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Corticotrophin releasing factor, but not alcohol, modulates norepinephrine release in the rat central nucleus of the amygdala

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Abstract

Alcohol misuse and dependence is a widespread health problem. The central nucleus of the amygdala (CeA) plays important roles in both the anxiety associated with alcohol (ethanol) dependence and the increased alcohol intake that is observed during withdrawal in dependent animals. We and others have shown the essential involvement of the corticotrophin releasing factor (CRF) system in alcohol's synaptic effects on the CeA and in the development of ethanol dependence. Another system that has been shown to be critically involved in the molecular underpinnings of alcohol dependence is the norepinephrine (NE) system originating in the locus coeruleus. Both the CRF and NE systems act in concert to facilitate a stress response: central amygdalar afferents release CRF in the locus coeruleus promoting widespread release of NE. In this study, we are the first to use fast-scan cyclic voltammetry to classify local electrically-evoked NE release in the CeA and to determine if acute alcohol and CRF modulate it. Evoked NE release is action potential dependent, is abolished after depletion of monoaminergic vesicles, differs pharmacologically from dopamine release, is insensitive to acute alcohol, and decreases in response to locally applied CRF. Taken together, these results indicate that NE release in the CeA is released canonically in a vesicular-dependent manner, and that while acute alcohol does not directly alter NE release, CRF decreases it. Our results suggest that CRF acts locally on NE terminals as negative feedback and potentially prevents hyperactivation of the CRF-norepinephrine stress pathway.

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Keywords

stress; alcohol; CRF; norepinephrine; amygdala; voltammetry

1. Introduction

Alcohol use disorder (AUD) is one of the leading preventable causes of death both due to direct toxicity and exacerbation of comorbidities (Bauer et al., 2014). Alcoholism, or the neurophysiologic dependence on alcohol (ethanol), is behaviorally described as the compulsory seeking and drinking of alcohol, progressive loss of regulatory control, and finally, a negative emotion state in the absence of alcohol (Koob, 2013). This negative emotion state is characterized by the recruitment of brain stress circuitry, particularly in the central nucleus of the amygdala (CeA), the principle output nucleus of the amygdala (Koob, 2008; Koob and Le Moal, 2008; Roberto et al., 2010, 2012).

Two of the predominant stress systems in the brain are the norepinephrine (NE) and corticotropin releasing factor (CRF) signaling systems. Norepinephrine and CRF signaling within the extended amygdala are involved in stress-induced reinstatement of drug-seeking and are also necessary for the enhanced anxiety that accompanies protracted abstinence from chronic drug exposure (Smith and Aston-Jones, 2008). Norepinephrine neurons principally originate in the locus coeruleus (LC) of the brainstem and project diffusely to many different areas of the brain where NE is involved in a variety of neuropsychiatric states and disorders (Sara, 2009), and increased firing of LC NE neurons results in a state of increased anxiety and stress (McCall et al., 2015). The extrahypothalamic CRF system also is widely distributed throughout the brain including in the CeA (Sakanaka et al., 1986; Stenzelpoore et al., 1994; Curtis et al., 2002). Importantly, these two stress systems interact. There are NE LC neurons that directly project to the CeA (Campese et al., 2017; Finnell et al., 2019) and CRF+ neurons in the CeA that correspondingly project to the LC (Reyes et al., 2008, 2011; McCall et al., 2015), where CRF transmission in the LC increases anxiety (Lee et al., 2008; McCall et al., 2015). Functional connectivity between these two areas has given rise to a theory in which the CeA and the LC are involved in a “feed-forward loop” which escalates the brain stress response (Koob, 1999). According to this theory, the LC NE neurons projecting to the CeA are among the first ones to be recruited following a stressful event and they activate CeA CRF+ cells which project back to the LC, increasing tonic firing of LC NE neurons. Chronic alcohol dysregulates these systems (Koob, 2015) and increased levels of extracellular CRF in the CeA have been measured following acute withdrawal from alcohol (Roberto et al, 2010).

Despite how well characterized the relationship between the LC and the CeA is in stress response, little is known about how local NE release in the CeA is regulated and whether it is dysregulated by acute and/or chronic alcohol exposure. Thus, we sought to characterize the effects of alcohol and CRF on locally-evoked NE release in the CeA using fast-scan cyclic voltammetry (FSCV), an electrochemical technique that allows for sub-second monitoring of neurotransmitter release and kinetics. Interestingly, we found that while acute alcohol has no effect on NE release, CRF decreased electrically-evoked NE release in both

naïve and dependent animals, indicating that CRF interactions in the CeA may provide local inhibition to the LC CeA feed-forward reciprocal loop.

2. Materials and Methods

2.1 Animals

Adult male Sprague Dawley rats (n = 45, 225 – 350 g) were obtained from Charles River (Raleigh, NC) and were given *ad libitum* access to food and water and maintained on a reverse light/dark cycle with lights on between 8 PM and 8 AM. All protocols and procedures were approved by The Scripps Research Institute and Brigham Young University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

2.2 Chronic Alcohol Treatment

Alcohol dependent rats (n = 6) were generated using the standard alcohol (ethanol) inhalation method of The Scripps Research Institute Alcohol Research Center in which rats were exposed to alcohol vapor for 5 – 7 weeks, 14 hours per day (Rogers et al., 1979; Roberto et al., 2004b, 2004a, 2010; Roberto and Siggins, 2006; Cruz et al., 2013; Kallupi et al., 2014). This procedure has been shown by us and others to generate alcohol dependence in rodents, as demonstrated by increased alcohol drinking behavior, anxiety-like behavior, and reward deficits (O'Dell et al., 2004; Gilpin et al., 2008; Roberto et al., 2010). On experimental days, alcohol dependent rats were maintained in the vapor chambers until slices were prepared. Slices were then maintained in alcohol-free solutions and all electrochemical recordings were performed in slices undergoing acute withdrawal (2–8 hours) as previously described (Kallupi et al., 2014; Roberto et al., 2004b, 2004a, Roberto et al., 2010, Varodayan et al., 2017b, 2017a). Blood alcohol levels (BALs) were measured 1–2 times per week from tail blood samples. The mean BAL was 151.70 ±10.46 mg/dL.

