Manipulation of Corticotropin-Releasing Factor Signaling Using DREADDs Reduces Binge-Like Ethanol Consumption of CRF-Cre Transgenic Mice

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Abstract

Binge-like ethanol consumption is characterized by the rapid achievement of blood ethanol concentrations (BEC) of ≥80 mg/dl and is associated with an increased risk of ethanol dependence. Evidence of this association is based on an overlap between neurobiological mechanisms active during ethanol dependence and those activated during excessive ethanol consumption prior to dependence. One such contributor is the corticotropin-releasing factor (CRF) system. Our laboratory has shown that antagonism of CRF receptors within the central amygdala (CeA) reduces binge-like ethanol consumption. The current study was designed to extend previous research by measuring ethanol consumption following the manipulation of CRF signaling in the CeA using Cre-induced inhibitory designer receptors exclusively activated by designer drugs (DREADDs) technology. Additionally, we tested whether manipulation of CRF signaling in the CeA modulates general rewarding behavior or specifically reduces binge-like ethanol consumption. Ethanol or sucrose, consumption of which is indicative of general rewarding behavior, was administered using a 4-day procedure that mimics binge-like drinking. On the first two test days, mice received drug injections to activate the DREADDs, thereby inhibiting CRF neurons within the CeA. Statistical analysis revealed that Gi-DREADD activation in the CeA reduced ethanol consumption but had no significant effect on sucrose consumption. These data suggest that the reduced ethanol consumption during the first two cycles was not merely a general rewarding behavior but was instead specific to ethanol consumption. Moreover, they suggest that inhibition of the CRF signaling pathway in the CeA remains a viable target for manipulating binge-like ethanol consumption.

Introduction

Binge drinking is a destructive pattern of heavy ethanol consumption characterized by the rapid achievement of blood ethanol concentrations (BECs) greater than 80 mg/dl (Council, 2004). Research indicates that binge drinking is associated with an increased risk of developing alcohol dependence, a severe yet common health concern (Jennison, 2004; McCarty et al., 2004). In its earliest stages, research on the neurobiological effects of binge-like ethanol consumption relied on rodent models that involved
voluntary consumption (McBride, 1998). However, rodents do not voluntarily consume enough ethanol under standard conditions to produce pharmacologically relevant BECs or to generate behavioral intoxication (Spanagel, 2000), both of which characterize human binge drinking. Rodent models have since been developed to more accurately model human binge drinking. One such model is the Drinking in the Dark (DID) paradigm which regularly produces BECs greater than 80 mg/dl and evidence of behavioral intoxication (Rhodes et al., 2005). Thus, the DID model simultaneously serves as a rodent correlate of human binge drinking as well as a useful tool for studying the neurobiological mechanisms that underlie binge-like ethanol consumption in mice (Sprow and Thiele, 2012).

Neurobiological mechanisms involved in excessive ethanol consumption prior to dependence overlap with those associated with ethanol dependence (Sprow and Thiele, 2012); thus, studies investigating the neurobiological systems that underlie binge ethanol consumption have the potential to shed light on the neurobiological changes that precede alcohol dependence. One such system is the corticotropin-releasing factor (CRF) system, which is implicated both in the regulation of ethanol dependence and excessive consumption. CRF is an amino acid peptide present in a wide variety of cell types, including neurons. CRF is most commonly associated with the general mammalian stress response, but considering the relationship between stress and alcohol consumption, it is not surprising that CRF signaling modulates ethanol consumption (Martin et al., 2010; Bahi, 2013). Previous research has illustrated that CRF plays a key role in excessive, dependence-like ethanol consumption in rats through the activation of G-protein coupled CRF-1 receptors (CRF1R) in the central nucleus of the amygdala (CeA) (Roberto et al., 2010). The CeA is known to play a fundamental role in physiological and behavioral responses to drug-related stimuli, and neuroadaptations within the CeA have been shown to play a central role in alcohol dependence (Gilpin, 2012). Our laboratory has built upon the aforementioned studies involving the CRF system in the CeA by showing that not only does ethanol dysregulate
the CRF system, but also that antagonism of CRF receptors within the CeA reduces binge-like ethanol consumption (Lowery et al., 2010).

