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Extended Amygdala to Ventral Tegmental Area Corticotropin-Releasing Factor Circuit Controls Binge Ethanol Intake

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Abstract

Background—Corticotropin-releasing factor (CRF) signaling at CRF1 receptors (CRF-1R) in the ventral tegmental area (VTA) can modulate ethanol consumption in rodents. However, the effects of binge-like ethanol drinking on this system have not been thoroughly characterized and little is known about the role of the CRF-2R or the CRF neurocircuitry involved.

Methods—The effects of binge-like ethanol consumption on the VTA CRF system were assessed following “drinking-in-the-dark” (DID) procedures. Intra-VTA infusions of selective CRF-1R and/or CRF-2R compounds were employed to assess the contributions of these receptors in modulating binge-like ethanol consumption (n=89). To determine the potential role of CRF projections from the bed nucleus of the stria terminalis (BNST) to the VTA, CRF neurons in this circuit were chemogenetically inhibited (n=32). Binge-induced changes in VTA CRF system protein and mRNA were also assessed (n=58).

Results—Intra-VTA antagonism of CRF-1R and activation of CRF-2R resulted in decreased ethanol intake which was eliminated by simultaneous blockade of both receptors. Chemogenetic inhibition of local CRF neurons in the VTA did not alter binge-like ethanol drinking, but inhibition of VTA-projecting CRF neurons from the BNST significantly reduced intake.

Conclusions—Here we provide novel evidence that A) blunted binge-like ethanol consumption stemming from CRF-1R blockade requires intact CRF-2R signaling and CRF-2R activation reduces binge-like drinking, B) inhibiting VTA-projecting BNST CRF neurons attenuates binge-like drinking, and C) binge-like ethanol drinking alters protein and mRNA associated with the

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VTA-CRF system. These data suggest that ethanol-induced activation of BNST-to-VTA CRF projections is critical in driving binge-like ethanol intake.

Keywords

Corticotropin-releasing factor; ventral tegmental area; drinking in the dark; binge drinking; ethanol; Extended Amygdala

Introduction

The transition from moderate controlled drinking to ethanol dependence is usually accompanied by intermittent bouts of binge consumption of ethanol, culminating in heavy, uncontrolled ethanol consumption. The National Institute of Alcohol Abuse and Alcoholism (NIAAA) defines a binge as a pattern of drinking characterized by consuming enough ethanol to rapidly achieve blood ethanol concentrations (BECs) in excess of 80 mg/dl [0.08%, (1)]. Because this pattern of drinking often leads to the development of ethanol dependence (2, 3), examining the systems that are recruited or dysregulated during repeated episodes of binge ethanol drinking provides an opportunity to understand the mechanisms involved and identify potential therapeutic targets to prevent the transition to ethanol dependence.

One system that has been strongly implicated in alcohol use disorders is corticotropin-releasing factor (CRF) and its receptors (4). CRF is a 41 amino acid peptide, canonically involved in the stress response, exerting its effects through two G protein-coupled receptors (GPCR), the CRF-1 receptor (CRF-1R) and the CRF-2R (5). CRF has a higher affinity for the CRF-1R than the CRF-2R, thus higher concentrations of CRF are necessary to activate both receptor subtypes (6). Evidence shows that CRF and the CRF-1R modulate dependence-induced ethanol intake (7-11), suggesting that CRF-1R signaling becomes dysregulated with dependence promoting drinking. However, recent evidence suggests that CRF signaling is also recruited during binge-like ethanol drinking prior to dependence (12, 13). Lowery-Gionta et al., (2012) showed that CRF protein levels were significantly increased in the central amygdala (CeA) following binge-like ethanol consumption and blockade of CRF-1R in the CeA blunted binge-like ethanol consumption. This is analogous to the observation that CeA infused CRF-1R antagonists protect against dependence-induced escalation of ethanol consumption (4, 8). Interestingly, Lowery-Gionta et al., (2012) reported that CRF protein levels in the ventral tegmental area (VTA) were also significantly increased after binge-like ethanol drinking. Consistently, recent data have demonstrated that intra-VTA CRF-1R antagonists can reduce escalated ethanol consumption in chronically drinking mice (14) and blunt binge-like ethanol drinking (15). Additionally, selective inhibition of CRF neurons in the bed nucleus of the stria terminalis (BNST), which projects to the VTA, also reduces binge-like ethanol consumption (16). And despite evidence that central CRF-2R agonists also reduce ethanol consumption (9, 12), the role of VTA CRF-2R activation, or the interaction between CRF-1R and CRF-2R, have not been explored.