2.3 Brain slice preparation and drug application

Rats were briefly anesthetized with 3–5% isoflurane and decapitated. The brains were rapidly dissected and placed in oxygenated (95% O_2, 5% CO_2), ice-cold high sucrose cutting solution (pH 7.3–7.4) consisting of (in mM): 206.0 sucrose, 2.5 KCl, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 1.2 NaH_2PO_4, 26.0 $NaHCO_3$, 5.0 D-glucose, and 5.0 HEPES (Roberto et al., 2003, 2004b, 2004a; Varodayan et al., 2017a, 2017b). Coronal brain slices (400 μm) were obtained using a Leica VT1200 S vibratome. Slices were then incubated in oxygenated artificial cerebral spinal fluid (ACSF) which contained the following (in mM): 130 NaCl, 3.5 KCl, 2.0 $CaCl_2$, 1.25 NaH_2PO_4, 1.5 $MgSO_4$, 24.0 $NAHCO_3$, and 10.0 D-glucose for 30 min at 37°C then 30 min at room temperature.

All drugs were obtained from Tocris Bioscience (Bristol, UK), unless otherwise notated. Drugs were bath-applied for slice voltammetry at the following concentrations (unless otherwise specified), which are based on K_i values (to attempt to prevent non-specific binding) when applicable: alcohol (44 mM; Pharmaco-Aaper, Brookfield, CT, USA), CRF (100 nM), yohimbine (25 μM), clonidine (10 uM), desipramine (50 μM), TTX (1 μM), and reserpine (10 μM).

2.4 Voltammetry recordings

For recording, slices were transferred to a recording chamber and continuously superfused with fresh, oxygenated ACSF (32–34 °C) at approximately 2 mL/min. Fast-scan cyclic voltammetry recordings were collected and analyzed using Demon Voltammetry and Analysis software (Yorgason et al., 2011a). Carbon fiber electrodes were custom made by aspirating a single carbon fiber into a borosilicate glass capillary tube (O.D. 1.2mm, I.D. 0.69mm; Sutter Instruments, Novato, CA) as previously described (Schilaty et al., 2014). The capillary tubes were then pulled using a model P-1000 horizontal electrode puller (Sutter Instruments). Under microscopic control, the carbon fibers were trimmed so that 100 – 150 μm of bare fiber protruded from the glass seal on the carbon fiber. During the recording, the electrode was linearly scanned (400 V/s) from −0.4 to 1.2 V and back to −0.4 V (Ag vs AgCl) and cyclic voltammograms were recorded at 10 Hz by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, Minneapolis, MN). Electrodes were conditioned prior to insertion into tissue by applying the triangular waveform at 60 Hz for 5–10 min (Yorgason et al., 2017). Low noise electrodes were positioned ~75 μm below the surface of the tissue in the medial subdivision of the CeA. Tissue was electrically stimulated using a bipolar stimulator (30 Hz, 30 P, 1 msec pulsewidth for NE in CeA; 20 Hz, 10 P, 4 msec pulsewidth for dopamine (DA) in striatum) to evoke catecholamine release. Due to the unique challenges of recording the small signals, some recordings showed stimulation artifact during electrical stimulation (e.g. prominent negative signal in Fig 5 traces). When recording NE in the CeA, the tissue was stimulated every 4 minutes, and when recording dopamine in the striatum, the tissue was stimulated every 2 minutes.

2.5 Data and Statistical Analysis

All catecholamine release was analyzed using Demon Voltammetry Analysis software. Evoked release was quantified at peak oxidation currents (peak heights/amplitude). Norepinephrine reuptake (clearance) was quantified using the time constant (τ) calculated by Demon Voltammetry using an single exponential fit function, and the goodness of fit was verified by calculating the Spearman correlation coefficient (Fig 1C) (Yorgason et al., 2011a). Norepinephrine concentrations were calculated by calibrating each electrode against 10 μM NE. Norepinephrine release was verified by comparing the color plot and voltammogram against the calibration voltammogram. Given the nearly identical oxidative potential of NE and DA, FSCV alone was not sufficient to discriminate between these two molecules: each experiment was verified to be either NE or DA pharmacologically as described previously (Park et al., 2011). Yohimbine, an $\alpha 2$ adrenergic receptor inhibitor, acts on the adrenergic autoreceptor and effectively disinhibits NE release – NE signals treated with yohimbine (25 μM) increased in amplitude whereas DA signals did not respond. Correspondingly, NE signals treated with desipramine (50 μM), a NET blocker, increased clearance time (τ), but desipramine had no effect on DA signals. While it is possible that $\alpha 2$ adrenergic receptors may be present on DA terminals in the CeA, potentially confounding this pharmacology-based discrimination method, a current literature search has not indicated that there is evidence for $\alpha 2$ adrenergic receptors on DA terminals in this region. When possible, the experimental pharmacology was applied first, and then washed out prior to species confirmation via either yohimbine or desipramine. This was done to prevent any potentiation or modulation of the experimental conditions. Exceptions to this are noted and

explained below. To ensure biological variability and avoid potential bias, slices for each experimental condition came from at least 3 different animals.