The current study extends previous research by measuring ethanol consumption following the manipulation of CRF signaling in the CeA using Cre-induced Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) technology. DREADDs are G-protein coupled receptors which are activated by small, drug-like molecules. They are particularly useful as a means of investigating and manipulating discrete populations of neurons in the brain, as they have reduced off-target effects and heightened functional selectivity compared to other psychopharmological methods, including agonist and antagonist ligands (Fortress et al., 2015; Lee and Roth, 2014). For this study, the Gi-DREADD, which induces neuronal silencing, was used to selectively inhibit CRF signaling in the CeA.

Materials and Methods

Animals

CRF-Cre transgenic mice, backcrossed with C57s, were bred in house and were 10-18 weeks of age at the onset of the study. These mice expressed the exogenous Cre-recombinase only in cells that expressed endogenous CRF (Martin et al., 2010). All mice were housed in individual plastic cages with constant access to Purina RMH 3000 chow. Water was available at all times except when the mice were given access to alcohol or sucrose. The animal colony room was kept at a temperature of approximately 22°C and operated on a 12 hour light: 12 hour dark cycle (lights on at 08:00 hours). All procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of North Carolina Institutional Animal Care and Use Committee (National Research Council, 2011).

Stereotactic Surgery and DREADDS
The study used surgically delivered Cre-inducible adeno-associated viruses to encode for designer receptors exclusively activated by designer drugs (DREADDs) as a means of investigating specific neurological changes associated with binge-like alcohol consumption (Fortress et al., 2015). The DREADDs were incorporated into the viral genome, so the neural cells of interest were exposed to the virus and subsequently expressed the designer receptor. Prior to surgery, mice received an anesthetic dose of a ketamine cocktail as well as a topical anesthetic. Stereotactically, surgery was used to bilaterally infuse either the inhibitory Gi-DREADD (AAV8-hSyn-DIO-hM4D-mCherry) or a control virus with a fluorescent tag (AAV8-hSyn-DIO-mCherry) at the following coordinates in the central amygdala: AP: -1.05 ML:+/-2.5 DV:-4.64. A Hamilton syringe was used to inject a total of 0.3 µl bilaterally at a rate of 0.1 µl per minute. After each injection, the virus was allowed to diffuse for ten minutes.

_Drinking-in-the-Dark Procedure_

**Figure 1.** Pictorial representation of the DID procedure timeline.

Ethanol was administered using the DID procedure, a well-established 4-day binge-like drinking model (Rhodes et al., 2005, 2007; Sparta et al., 2008; Lowery et al., 2010). Animals were given access to a 20% (v/v) ethanol solution three hours into the dark cycle on each of the 4 days. Tap water and 95% ethyl alcohol were used to prepare the 20% (v/v) ethanol solution. The ethanol was available for a period of two hours on days 1-3. Day 4 was classified as the binge test day, and
the ethanol was available for 4 hours. Animals underwent 3 consecutive weeks of ethanol testing followed by an open field test and one week of sucrose testing. Sucrose was administered using the DID procedure and served as a control to examine the specificity of the inhibition of CRF neurons to ethanol consumption. Immediately preceding the DID procedures in week 1 and 2 of the ethanol test and the sucrose test, mice were injected intraperitoneally with 0.1 mg/kg clozapine-N-oxide (CNO) to induce Gi activation which in turn induced neuronal silencing in CRF neurons of the CeA expressing the DREADDs. However, immediately preceding week 3 of ethanol testing, mice were injected with saline instead of CNO to measure ethanol consumption in the absence of manipulation of CRF signaling.