The function of CRF signaling in the VTA is of particular interest because of the role that the VTA plays in regulating the reinforcing properties of drugs and ethanol (17-20), yet the

mechanisms by which CRF signaling in the VTA modulates binge-like ethanol intake is still largely unknown. Herein, we show that A) blunted binge-like ethanol drinking stemming from CRF-1R blockade requires intact CRF-2R signaling and CRF-2R activation reduces binge-like drinking, B) that silencing VTA-projecting CRF neurons originating in the BNST attenuates binge-like drinking, and C) a history of binge-like ethanol drinking alters protein and mRNA expression of candidate target of the VTA CRF system.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) aged 8-10 weeks at the start of experimentation were used, with the exception of the *in vivo* chemogenetic experiment where male CRF-ires-Cre (CRF-Cre) mice (positive for the expression of Cre under the CRF promoter as determined by standard PCR genotyping protocols) at least 10 weeks of age were used. CRF-Cre mice were generated as previously described in detail (16, 21).

Drugs and Solutions

Details regarding drugs, doses and solutions are described in detail in the supplemental materials.

Drinking in the Dark Procedure

Binge-like ethanol consumption was induced using a standard 4-d DID protocol (22, 23). Approximately 3h into the dark cycle, home cage water bottles were removed and animals were given access to a bottle containing 20% ethanol for 2h on days 1-3 (Training Days, TD), and for 2-4h on day 4 (Binge Test, BT). Immediately after the BT, approximately 30 μ l of tail blood were collected by nicking the lateral tail vein and BECs determined using the Analox Analyzer (Analox Instruments, Lunenburg, MA). See supplement for additional details.

In Vivo Pharmacology

Intra-VTA CRF-1R antagonism—An initial cohort of C57BL/6J mice (n=20) experienced one cycle of ethanol DID to establish baseline drinking, and were subsequently implanted with bilateral cannulae aimed at the VTA. After one week of recovery mice experienced a second cycle of DID, and were rank-ordered based on average consumption on days 1-3 and assigned to receive an intra-VTA microinfusion of either antalarmin or vehicle approximately 1h prior to ethanol access on the BT, such that ethanol consumption was equated between treatment conditions. Because a number of mice for this experiment lost cannulae, mice experienced a third cycle of DID and were assigned to receive either vehicle or antalarmin in a Latin Square design [final n=10/treatment after excluding cannulae loss (n=4) and placements outside of the VTA (n=6)]. These mice then experienced 2 cycles of DID with sucrose in a Latin Square design (final n=10/treatment).

Intra-VTA CRF-2R activation—To examine the effects of CRF-2R activation in the VTA on binge-ethanol consumption, a second cohort of mice (n=20) experienced one cycle of ethanol DID, and were subsequently implanted with bilateral cannulae aimed at the VTA.

After a second cycle of DID, and approximately 1h prior to ethanol access on the BT they received an infusion of either Urocortin III (Ucn3) or vehicle (n=9-10 per treatment). A sufficient number of mice retained their cannulae (n=19), and thus a Latin Square design was unnecessary. These mice then experienced one cycle of sucrose DID and received the opposite treatment they received during the ethanol DID.

Anatomical control site for CRF-1R antagonism and CRF-2R activation—To ensure that the effects of CRF-1R antagonism and CRF-2R activation were specific to the VTA and not due to drug traveling up the injector path, a third cohort of animals (n=16) experienced once cycle of ethanol DID, and were then implanted with cannulae aimed at a region approximately 2mm dorsal to the VTA. During a second cycle of DID mice were assigned to receive either antalarmin or vehicle (n=8/treatment). On the third cycle of DID mice that previously received drug, received vehicle, and mice that previously received vehicle, received Ucn3 (n=8/treatment).

