting that tonic activation of CRF₁ may be specific to dependent males.

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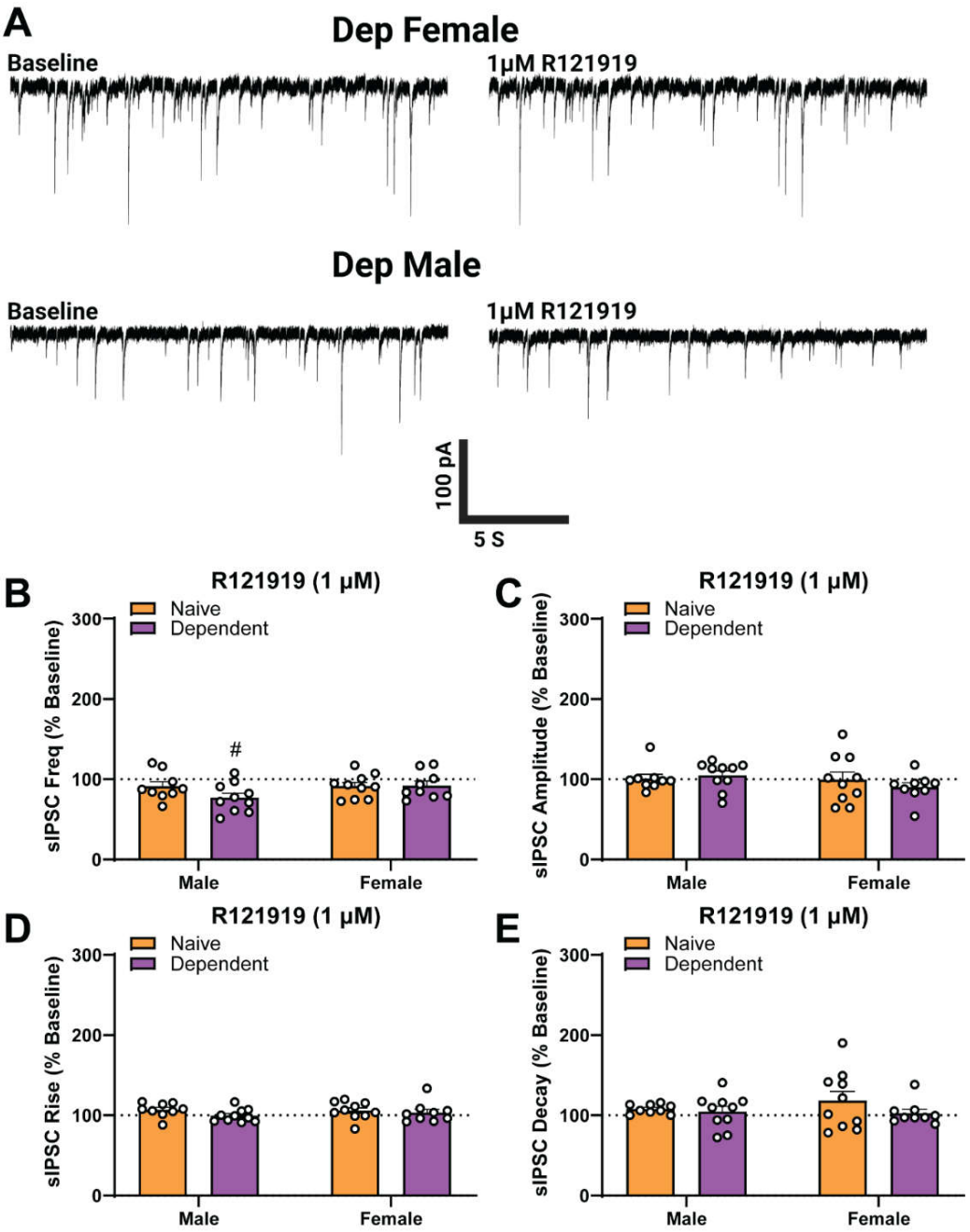

Figure 6. Alcohol dependent males, but not females, have a basal CRF$_1$-mediated tone in the CeA. (**a**) Representative GABA$_A$-mediated sIPSCs from CeA neurons of dependent female (upper) and male (lower) rats at baseline (left) and during acute application of R121919 (1 μM; right). There was no main effect of Sex or Alcohol Exposure and no interaction effect of R121919 on sIPSC frequency (**b**), amplitude (**c**), rise time (**d**), or decay time (**e**). Changes from baseline sIPSC properties were assessed using one sample t-tests where # denotes $p < 0.05$. Group differences in the sIPSC responses to R121919 were assessed using two-way ANOVA test but were not observed. n = 9–10 neurons per group; N = 5-6 animals per group; bars represent *Mean* ± *SEM*.