Approximately 60 µl of tail blood was collected from each animal immediately following the end of the binge drinking period on day 4 of each test week. The samples were used to determine individual BECs using an Analox Analyzer (Analox Instruments).

Open Field Testing

Mice used in the DID tests were split into two groups, receiving either intraperitoneal CNO or saline, and subsequently subjected to open-field locomotor activity tests. The purpose of the open-field tests is to measure anxiety-like behavior, as avoidance of the central area of the chamber reflects a heightened state of anxiety (Choleris et al., 2001; Fee et al., 2004). The open field tests were conducted identical to previous reports using locomotor activity chambers made of clear Plexiglass and measuring 40.64 x 40.64 x 30.48 cm (Sparta et al., 2008). Testing sessions were 4 hours long, with measurements taken every 5 minutes. Total distance traveled (cm) was measured to assess the effect of CNO induced Gi activation in the CeA on locomotor activity, and center distance (cm) and time were used to examine the effects of silencing CeA CRF neurons on anxiety.

Placement Checks

At the conclusion of the study, mice were euthanized with a sodium pentobarbital overdose and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde.
The brains were extracted, sliced in 40 µm sections using a vibrating microtome (Leica VT1000S; Wetzlar, Germany), and mounted. The placement of the virus was visualized and confirmed to be in the CeA using an Olympus BX-51 fluorescent microscope set to a 100x magnification.

Data Analysis

Two tailed t-tests were performed to determine if Gi-DREADD activation had a significant effect on ethanol consumption. Two-way ANOVAs were used to determine if CNO induced Gi activation in the CeA had an effect on locomotor activity and to examine the effects of silencing CeA CRF neurons on anxiety.

Results

DID Trials

During the first two weeks of ethanol testing, mice received CNO injections, inhibiting CRF neurons within the CeA. T-tests were used to determine if Gi-DREADD activation had a significant effect on ethanol consumption. Gi-DREADD activation in the CeA resulted in reduced ethanol consumption ([Test 1: Gi = 2.875 ± 0.3775 g/kg ethanol; control = 5.036 ± 0.3765 g/kg ethanol; p=0.0006]; [Test 2: Gi = 3.384 ± 0.2911 g/kg ethanol; control = 5.009 ± 0.4624 g/kg ethanol; p=0.0075]). Accordingly, there was also a significant reduction in BECs in test 1; however, there was no significant reduction in BECs in test 2 ([Test 1: Gi = 35.87 ± 8.71 mg/dl; control = 73.92 ± 11.68 mg/dl; p=0.0167]; [Test 2: Gi = 40.93 ± 10.49 mg/dl; control = 71.63 ± 12.82 mg/dl; p=0.0787]).
Figure 1. Two tailed t-tests indicated a significant reduction in ethanol consumption upon Gi activation in the CeA for test 1 (p = 0.0006; n=11) and test 2 (p=0.0075; n=11).

Figure 2. Two tailed t-tests indicated a significant reduction in BECs upon Gi activation in the CeA for test 1 (p=0.0167; n=11), but no significant reduction for test 2 (p=0.0787; n=11).

To determine if CNO had an effect independent of the virus, mice received a saline injection prior to the third round of ethanol testing. No significant difference in ethanol consumption was observed between the experimental and control group under these conditions (Test 3: Gi = 4.250 ± 0.2980 g/kg ethanol; control = 4.541 ± 0.5232 g/kg ethanol; p= 0.6339). Additionally, there was no significant difference in BECs under these conditions (Test 3: Gi = 69.07 ± 13.27 mg/dl; control = 71.83 ± 10.88 mg/dl; p=0.8743).
Figure 3. A two tailed t-test indicated no significant difference in ethanol consumption in mice treated with saline (p = 0.6339; n=10).

Figure 4. A two tailed t-test indicated no significant difference in BECs in mice treated with saline (p=0.8743; n=10).