Statistics were performed using JMP version 14 (JMP, SAS Institute Inc., Cary, NC) and data was visualized using Igor Pro 7 (Wavemetrics, Lake Oswego, OR). For groups of two variables, statistical significance was calculated using an unpaired, two-tailed Student's *t* test. For groups of more than two variables but with only one factor, significance was determined using a one-way ANOVA, combined with either Tukey's HSD test for post hoc analysis or Dunnett's post hoc analysis in cases of multiple drugs all being compared back to the original baseline. For all figures, error bars represent the standard error of the mean (SEM), numbers in parentheses at the base of histograms are indicative of the total number of experiments for that condition, and *, **, *** denote significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3. Results

3.1 Norepinephrine signals increase in amplitude when treated with yohimbine and in clearance time when treated with desipramine.

Since NE and dopamine (DA) are identical when measured with FSCV, it was first necessary to reliably differentiate between these two monoamines. To address this, voltammetry was used in concert with desipramine (a NET blocker) and/or yohimbine (an α2 adrenergic receptor antagonist) to verify for the presence of NE (see section 2.5 for methodological details). Bath application of desipramine (50 μM) (Millan et al., 2001) increased NE signals in the CeA showed increased clearance time (τ) of 290 ± 82% compared to baseline clearance rates (Fig. 1A–D; $F_{1,8}$=4.831, $p < 0.05$). Norepinephrine signal response to desipramine contrasts sharply with known DA signal response in the NAc, which failed to respond at all (Fig. 1D,E; $F_{1,10}$=0.543, $p = 0.49$, ns). To investigate the potential of DAT being involved in this mechanism, we tested GBR 12909 (300 nM) on NE signals. GBR 12909 did not have an effect on the NE signal ($F_{1,22}$=0.00516, $p = 0.943$ ns) in the CeA. Thus, uptake in the CeA appears to be mediated mostly via the NET. Predominantly NE signals responded to 25 μM yohimbine (Schwartz and Clark, 1998) with a signal amplitude increase to 150 ± 10% of baseline that reversed upon wash (Fig. 1F,G; $F_{2,14}$=6.968, $p < 0.01$). Thus, blocking the adrenergic α2 receptor results in greater NE release in the CeA, likely by decreasing autoreceptor feedback inhibition. Signals that failed to respond to either yohimbine or desipramine were considered to be predominantly dopaminergic.

3.2 Clonidine decreases the NE signal, which can then be reversed by yohimbine

We then tested to see if clonidine (an α2 adrenergic receptor agonist) would inhibit NE expression due to the location of α2 adrenergic receptors on presynaptic adrenergic terminals and their role as autoreceptors (Starke et al., 1989). As expected, treatment of a NE signal with 10 μM clonidine (Jarrott et al., 1979) resulted in a decrease in signal amplitude to 66 ± 8% of baseline amplitude. Importantly, immediately after treatment with clonidine (10 μM), slices were treated with yohimbine (25 μM), which caused the NE signals to rebound to baseline levels (Fig. 2; $F_{2,15}$= 6.46, $p < 0.01$).

3.3 Norepinephrine release is dependent on both vesicles and action potentials

Reserpine (a VMAT blocker) is known to deplete monoaminergic vesicles (Beckstead et al., 2004). Norepinephrine signals treated with reserpine (10 μM) were decreased to 22 ± 11% of baseline (Fig. 3A,B; $F_{1,3}$=113.5, $p < 0.001$), indicating that release is mainly dependent on functional vesicles. Similarly, 1.0 μM TTX (a voltage-gated Na^+ channel inhibitor) decreased the NE signal in the CeA to 10 ± 8% of baseline amplitude (Fig. 3C,D; $F_{1,3}$=17.64, $p < 0.0001$), demonstrating that NE release depends on action potentials. Since both reserpine and TTX are relatively irreversible antagonists, it was necessary to pharmacologically verify monoamine species prior to experimental condition. It is unlikely that this prior treatment affected the results as neither desipramine nor yohimbine are known to directly affect vesicular packaging or action potential generation and both drugs were washed out for at least 20 minutes prior to reserpine and TTX testing.

3.4 Acute alcohol does not alter NE release in CeA

Alcohol has been shown to have no effect on NE release in the basolateral amygdala (BLA) when NE is measured by microdialysis (Karkhanis et al., 2015), and is known to decrease DA release (measured by FSCV) in the NAc shell, which is part of the extended amygdala complex (Schilaty et al., 2014; Yorgason et al., 2015). However, to our knowledge, it is not known whether acute alcohol can impair NE release in the CeA and we are the first to investigate this. Here, we applied a dose that produces a maximal response on GABA release in the CeA (44 mM; Roberto et al., 2004b, 2003). Alcohol did not alter NE release (Fig. 4A,B; $F_{2,13}$=0.0255, p =0.97 ns) in the CeA of alcohol naïve rats. We then tested a higher concentration of alcohol (66 mM) and found that it was also ineffective in altering NE release ($F_{2,13}$=1.153, p =0.301 ns). Interestingly, when treated with acute 44 mM alcohol, signals insensitive to yohimbine (determined to be not NE) decreased to 52± 6% of baseline (Fig. 4C,D; $F_{1,14}$=14.83, $p < 0.01$). Given that DA and NE have an identical electrochemical signature, we assume that this non-NE catecholamine is DA. This acute alcohol-induced decrease in signal in the CeA resembles the effect of EtOH on evoked DA release in the striatum (Schilaty et al., 2014; Yorgason et al., 2015), strengthening the conclusion that the signal is predominantly DA. Indeed, alcohol (66 mM) decreased DA signals in the NAc to 73% of the baseline signal ($F_{2,10}$=11.11, $p < 0.05$). Together, this data shows that CeA NE release is relatively insensitive to alcohol's inhibitory effects.