Simultaneous CRF-1R and CRF-2R antagonism—To examine the relative contribution of each receptor to the reduction in binge-like ethanol consumption, a fourth cohort of mice (n=44) experienced one cycle of ethanol DID, and were subsequently implanted with bilateral cannulae aimed at the VTA. After recovery, the mice experienced a second cycle of DID and were assigned to receive vehicle (n=11), the CRF-1R antagonist, NBI-35965 (n=11), the CRF-2R antagonist, K-41498 (n=11), or both NBI-35965 and K-41498 simultaneously (n=11).

In Vivo Chemogenetic Manipulation

Inhibition of BNST to VTA projecting CRF neurons—Given previous evidence that $G_{i/o}$ -coupled designer receptors exclusively activated by designer drug (DREADDs (24)) cause functional inhibition of CRF neurons when transfected into CRF-Cre mice (16), we decided to examine the role of CRF neurons projecting from the BNST to the VTA. CRF-Cre mice (n=16) experienced one cycle of ethanol DID prior to surgery. Mice were then injected with either a Cre-dependent control vector (AAV8-hSyn-DIO-mCherry, n=8) or the Cre-dependent $G_{i/o}$ -coupled DREADD vector (AAV8-hSyn-DIO-hM4d-mCherry, n=8) into the dorsolateral BNST. Mice were also implanted with bilateral cannulae aimed at the VTA to allow for selective activation of the DREADD infected terminals projecting from the BNST to the VTA (25-27). After allowing approximately 5 weeks for maximal viral vector transduction, DREADD protein expression, translocation and incorporation, the mice then underwent 2 more cycles of DID. During cycle 2, all animals received intra-VTA microinjections of vehicle (1% DMSO in 0.9% saline) on Day 2 followed by a low dose of CNO (300 pmol) on day 4. Because the 300 pmol dose of CNO was ineffective, during cycle 3, all animals received intra-VTA microinjections of vehicle on day 2 and a higher dose of CNO (900 pmol) on day 4. To determine whether the increased CRF mRNA in the VTA after DID, reported herein, plays a functional role, an additional cohort of CRF-Cre mice were injected with either the control or Gi-DREADD vector (n=8/vector) directly into the VTA and given systemic CNO (3mg/kg, IP) or vehicle just prior to the BT (See supplemental materials for details). *qRT-PCR*

To examine the effects of binge-ethanol consumption on gene expression of CRF in the BNST and VTA, and CRF-1R and CRF-2R in the VTA, mice experienced one cycle of DID, receiving either 20% ethanol (n=16) or tap water (n=16) as described above. Immediately after the BT on day 4, animals were sacrificed by rapid decapitation, trunk bloods were taken to determine BECs, and bilateral punches of BNST and VTA tissue were collected. Using standard qRT-PCR protocols, changes in CRF, CRF-1R and CRF-2R gene expression were determined and gene expression was quantitated using the 2^{-CT} method, and are reported as fold change in RNA product. See supplemental materials for details on qRT-PCR procedures.

Western Immunoblotting

To examine the effects of binge-ethanol consumption on protein expression of CRF-1R, CRF-2R and ERK1/2 activation in the VTA, a separate cohort of mice experienced one cycle of DID, receiving either 20% ethanol (n=8-10) or tap water (n=8-10) as described above. Immediately after the BT on day 4, animals were sacrificed by rapid decapitation, trunk bloods were taken to determine BECs, and bilateral punches of VTA tissue were taken and whole-cell lysates were generated. Blots were incubated with primary antibodies raised against CRF-1R, CRF-2R, ERK1/2, phosphor-ERK1/2, and β -Actin/GAPDH, and either HRP-conjugated secondary antibodies for ECL detection or IRDye secondary antibodies (LI-COR Biosciences, Lincoln, NE). Band densities were quantified using Image Studio (LI-COR). See supplemental materials for details.