In the final round of testing, mice were given access to sucrose solution in the place of ethanol to test the specificity of Gi DREADD manipulations in the CeA and to determine if manipulating CRF signaling had an effect on general reward ing tendencies. A t-test showed no significant effect of Gi DREADD activation on sucrose consumption (Test 4: Gi = 8.997 ± 1.503 g/kg sucrose; control = 8.333 ± 1.225 g/kg sucrose; p= 0.7436).

Figure 5. A two tailed t-test indicated no significant difference in sucrose consumption upon Gi activation in the CeA (p=0.7436; n=11).
Open Field Tests

Mice received either an intraperitoneal CNO or saline injection and were subjected to open-field locomotor activity tests. Total distance traveled was measured to assess the effect of CNO induced Gi activation in the CeA on locomotor activity, and center distance and time were used to examine the effects of silencing CeA CRF neurons on anxiety. For center distance and total distance, a main effect of time was observed [(Center distance: F=6.36; p<0.0001); (Total distance: F=5.80; p<0.0001)], but there was no main effect of Gi activation in the CeA [(Center distance: F=2.31; p=0.1451); (Total distance: F=0.79; p=0.3859)]. For center time, no main effect of Gi activation (F=1.35; p=0.2599) or of time was observed (F=1.01; p=0.4478).

Figure 6. A two way ANOVA indicated a main effect of time but no significant interaction or main effect of Gi activation in the CeA in total distance.
Figure 7. A two way ANOVA indicated a main effect of time but no significant interaction or main effect of Gi activation in the CeA in center distance.
Figure 8. No interaction or main effects were observed in center time.

Discussion

The current study confirms previous findings that modulation of the CRF system in the CeA plays a role in alcohol consumption. The results showed that CRF neuronal inhibition in the CeA using Cre-induced DREADD technology reduced binge-like ethanol consumption. There was no significant difference in ethanol consumption in mice treated with saline, indicating that CNO did not have an effect independent of the virus. Additionally, there was no significant effect of Gi DREADD activation on sucrose consumption, suggesting that the effects of CRF receptor antagonism in the CeA is specific to ethanol consumption and does not affect general rewarding tendencies.

The results of the open field tests indicated that CRF receptor antagonism in the CeA had no effect on locomotor activity or anxiety. Together, the results of the DID tests and the open field tests suggest that inhibition of the CRF signaling pathway in the CeA specifically, reduces binge-like
ethanol consumption without decreasing activity or increasing anxiety. Thus, it remains a viable target for manipulating binge-like ethanol consumption without other negative consequences.

The mounting evidence for the role of CRF in excessive, binge-like ethanol consumption suggests an overlap between the neurobiological systems involved in the general mammalian stress response and those involved in the regulation of excessive alcohol consumption and dependence. The fundamental role of CRF receptors in the regulation of stress and alcohol dependence is well-documented, but recent studies have shown that peptides other than CRF, including a variety of urocortins, may also act on CRF receptors to modulate both stress and alcohol consumption (Ryabinin et al., 2012). Among others, the aforementioned studies demonstrate that stress is a risk factor for alcoholism. Thus, the neurobiological relationship between stress and alcohol consumption merits further study, as it could provide new insight into why people drink, how alcohol affects their behavior, and why some people become dependent on alcohol.

The current findings are limited to the role of CRF in the CeA, so it would be interesting to investigate the potential of manipulating CRF signaling in other regions of the amygdala to influence ethanol consumption. Specifically, the basal lateral amygdala (BLA) is known to have glutamatergic projections to the CeA (Ren et al., 2010), which could allow for upstream targeting of the CRF system. In conclusion, the current study sheds light on the role of the CRF signaling in the CeA in ethanol consumption. On a broader scale, it implicates CRF signaling in other parts of the brain as well as additional signaling pathways involved in the stress response in the regulation of excessive alcohol consumption.

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References