3.5 CRF decreases CeA NE release in both alcohol-naïve and alcohol-dependent rats

Given the complex interaction between the CRF and NE systems, we tested whether CRF would influence locally-evoked NE release. We hypothesized that acute application of CRF would increase NE release in the CeA and that this effect would be enhanced in alcohol-dependent animals. In contrast to our hypothesis, we found that CRF (100 nM; Roberto et al., 2010) decreased NE release in the CeA of alcohol-naïve rats to 82 ± 7% of baseline levels (Fig. 5A,B; $F_{1,3}$= 9.1, $p < 0.01$). In a separate set of experiments, we applied CRF first followed by CRF co-applied by CRF1 receptor antagonist R121919 (1.0 μM). We found that the CRF induced-inhibition of NE release was reversed with R121919 (Fig. 5C; $F_{2,12}$= 11.17, $p < 0.05$). Since CRF is known to play a role in alcohol dependence and drug-seeking behavior (Weiss et al., 2001), we also tested the effect of CRF on NE release in the CeA of

alcohol-dependent rats. Similarly, in alcohol dependent rats, CRF (100 nM) also decreased NE release to 80 ± 5% of baseline (Fig. 5D,E; $F_{1,4}$=158.6, $p < 0.01$). Interestingly, the magnitude of the CRF-induced decrease in the NE signaling was comparable in the two groups.

Additionally, comparable baseline NE peak amplitudes were observed in CeA of naïve and alcohol dependent animals, with average of 0.29 ± 0.09 μM NE and 0.27 ± 0.05 μM NE respectively (data not shown). There was no difference between baseline levels of release in alcohol-naïve and alcohol-dependent animals ($F_{1,5}$=4.69, $p = 0.84$, ns, data not shown), suggesting that alcohol dependence does not affect baseline release of NE as measured by FSCV. The apparent difference in baseline NE release in the timecourses (Fig. 5 A,D) is limited to the specific representative traces.

4. Discussion

Alcoholism is a chronic relapsing disorder characterized by a compulsion to seek and consume alcohol and has been linked to the dysregulation of brain emotion systems, including both those that mediate reward and those that mediate stress. Alcohol dependence is characterized by an increased negative emotion state (Koob, 2008; Koob and Le Moal, 2008), and recruitment of the NE system of the LC and the CRF system of the CeA (Koob, 1999; Haass-Koffler et al., 2018). Interactions between these two stress systems have been studied and there is evidence for a feed-forward loop between the CeA and the LC, giving the potential for a rapid stress response (Koob, 1999; Lee et al., 2008; Reyes et al., 2008, 2011; McCall et al., 2015). While stress-induced NE release occurs in both the BLA and CeA (Galvez et al., 1996; Quirarte et al., 1998; Hatfield et al., 1999), little is known about the local regulatory control on NE release in the CeA and potential CRF modulation. Thus, in this study, we used FSCV to measure real-time, electrically-evoked release of NE and investigate the effects of acute CRF on NE transmission in the CeA of both alcohol naïve and dependent rats. To the best of our knowledge, we are the first to identify electrically-evoked NE release in the CeA of both alcohol-naïve and alcohol-dependent rats and characterize the physical nature of this NE release. Similar to what was observed in the BNST (Park et al., 2011), blocking NE reuptake and α2 autoreceptors resulted in prolonged signals with larger release. However, a direct comparison between the previous *in vivo* BNST study and the present CeA study is not possible since *in vivo* and *in vitro* slice preparations can produce very different results in release and uptake even in the brain region (for example, compare (Yorgason et al., 2011b, 2016).

In characterizing the NE signal, we used reserpine, a VMAT inhibitor which causes terminals to become depleted of monoamines, as vesicles require constant VMAT activity to maintain the pH gradient to hold monoamines in vesicles (Freyberg et al., 2016). Notably, reserpine reduced evoked NE signals in CeA to 22% of baseline levels, suggesting that this type of neurotransmission is vesicle dependent. Additionally, blockade of voltage-gated sodium channels using TTX reduced signals to 10% baseline levels, suggesting that these NE vesicles are dependent on canonical action potentials.

While we had originally hypothesized that CRF would increase NE transmission, we found that acute, bath-applied CRF decreases electrically-evoked NE transmission in the CeA of both alcohol-naïve and alcohol-dependent animals, and effect that was blocked in naïve rats with CRF1 receptor antagonism. This suggests that CRF locally inhibits NE release in the CeA. One speculation is that CRF acts on CRF1 and/or CRF2 receptors on presynaptic CeA NE terminals directly modulating NE transmission, but CRF could also act by modulating NE release via multiple indirect synaptic effects. We have previously showed that CRF increases locally evoked GABA transmission in the CeA via CRF1 receptors (Roberto et al., 2010; Varodayan et al., 2017b). In contrast, CRF decreases locally evoked glutamate release mainly via CRF1, and only partially via CRF2, receptors in CeA (Varodayan et al., 2017a). Notably, CRF-induced enhancement of CeA GABAergic transmission in alcohol dependent rats was greater than the effect observed in naïve animals (Roberto et al., 2010), whereas we did not observe differences in the CRF-induced evoked glutamatergic responses in the CeA of naïve versus alcohol-dependent rats (Varodayan et al., 2017a). Similar to the effects of CRF on evoked glutamate transmission, the effect of CRF on evoked NE response was not different between alcohol naïve and dependent animals. Thus, the present results indicate that the CRF system modulating NE terminals may undergo compensatory neuroadaptations that contribute to the maintenance of homeostasis during chronic ethanol exposure.