STATISTICAL ANALYSIS

All data are presented as the mean $\pm$ SEM. Unpaired t-tests (two-tailed), with Welch's correction where appropriate, were used to assess differences in mRNA and protein expression. BECs and ethanol consumption at both the 2-hr or 4-hr time-points were analyzed using unpaired t-tests (two-tailed) or repeated-measures (RM) ANOVAs (with Tukey's posthoc tests). In the case of experiments where a Latin Square design was employed, treatment order was included as a factor to ensure there were no drug carryover or order effects.

RESULTS

Intra-VTA CRF-1R antagonism and CRF-2R agonism significantly reduce binge-like ethanol consumption

Intra-VTA CRF-1R antagonism with the selective CRF-1R antagonist, antalarmin (2.4 nmol), significantly decreased binge-like ethanol consumption across the 4-hr BT (see Figure 1A). A RM ANOVA analyzing drinking across the 4 hour session with Time (2 vs. 4 hr) as a within-subjects factor and Drug (vehicle vs. antalarmin) and Order of drug treatment (1st vs. 2nd) revealed a significant main effect of Drug [$F_{1,16} = 12.731$; $p = 0.003$], but no effect of Time or Order, and no interaction effects (all $p > 0.15$), indicating that the reduction in ethanol consumption was not influenced by drug treatment order. There was a nonsignificant reduction in BECs in animals treated with intra-VTA antalarmin as seen in Figure 1B [$t_{18} = 1.215$, $p = 0.2399$]. Antagonism of the CRF-1R with antalarmin in the VTA

had no effect on binge-like consumption of the 10% sucrose control solution as is evident in Figure 1C [$t_{18} = 0.7118$, $p = 0.4857$], suggesting that the effects of CRF-1R antagonism were specific to ethanol.

As is evident in Figure 2A, a two-way RM ANOVA revealed a significant main effect of drug [$F_{1,17} = 6.147$; $p = 0.0239$], but no effect of time and no drug $\times$ time interaction demonstrating that intra-VTA administration of the CRF-2R agonist, Ucn3 (60 pmol), significantly reduced binge-like ethanol consumption across the 4 hr test, but did not significantly reduce corresponding BECs [Figure 2B; $t_{17} = 1.113$, $p = 0.2812$] and had no effect on sucrose consumption [Figure 2C; $t_{17} = 0.09602$, $p = 0.9247$]. Additionally, to ensure that the effects of CRF-1R antagonism and CRF-2R activation were site-specific, we microinjected antalarmin and Ucn3 into an anatomical control region dorsal to the VTA where drug may have naturally spread during the insertion or removal of the injector cannulae. Neither antalarmin, nor Ucn3, had any effect on binge-like ethanol consumption or associated BECs, indicating that the effects of CRF-1R antagonism and CRF-2R activation are specific to VTA infusions (see Supplemental Results, Figure S2).

We next determined if the ability of CRF-1R blockade to reduce binge-like ethanol drinking required intact CRF-2R signaling in light of the observation that activation of the CRF-2R in the VTA also blunted binge-like ethanol drinking. To this end we employed a double-pharmacology strategy whereby we micro-injected animals with vehicle, the CRF-1R antagonist, NBI-35965, the CRF-2R antagonist, K-41498, or both antagonists simultaneously. Here, a two-way RM ANOVA revealed a significant main effect of time [$F_{1,40} = 373.4$; $p < 0.0001$], and a significant drug $\times$ time interaction [$F_{3,40} = 5.133$; $p = 0.0043$] and posthoc analyses indicate that, consistent with antalarmin, the CRF-1R antagonist, NBI-35965, caused a significant reduction in binge-like ethanol consumption in the first 2hr of the BT compared to all other drug treatments (Figure 3A), an effect that was not evident at the end of the 4hr BT (see Figure 3B). As predicted CRF-2R antagonism with K-41498 had no effect on binge ethanol consumption, but when CRF-2Rs were antagonized simultaneously with CRF-1Rs the ability of CRF-1R antagonism alone to reduce ethanol consumption was no longer evident. Finally, there were no group differences in BECs (see Figure 3C).