3. Discussion

In this study, we identified distinct, sexually dimorphic responses of the CRF system on GABAergic synapses in the CeA of naïve and alcohol dependent rats (see schematic in Figure 7). In brief, we found that CeA neurons of naïve male and female rats display similar baseline presynaptic GABAergic inputs and postsynaptic receptor function. However, alcohol dependence induced an increase in the baseline decay times of postsynaptic GABA$_A$ receptor-mediated currents in CeA neurons of both sexes. We recapitulated our

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previous work [17, 33, 34] showing that a maximal concentration (200 nM) of CRF increased action-potential dependent GABA release in the CeA of naïve and dependent male rats but found naïve female CeA unresponsive to CRF. We then characterized the effects of high and low CRF concentrations in each sex, which confirmed that naïve females do not respond to CRF. Interestingly, alcohol dependence induced CRF responsivity in females to a similar degree observed in dependent males such that no sex difference was observed. We hypothesized that this heightened responsivity to CRF was due to changes in CRF_1 expression in GABAergic neurons in the CeA, which was confirmed via *in situ* hybridization. Lastly, selective antagonism of CRF_1 with R121919 revealed that tonic CRF_1 activation, which regulates GABA release, occurred only in dependent males. R121919 had no effect on naïve or dependent females suggesting a lack of tonic CRF signaling in females.

We recently reported the acute alcohol insensitivity of GABAergic CeA neurons in naïve females, which was maintained in alcohol dependent females except at the highest concentration (88 mM) of ethanol [30]. Here, we found a similar profile in the CRF system such that naïve female CeA neurons were unresponsive to acute CRF, and alcohol dependence induced responsivity in these CeA neurons to high but not low concentrations of CRF. Other studies have reported similar sexually dimorphic effects of the CRF system in CeA. For example, Rouzer et al found a Sex x Age interaction in basal spontaneous GABA synaptic transmission within the rat CeA, but only age differences in action potential-independent GABA release [34]. However, sex differences mediated by the selective CRF_1 agonist Stressin-1 emerged: opposing effects of Stressin-1 were observed in the action potential-independent GABA release of adolescent and adult males, while in the females there was no change in the response between adolescents and adults [34]. Furthermore, in a study using transgenic CRF_1 reporter mice, voluntary alcohol drinking increased the sensitivity of CRF_1-positive neurons in the CeA to the effects of acute alcohol in males but not females. In contrast, alcohol drinking increased acute CRF sensitivity of these neurons in both males and females [35]. Tonic CRF activity was also found to be sexually dimorphic, as the CRF_1 antagonist R121919 decreased GABA release in water and alcohol drinking females, but not in either male group [35]. A study by Retson and colleagues also reported sex differences in the CRF system of the rat CeA, which found that alcohol drinking activated CeA CRF neurons and enhanced the response of these neurons to stress selectively in male but not female rats [36].

Here, we found that alcohol dependence increased the CRF responsivity of GABAergic synapses in females while male responses remained elevated. Our results also showed that CRF_1 antagonism using R121919 significantly decreased (action-potential dependent) GABA release in dependent males, revealing a basal activity of these receptors in the modulation of CeA GABA transmission after chronic ethanol exposure. In contrast, CRF_1 antagonism did not alter basal CeA GABA transmission in either naïve or dependent female rats. A similar difference was seen for action-potential independent GABA release by Rouzer et al, where the CRF_1 selective antagonist NBI 35965 increased GABA release in adult male but not female rats [34]. To that end, one limitation of the current study is that we did not investigate action-potential independent GABA transmission, which should be addressed in future work.

Some of the discrepancies across studies may be explained by the differential effects produced by alcohol paradigms (i.e., voluntary alcohol drinking vs. vapor-induced alcohol dependence) the age of the study animals, and the properties of the different CRF_1 agonists and antagonists. However, another important factor to consider is the composition of the cell population being studied, which can be associated with distinct responses. For instance, the Agoglia et al. study specifically targeted CRF_1+ neurons in mice [35], while our electrophysiology data come from neurons of unknown CRF_1 expression in rats. As our *in situ* hybridization data shows, naïve females have fewer GABAergic neurons expressing CRF_1. Thus, our selection of neurons in females were less likely to be CRF_1+ than in males. It is possible that the sensitivity of rat CeA CRF_1+ neurons to CRF system

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agonism and antagonism matches that seen in mice, although re-examination of these effects in labeled CRF_1+ neurons of rats would need to be done in future studies to be certain.