Our voltammetry data showing CRF-induced decrease of evoked NE in CeA contrasts with the findings of Su et al, who reported that CRF causes release of NE in the CeA (Su et al., 2015). To understand this discrepancy, it is necessary to recognize the difference in experimental conditions between these two studies. The current study was conducted solely in brain slices that largely eliminate macrocircuitry (afferent and efferent projections are sheared during the slice preparation, which includes loss of NE terminal regulation from the soma), whereas Su et al were using microdialysis in freely moving animals. Apart from the trauma induced by cannulation, the macrocircuitry of the feed-forward CeA-LC loop was intact. This limitation (lack of intact macrocircuitry) likely accounts for the difference between these two studies, but also affords valuable insights into the local, regulatory control on NE release in the CeA.

It is also important to note that acute alcohol had no effect on NE transmission in the CeA, when measured here using FSCV, similar to what has previously been shown in the BLA (Karkhanis et al., 2015). While alcohol clearly affects transmission of the biochemically-related DA in the striatum when measured with either FSCV or microdialysis, this effect appears to be indirect, likely through glycinergic or GABAergic intermediaries (Jonsson et al., 2014; Schilaty et al., 2014; Yorgason et al., 2015). While presumed DA signals in the CeA are affected by alcohol, it is important that there is not a similar mechanism affecting NE release, and that although alcohol is involved in a stress response, it does not appear to directly affect NE transmission in the CeA. Despite this lack of direct effects of alcohol on NE transmission, acute alcohol increases GABAergic transmission in the CeA via increased GABA release (Roberto et al., 2003, 2004a), likely diminishing CeA activity to the LC, and slowing CRF-containing driven LC NE activity. Additionally, while we speculate that CRF1 activation mediates this increase in GABA release, we can't rule out a direct activation of CRF1 by alcohol. In our *in vitro* slice preparation without TTX, the local circuitry is maintained, thus, it is possible that other targets modulated by alcohol, including voltage-

gated calcium channels () or other neuropeptidergic systems may blunt/counteract an alcohol effect on NE transmission. Finally, there could be other brainstem NE sources that may influence our measurements.

Both NE and CRF play crucial roles in behavioral aspects of addiction, especially the anxiogenic effects of drug withdrawal (Menzaghi et al., 1994; Heinrichs et al., 1995; Delfs et al., 2000). CRF release (measured using microdialysis) in the CeA is increased during withdrawal in alcohol-dependent animals (Merlo Pich et al., 1995), and appears to contribute to withdrawal-related anxiety. This withdrawal-related anxiety can be reduced by injection of CRF receptor antagonists into the CeA (Rassnick et al., 1993). Additionally, acute restraint stress induced rapid elevation in extracellular NE content in rat CeA (Galvez et al., 1996; Inglis and Moghaddam, 1999; Khoshbouei et al., 2002; Reznikov et al., 2007, 2009). Based on these studies, we hypothesize that NE and CRF may act in concert in the CeA to modulate stress-related components of alcohol addiction.

In conclusion, CRF decreases electrically-evoked NE release in the CeA by increasing inhibitory and/or decreasing excitatory control over the NE terminals in CeA. It is possible that CRF is released somatodendritically in the CeA as has been previously characterized (Dabrowska et al., 2013), providing a tight regulation of a CRF-evoked NE response from local CRF negative feedback in the CeA. In addition, local application of exogenous CRF modulates both GABAergic and glutamatergic signaling in the CeA (Roberto et al., 2010; Silberman and Winder, 2015; Varodayan et al., 2017a). Taken together, this indicates that increased CRF receptor activity in the CeA may increase anxiety-like behaviors without driving a global, NE-driven stress response through the LC. The CeA is a hub for negative emotional processing (reviewed in Gilpin, Herman & Roberto 2015) and serves as the major output nucleus of the entire amygdala complex, projecting to additional regions that regulate stress and anxiety-related behaviors. Therefore, CRF regulation of the NE system in the CeA may represent a novel mechanism within a key stress circuit that can lead to potential additional targets for alcohol-dependence treatment.

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Highlights

- Evoked norepinephrine release in the CeA is vesicle and action potential dependent
- CRF decreases norepinephrine release in the CeA
- Norepinephrine release in the CeA is insensitive to alcohol