In vivo chemogenetic inhibition of CRF projections from the BNST to VTA blunts binge-like ethanol drinking

Injection of channelrhodopsin viral vector (AAV5-EF1a-DIO-hChR2-EYFP) into BNST of CRF-cre mice (see supplement for details) was found later to be expressed in both the BNST neurons and VTA terminals (see Supplemental Results, Figure S3), confirming the BNST-to-VTA projection of CRF neurons. Having established this pathway as a viable candidate for releasing CRF in the VTA, we next examined how chemogenetic inhibition of this pathway altered drinking. In an independent test prior to the BT day, intra-VTA microinjections of vehicle was not associated with differences in binge-like ethanol consumption between either CRF-cre mice infected with the control virus versus G_i-DREADD virus in the BNST [Figure 4A; $t_{1,14} = 0.7636$, $p = 0.4578$]. Importantly, during the BT, intra-VTA microinjections of the selective DREADD agonist, CNO (900 pmol), prior to the 2hr BT

caused a significant reduction of binge-like ethanol drinking in CRF-Cre mice infected with the G_i -DREADD in the BNST relative to CRF-cre mice infected with the control virus [Figure 4B; $t_{14} = 2.641$, $p = 0.0194$]. Further, on the BT day, while G_i -DREADD mice treated with CNO exhibited BECs below 80 mg/dl, there was no significant difference between virus groups [Figure 4C; $t_{14} = 0.8216$, $p = 0.4251$]. In contrast, G_i -DREADD-induced inhibition of VTA CRF neurons did not impact binge-like ethanol drinking (see Supplemental Results, Figure S4), suggesting that while there are increases in CRF transcript in the VTA (see below) these neurons do not play a substantive role in regulating binge-like ethanol consumption.

Binge-like ethanol consumption alters mRNA and protein for CRF signaling molecules in the VTA

One 4-day cycle of ethanol DID (see Supplemental Results for consumption and BEC data, Figure S5A) did not alter CRF mRNA in the BNST [$t_{10} = 1.269$, $p = 0.2333$; Figure 5A], but significantly increased CRF mRNA in the VTA [$t_{5.401} = 3.203$; $p = 0.0215$ (with Welch's correction); Figure 5B]. Additionally, there were no significant effects on mRNA for the CRF-1R [$t_{13} = 1.455$; $p = 0.1695$; Figure 5C] or the CRF-2R [$t_{13} = 0.03538$; $p = 0.9723$; Figure 5D]. To determine the effects of binge-like ethanol consumption on CRF receptor protein expression, Western immunoblotting was performed on VTA tissue from mice that experienced one cycle of DID (see Supplemental Results for consumption and BEC data, Figure S5B). Here, binge-like ethanol drinking produced a significant decrease in CRF-1R protein expression in the VTA [$t_{11} = 2.439$, $p = 0.0329$; Figure 6A], but had no impact on CRF-2R expression [$t_{13} = 0.133$, $p = 0.8962$; Figure 6B]. To assess whether the downregulation in CRF-1R protein was reflected in activity associated with the receptor, downstream signaling molecules associated with CRF-1R signaling were also analyzed. In line with decreased CRF-1R expression there was a trend toward a decrease in ERK1/2 activation (i.e., ratio of phosphorylated ERK1/2 to total ERK1/2) in the VTA [$t_{11} = 1.878$, $p = 0.0871$; Figure 6C], but no change in total ERK1/2 expression [$t_{11} = 1.433$; $p = 0.1797$; Figure 6D].

DISCUSSION

Here we show that intra-VTA CRF-1R antagonism and CRF-2R activation both significantly decreased binge-like ethanol consumption, and that reductions in binge-like drinking due to CRF-1R blockade were dependent on intact CRF-2R signaling. Additionally, we show that CRF projections from the BNST to the VTA, but not local VTA CRF neurons, are involved in modulating binge-like ethanol drinking. Finally, binge-like ethanol consumption was sufficient to induce increased CRF mRNA expression within the VTA, associated with a significant decrease in CRF-1R protein levels. Together, these data provide a more complete understanding the role of CRF receptors within the VTA and the neurocircuitry that modulate binge-like ethanol drinking.