However, in light of recent work [37], another possibility is that the animal models used (rats vs. mice) could be responsible for these differences. The alcohol-induced increase in GABA release upon acute CRF application in the CeA is an effect we have previously demonstrated in many species including mice, rats, and non-human primates [10, 17, 31, 32, 38]. In rats, this increase in GABA in the CeA has been implicated in excessive alcohol drinking of dependent animals, and this effect is mediated by locally projecting CRF neurons in the CeA [39-41]. However, in mice, chemogenetic activation or inhibition of these local CeA CRF neurons had no effect on alcohol consumption [37]. Thus, the involvement of the CeA CRF system in alcohol dependence may be species dependent, and this difference in CRF system functioning could explain the discrepancies between the rat and mouse data, including the dichotomous sex differences.

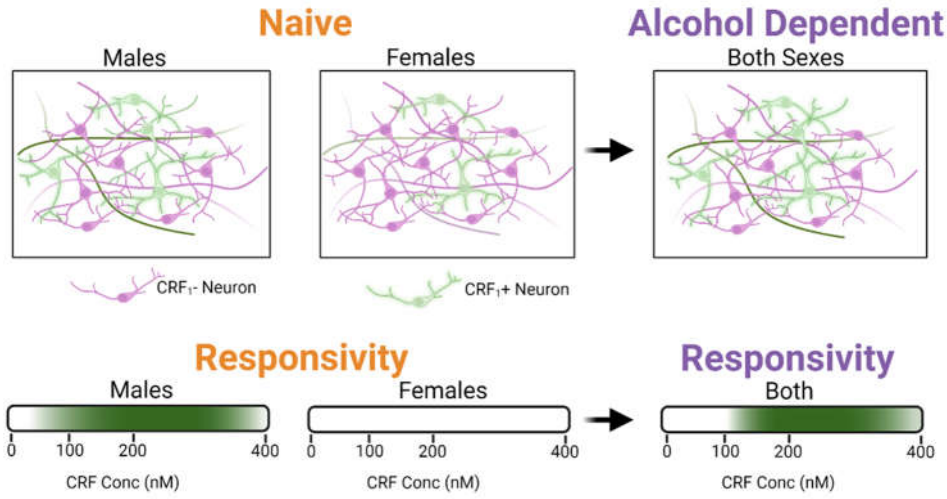

Figure 7. Summary of synaptic changes observed in the CeA during alcohol dependence in male and female rats. All neurons represented in the top figure are GABAergic. Green denotes CRF_1+ neurons, while purple denotes CRF_1- neurons. Naïve females have lower CRF_1 expression on GABAergic neurons and are less responsive to CRF than naïve males. In alcohol dependent females, CRF_1 expressing cell populations and CRF responsivity increase, similar to levels observed in dependent males. Created with BioRender.com.

Overall, our findings provide insight into the function of the CRF/CRF_1 system in the CeA of females and identify maladaptations in this system that occur during alcohol dependence.

4. Materials and Methods

Animals

Female (N = 45) and male (N = 40) Sprague Dawley rats (Charles River, Raleigh, NC) weighed on average 257.2 ± 9.3 g and 354.85 ± 16.9 g respectively at time of sacrifice. Estrous cycle was determined before euthanasia, but not selected for. All rats were housed in a temperature- and humidity-controlled room on a 12-hour light/dark cycle with food and water available ad libitum. Alcohol dependent rats (average blood alcohol level 172.68 ± 17.4 mg/dL at time of sacrifice) were generated by exposure to alcohol vapor daily (14 h alcohol vapor; 10 h air vapor) for 5 – 7 weeks [30, 42-44] Female (N = 6) and male (N = 6) Wistar rats (Charles River, Raleigh, NC) weighed on average 253.17 ± 12.7 g and 387.67 ± 24.5 g, respectively, at time of sacrifice. All procedures and care 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 of The Scripps Research Institute.