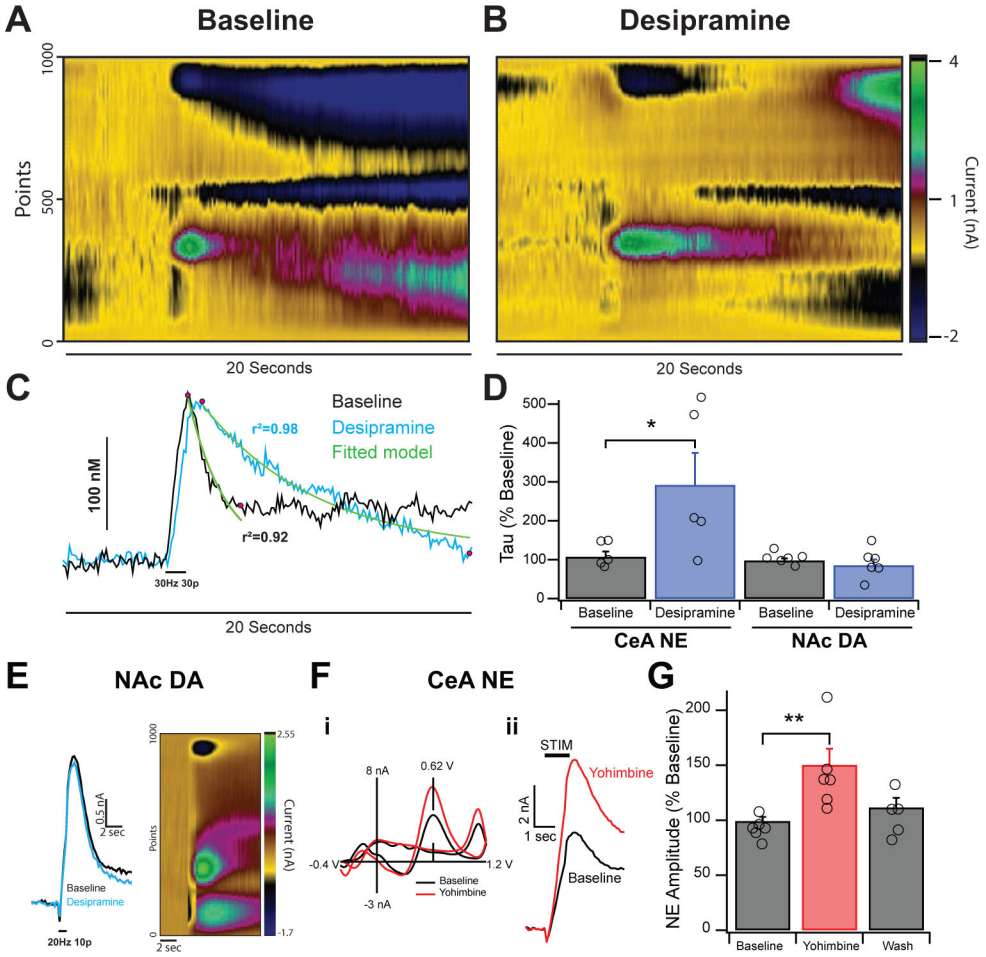

Figure 1: Effects of Desipramine and Yohimbine on Evoked NE Release in the CeA.

A&B. Colorplots depicting electrically evoked (30 Hz 30 pulse) NE release in the CeA before and after desipramine (50 μM). Stimulation triggers rapid release of neurotransmitter, which peaks and levels decay slowly to baseline. **C**. Current vs Time traces from colorplots in A&B at peak oxidation potentials. Traces were modeled using a single exponential fit before and after desipramine. Curve fits (shown in green) were based off a region of interest between the peak response and where the initial decay had stabilized (highlighted with pink dots). Reuptake times were calculated by averaging the half-life constant *tau.* **D.** Desipramine increased reuptake times of NE signals (left) to approximately 290% of baseline without affecting peak height. Desipramine failed to increase the half-life constant for exclusively DA signals (right) in the striatum. **E.** Stimulated DA release in the NAc showing no effect of desipramine on reuptake. **F**. Inset **i** shows the characteristic voltammogram of NE with a primary oxidative peak at 0.62 V and Yohimbine's effects on NE release. Amplitude of this peak is directly correlated with volume of NE released from tissue. Inset **ii** shows current vs time plots of electrically-evoked NE release. **G.** Yohimbine (25 μM) increased the NE signal to approximately 150% of baseline levels, an effect that washed out after removal of drug. Error bars represent the SEM and * and ** indicate a significance level of $p < 0.05$ and 0.01 respectively.

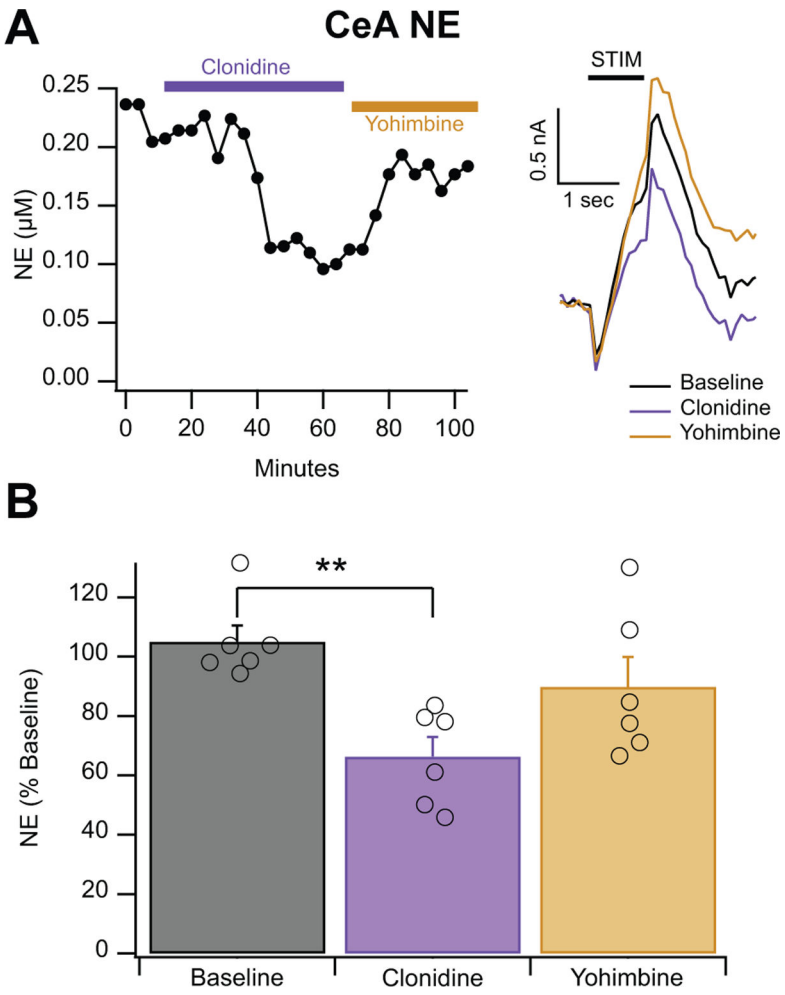

Figure 2: Clonidine Suppresses Evoked NE Release, which can then be Restored by Yohimbine.