Our results replicate previous work (15) showing that CRF-1R antagonism in the VTA blunts binge-like ethanol drinking without altering consumption of a sucrose solution. While we have previously shown that ventricular infusion of the CRF-2R agonist, Ucn3, blunts

binge-like ethanol drinking (12), the current work identifies a locus of action as VTA-infusion of Ucn3 selectively blunted binge-like ethanol drinking. As intended, the subthreshold dose of the CRF-2R antagonist K-41498 did not alter ethanol intake allowing for the determination of CRF-2R involvement in CRF-1R effects on consumption [though a recent report showed that CRF-2R antagonist, Astressin-2B, had biphasic effects on ethanol intake (28, 29)]. Interestingly, simultaneous blockade of CRF-1R and CRF-2R prevented CRF-1R antagonist-induced reduction of binge-like ethanol drinking. While these observations suggest that the ability of CRF-1R blockade to reduce binge-like ethanol drinking requires intact CRF-2R signaling, it should be noted that despite the high selectivity of the CRF receptor ligands used in these experiments, the differences in pharmacokinetics and the time-course of the effects seen may imply that other mechanisms are engaged. However, given the consistency of our findings that two different CRF-1R antagonists decreased ethanol consumption with data previously reported by Sparta et al., (2013), and the robust blockade of this effect when CRF-2 receptors were antagonized, we believe that the dependence of the CRF-1R on intact CRF-2R signaling to be the most plausible explanation, given the data available.

We have recently shown that CNO activation of Gi-DREADDs in CRF neurons of the BNST both functionally inhibit the activity of these neurons *in vitro* and blunt binge-like ethanol drinking *in vivo* (16). BNST CRF neurons project to the VTA (30) and release GABA (T.L. Kash, Personal Communication) and have recently been shown to be exclusively co-localized with GABA by immunohistochemical, transcriptomic, and electrophysiological techniques (31). Additionally, inhibition of GABAergic signaling locally in the posterior VTA has been shown to blunt binge-like ethanol drinking (32). Thus, we predicted that silencing VTA-projecting CRF/GABAergic neurons from the BNST would blunt binge-like ethanol drinking. Using the channelrhodopsin viral vector AAV5-EF1a-DIO-hChR2-EYFP as an anterograde tracer in CRF-cre mice, we showed that BNST CRF neurons do in fact provide dense projections to the VTA. Importantly, when the Gi-DREADD was transduced in the BNST and CNO infused into the VTA, mice exhibited blunted binge-like ethanol drinking that was not evident in mice expressing the control virus in the BNST or following vehicle administration. And while we do not have definitive electrophysiological evidence of terminal CNO effects, as *in vivo* and *ex vivo* validation of such is inherently challenging, a number of recent studies have shown that terminal application of CNO does indeed alter behavior (25-27). Additionally, since this same treatment did not impact sucrose consumption, our results provide the first direct evidence that a CRF/GABAergic pathway form the BNST to the VTA selectively modulates binge-like ethanol drinking.

Increased BNST to VTA CRF release could feasibly explain our previous work showing increased VTA CRF immunoreactivity stemming from binge-like drinking (13), as evidence suggests that CRF in the VTA originates from projection neurons of structures such as the BNST, CeA and the paraventricular nucleus of the hypothalamus (PVN) (30). And until recently, there was little evidence that neurons within the VTA produced CRF. However, we provide novel evidence showing that binge-like ethanol drinking promotes increased CRF mRNA within the VTA (but not BNST). These observations are consistent with recent data showing that nicotine use and withdrawal also increase CRF mRNA in the VTA in a subpopulation of dopaminergic neurons (33). Grieder and colleagues (2014) also

demonstrated a functional role for these CRF/dopaminergic VTA neurons by showing that silencing of CRF mRNA in the VTA prevents the aversive effects of nicotine withdrawal and abstinence-induced escalation of the nicotine intake. However, given that inhibition of the intra-VTA CRF neurons using Gi-DREADDs did not alter binge-like consumption of ethanol in the present study, it appears that, while CRF neurons within the VTA are engaged by acute binge-like ethanol consumption, this population of neurons does not appear to modulate this behavior. We surmise that CRF/TH positive neurons in the VTA likely play a more significant role in other aspects of ethanol reward or given their involvement in other stress-related aspects of drug addiction, they may serve to promote relapse during episodes of abstinence in dependent models, which has yet to be explored. One caveat to this argument is that the Gi-DREADDs require functional characterization in the VTA of CRF-cre mice. Additionally, while we found no changes in CRF receptor mRNA in the VTA, binge-induced reductions of CRF-1R protein was significant. It has been reported that increases in CRF signaling promote the internalization and recycling of the CRF-1R (34-37), and thus the reduction in protein expression seen here likely reflects a compensatory response to counter increased CRF signaling