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Electrophysiology

Preparation of acute brain slices and electrophysiological recordings were performed as previously described [17, 30, 45, 46]. Rats were deeply anesthetized with isoflurane (3-5%) before decapitation and brain isolation. Coronal CeA slices (300 μm) were prepared using a Leica VT1200 vibratome (Leica Biosystems, Deer Park, IL, USA) in an ice-cold high-sucrose cutting solution (sucrose 206 mM; KCl 2.5 mM; $CaCl_2 \bullet 2H_2O$ 0.5 mM; $MgCl_2$ 7 mM; NaH_2PO_4 1.2 mM; $NaHCO_3$ 26 mM; glucose 5 mM; HEPES 5 mM). Slices were incubated and superfused (flow rate of 2–4 ml/min) with carbogen (95% O_2/5% CO_2) equilibrated artificial cerebrospinal fluid (aCSF; in mM: 130 NaCl, 24 $NaHCO_3$, 10 glucose, 1.5 $MgSO_4 \bullet 7H_2O$, 3.5 KCl, 1.25 $NaH_2PO_4 \bullet H_2O$, 2 $CaCl_2 \bullet 2H_2O$). Whole-cell patch-clamp recordings of GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) were performed in neurons from the medial subdivision of the CeA clamped at −60 mV. Patch pipettes (3 to 6 MΩ) were filled with an internal solution composed of (in mM): 145 KCl, 0.5 EGTA, 2 $MgCl_2$, 10 HEPES, 2 Mg-ATP, and 0.2 Na-GTP. For animal variability, each experimental group contained neurons from a minimum of 3–4 rats. GABAergic activity was pharmacologically isolated with 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), 30 μM DL-2-amino-5-phosphonovalerate (DL-AP5), and 1 μM CGP 55845A. In all experiments, cells with a series resistance greater than 20 MΩ were excluded from analysis, and series resistance was periodically monitored during gap-free recording with a 10 mV pulse. Cells in which series resistance changed more than 25% during the experiment were excluded from analysis. Data were analyzed using Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) with 3-min bins of gap-free recording. All drugs were applied by bath superfusion.

Drugs

CRF, CGP 55845A, DL-AP5, and DNQX were obtained from Tocris (Ellisville, MO). Drugs were added to the aCSF from stock solutions to obtain known concentrations in the superfusate. Stock solutions of AP-5, CGP 55845A and CRF were prepared in distilled water, while DNQX and R121919 hydrochloride (R12) were dissolved in 100% DMSO. All drugs were applied to the bath solution to achieve the final desired concentrations. The final DMSO concentration in the bath solution did not exceed 0.15%.

In situ hybridization and confocal microscopy

Male and female wistar rats (3 per treatment group) were anesthetized with isoflurane and transcardially perfused with ice cold phosphate-buffered saline (PBS) followed by Z-fix (Fisher Scientific, Waltham, MA). Brains were dissected, immersion fixed in Z-fix at 4°C for 24 hr, cryoprotected in 30% sucrose in PBS at 4°C for 24-48 hr, flash frozen in isopentane on dry ice, and stored at -80° C. Brains were then sliced on a cryostat into 20 μm thick sections, mounted on SuperFrost Plus slides (Fisher Scientific, 1255015), and stored at -80°C until use. In situ hybridization was performed using RNAscope fluorescent multiplex kit (ACD Biotechne, Catalog No. 320850) as previously described [47]. Briefly, target retrieval pretreatment was preformed according to the RNAscope manual, where slides were submerged at 95-98°C for 10 min in target retrieval buffer (ACD, Catalog No. 322000), immediately rinsed in distilled water, and then dehydrated in ethanol (stored at -80°C if needed), followed by incubation at 40°C for 20 min with protease IV. Next, the RNAScope Fluorescent Multiplex Reagent Kit User Manual was followed exactly. Lastly, slides were mounted and coverslipped with Vectashield+DAPI (Fisher Scientific). The probes used from ACD Biotechne were as follows: 3-plex negative control (ACD, Catalog No. 320871), *Crhr1* (ACD, Catalog No. 318911-C1), and *Gad2* (ACD, Catalog No. 435801-C2).

Images of the CeA were acquired with a Zeiss LSM 780 laser scanning confocal microscope (40X oil immersion, 1024x1024pixel, 5-μm z-stacks). All microscope settings were kept the same within experiments. Quantification was performed with the image analysis software CellProfiler [48] using the recommended guidelines for analysis of RNAscope images by ACD Biotechne with background (negative control) subtraction.

Nuclei were considered positive if they contained one or more puncta after thresholding out any background. Images were visually inspected for accuracy if required. The percent of positive nuclei was calculated for each probe, and in instances of multiple treatment group comparisons, data was normalized to the control/naïve group to show relative values. Outliers were detected with Grubb's test. Analysis was performed on raw images, and brightness/contrast/pixel dilation are the same for all representative images shown per figures.

Statistical Analysis

Data are presented as *Mean* ± *SEM* of either raw values or were normalized to the baseline values, and *n* refers to the number of cells, while *N* refers to the number of used rats (exact values are indicated for each experiment). To avoid pseudo-replication (i.e., collecting multiple samples from an individual animal), no more than 3 data points were collected from any single animal. The criterion for statistical significance was $p < 0.05$. Statistical analyses were performed in Prism 9 (GraphPad Software, La Jolla, CA, USA). Data were analyzed with two-way ANOVA (Alcohol Exposure × Sex or Sex × CRF Concentration) followed by Šídák's post hoc comparisons (when appropriate.) Changes over baseline (e.g., agonist/drug responses) were assessed using one sample t-tests where indicated.

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