A. Representative recordings of clonidine (10 μM) followed by yohimbine (25 μM). Clonidine represses the NE signal, which is then restored by yohimbine. Inset shows representative traces. **B.** Cumulative histogram of showing clonidine (10 μM) reduces the NE signal to approximately 66% of baseline and the restoration of the signal by yohimbine (25 μM). Error bars represent the SEM and ** indicates a significance level of $p < 0.01$.

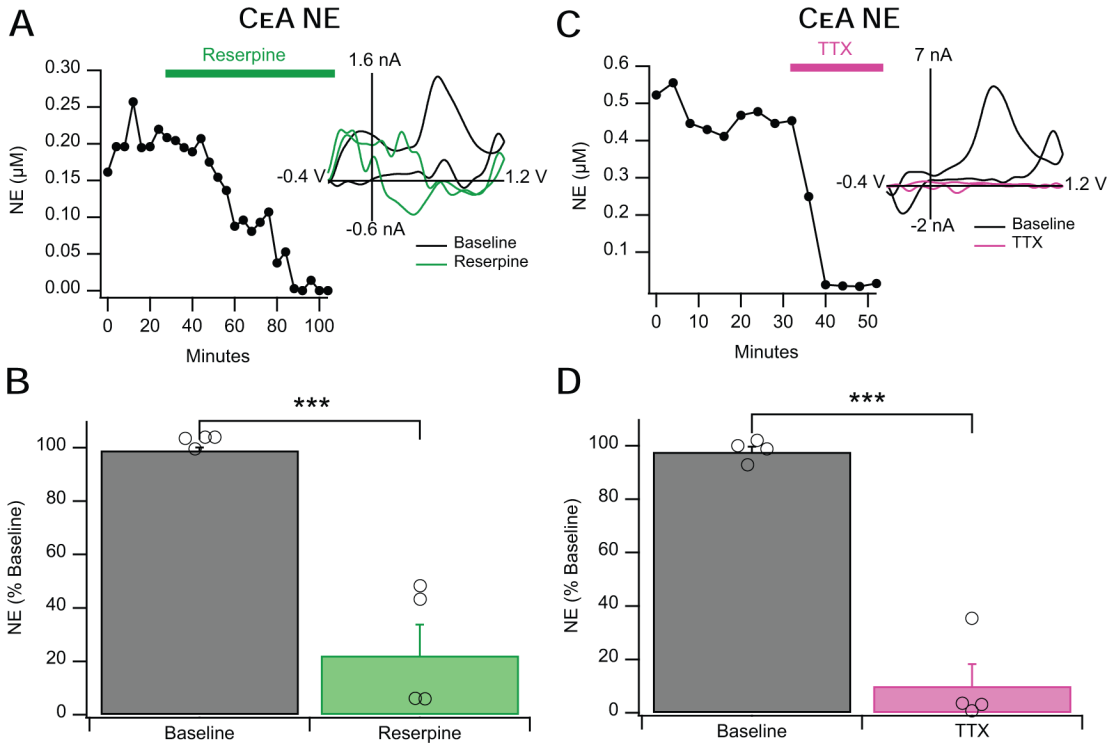

Figure 3: Reserpine and TTX Abolish Evoked NE Release in the CeA.

A&B. Reserpine (10 μM) attenuates evoked NE release in the CeA to approximately 22% of baseline levels. The left inset shows a representative time course of reserpine's action and the right inset shows representative voltammograms. **C&D.** TTX (1.0 μM) similarly decreases evoked NE release to approximately 10% of baseline release levels. The left inset shows a representative time course of TTX's action and the right inset shows representative voltammograms. Error bars represent the SEM and *** indicates a significance level of $p < 0.001$.