The mechanism by which CRF-1R and CRF-2R interact in the modulation of binge-like ethanol drinking is worth considering. Since BNST-to-VTA CRF neurons likely entail a GABAergic phenotype, and inhibition of GABA signaling in the VTA blunts binge-like ethanol drinking (32), it is likely that binge-like ethanol drinking triggers activity of BNST-to-VTA CRF/GABAergic neurons and activation of these neurons promotes continued binge drinking (consistent with our chemogenetic observations). We speculate that CRF-1R and CRF-2R function as heteroreceptors modulating binge-like ethanol drinking via presynaptic regulation of GABA release. In fact, evidence suggests that presynaptic CRF-1R stimulates GABA release from neurons in the amygdala (4, 38), and in the VTA of rats subjected to stressor/cue-induced reinstatement of cocaine-seeking following a chronic cocaine exposure presynaptic CRF-2R blunts GABA release (39). Thus, we hypothesize that presynaptic CRF-1R stimulates GABA release from CRF/GABA projection neurons while CRF-2R blunts GABA release in these same neurons. When CRF-1R is blocked, the ability of the CRF-2R to inhibit or “brake” binge-induced GABA release is unopposed. Exogenous application of the selective CRF-2R agonist, Ucn3, would theoretically potentiate this braking mechanism. On the other hand, when both CRF receptors are blocked, binge-induced GABA signaling from GABAergic projection neurons to the VTA remains unregulated and drives continued binge-like ethanol drinking, perhaps by inhibiting VTA GABA interneurons (that normally inhibit phasic DA firing) and promoting the CRF-induced enhancement of glutamatergic activation of dopamine neurons as proposed by Sparta et al. (15). Viewed this way, the present pharmacological and chemogenetic data can be interpreted with consistency (schematic shown in Figure 7). However, additional work is needed to determine the exact mechanism by which CRF receptor signaling in the VTA modulates binge-like ethanol intake.

In summary, here we provide novel evidence for a role of the CRF-2R in the VTA, and an interaction between CRF-1R and CRF-2R, in the modulation of binge-like ethanol drinking. While binge-like ethanol drinking triggers the activity of local CRF neurons, these neurons do not appear to modulate drinking. The CRF signaling in the VTA that modulates binge-

like ethanol drinking arises from a circuit originating in the BNST and likely involves heteroregulation of GABA signaling from the same neurons. These observations reinforce the idea that targets aimed at the central CRF system may be therapeutic in treating alcohol use disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Intra-VTA antagonism of the CRF-1 receptor significantly reduces binge-like ethanol consumption

(A) Bilateral infusion of the CRF-1R antagonist antalarmin (2.4 nmol/0.5µl/side) into the VTA significantly ($p = 0.0025$) decreased binge-like ethanol consumption across the 4-hr BT (mean Ethanol consumption in g/kg + SEM). (B) Corresponding blood ethanol concentrations (BECs) achieved during the 4-hr BT (mean + SEM) were not significantly reduced. (C) Intra-VTA antalarmin did not alter binge-like consumption of a 10% sucrose solution (mean + SEM). (D) For ease of visualization, injector tip placements are shown

only for those animals that had bilateral hits in the VTA ($n = 10$). Missed placements were excluded from analysis.

Figure 2. Intra-VTA activation of the CRF-2 receptor significantly reduces binge-like ethanol consumption

(A) Bilateral infusion of the CRF-2R agonist Urocortin-III (UCN3, 60 pmol/0.4 μ l/side) into the VTA significantly (**p* = 0.024) decreased binge-like ethanol consumption across the 4-hr BT (mean Ethanol consumption in g/kg + SEM). (B) Corresponding blood ethanol concentrations (BECs) achieved during the 4-hr BT (mean + SEM) were not significantly reduced. (C) Intra-VTA UCN3 did not alter binge-like consumption of a 10% sucrose solution (mean + SEM). (D) For ease of visualization, injector tip placements are shown

only for those animals that had bilateral hits in the VTA ($n = 19$). Missed placements were excluded from analysis.