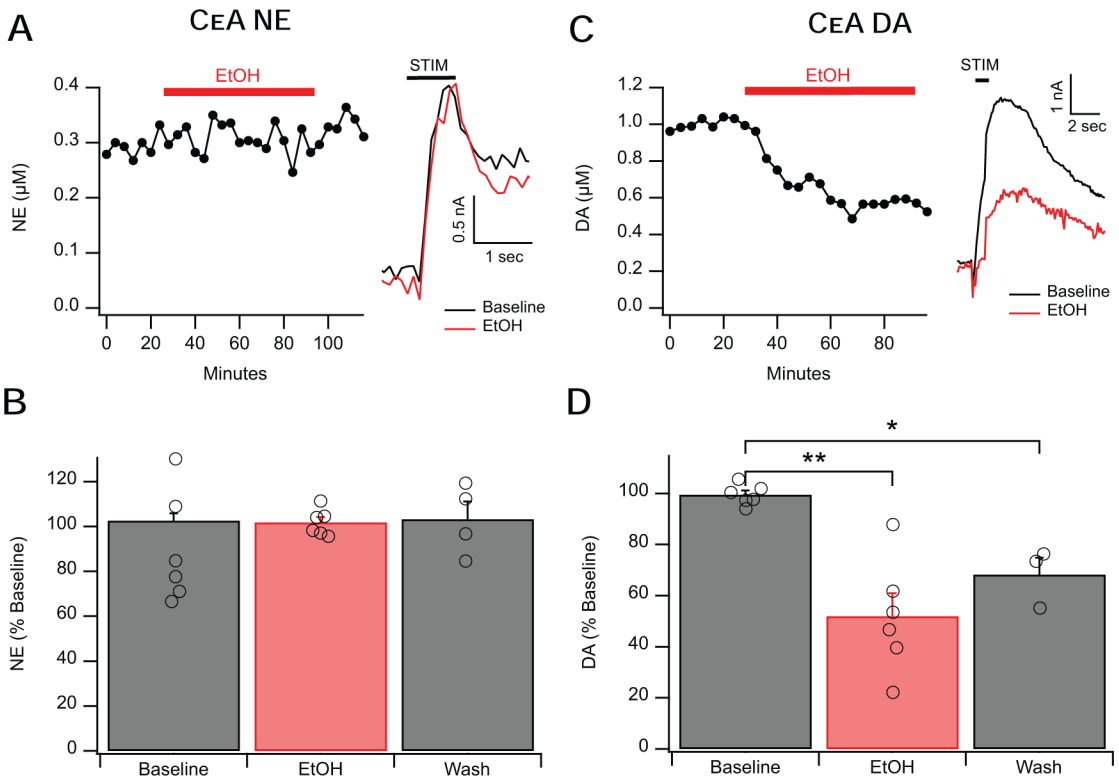

Figure 4: Alcohol does not Affect NE Release in the CeA.

A&B. Alcohol (44 mM) failed to affect evoked NE release in the CeA. The left inset shows a representative time course of alcohol's action and the right inset shows representative current vs time traces. **C&D.** Alcohol (44 mM) decreased evoked DA release in the CeA to approximately 52% of baseline release levels, an effect which did not fully wash out. The left inset shows a representative time course of alcohol's action and the right inset shows representative current vs time traces. Error bars represent the SEM, numbers in histograms represent total number of experiments for the experimental condition. * and ** indicate a significance level of $p < 0.05$ and 0.01 respectively.

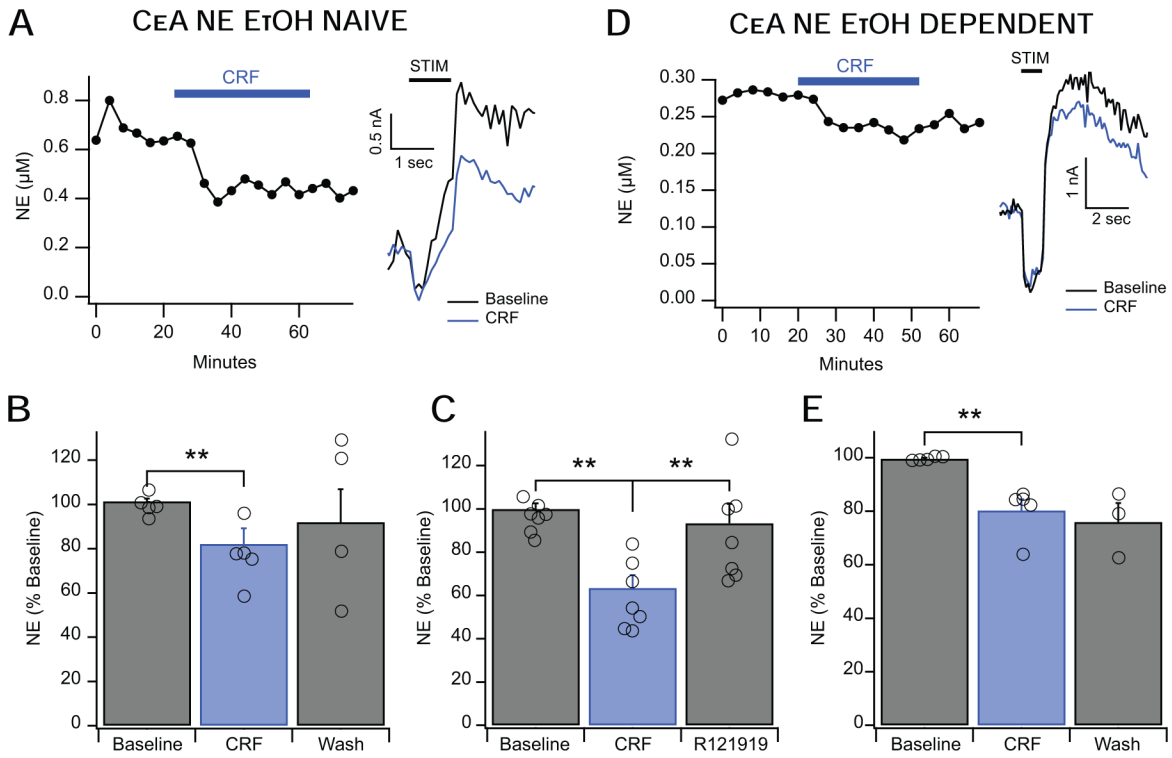

Figure 5: CRF Decreases Evoked NE Release in the CeA.

A&B. CRF (100 nM) decreased NE release in the CeA of alcohol-naïve animals to approximately 82% of baseline release levels with moderate (but statistically insignificant) washout. The left inset shows a representative time course of CRF's action and the right inset shows representative current vs time traces. **C.** After CRF application, R121919 (1 μM) co-applied with CRF fully reversed the CRF-induced decrease in NE signal. **D&E.** CRF (100 nM) similarly decreased NE release in the CeA of alcohol-dependent animals to approximately 80% of baseline release levels, and this effect failed to wash out. The left inset shows a representative time course of CRF's action and the right inset shows representative current vs time traces. It is important to note that while the representative insets show a difference in baseline levels of NE release between alcohol naïve and dependent animals, this effect is limited to the selected representative examples. This effect is averaged out when combined with aggregate data (see section 3.5). Error bars represent the SEM and *,** indicates a significance level of $p < 0.05$ and $p < 0.01$, respectively.