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Figure 3. Simultaneous blockade of the CRF-1R with the antagonist NBI-35965 and the CRF-2R with antagonist K41498 has no effect on binge-like ethanol consumption

(A) Intra-VTA injections of the CRF-1R antagonist NBI-35965 (30 pmol/0.3μl/side) reduced binge-like ethanol consumption at the 2-hr timepoint (* $p=0.0298$), but the effect was no longer evident at the 4-hr timepoint (B), and due to the transient nature of the effect, was also not reflected in the BECs (C). Importantly, the CRF-2R antagonist, K41498 (50 pmol/0.3μl/side) alone did not alter drinking at any of the timepoints, and interestingly, when NBI-35965 and K41498 were administered simultaneously, the ability of NBI-35965 to reduce ethanol consumption was no longer evident. All data are presented as mean $\pm$ SEM.

Figure 4. Inhibition of CRF projections from the BNST to the VTA blunts binge-like ethanol consumption

(A) Intra-VTA microinjections of vehicle (1% DMSO in saline, 0.3 μ l/side) in CRF-Cre mice injected with either a Cre-dependent G_i -DREADD (hM4d-Gi) or a control vector into the BNST did not alter Ethanol consumption during a 2-hr test (mean Ethanol consumption in g/kg + SEM). (B) Microinjections of CNO (900 pmol/0.3 μ l/side) did, however, significantly (* $p = 0.0194$) reduce binge-like ethanol consumption in hM4d-Gi mice relative to control virus mice. (C) Corresponding BECs achieved during the 2-hr BT (mean + SEM) showed only a trend toward a reduction in the hM4d-Gi. (D) Confocal images of mCherry expression in CRF-Cre neurons expressing the G_i -DREADD in the BNST are shown at 10 $\times$ magnification (scale bar = 100 μ m) and 40 $\times$ (inset, scale bar = 50 μ m). (E) Intra-VTA cannulae placements are shown only for those animals that had bilateral DREADD or control vector expression in the BNST and bilateral hits in the VTA (n=16).

Figure 5. One cycle of binge-like ethanol consumption alters CRF mRNA in the VTA, but not the BNST

(A) qRT-PCR analysis of CRF mRNA after one cycle of DID showed binge-like ethanol consumption did not alter CRF transcripts in the BNST (water controls $n = 7$, ethanol $n = 5$).

(B) However, it did significantly increase CRF mRNA expression approximately 8-fold in the VTA (* $p = 0.0215$; water controls $n = 8$, ethanol $n = 6$).

(C & D) Binge-like ethanol consumption had no significant effect on mRNA for either the CRF-1 or CRF-2 receptor in the VTA (water controls $n = 8$, ethanol $n = 7$).

Figure 6. One cycle of binge-like ethanol consumption reduces CRF-1R and ERK1/2 activation in the VTA

(A) Western blot analysis of CRF-1R protein showed that the trend toward an increase in CRF-1R message did not translate to a functional change in protein expression, as one cycle of DID lead to a significant decrease in CRF-1R protein in the VTA ($*p = 0.0329$). (B) However, binge-like consumption of ethanol had no effect on CRF-2R protein expression in the VTA. (C) In line with the decrease in CRF-1R, one cycle of DID also resulted in a trend toward a decrease in ERK 1/2 activation, determined by a decreased ratio of phosphorylated

ERK 1/2: total ERK 1/2 in ethanol drinking animals compared to controls ($p = 0.0871$), with no significant changes in total ERK 1/2 expression relative to β -Actin (**D**).

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Figure 7. Schematic of VTA-projecting CRF/GABA neuron of the BNST

We hypothesize that in addition to acting as a postsynaptic receptor, CRF-1R also functions as a heteroreceptor located on CRF/GABAergic projections from the BNST. Alcohol-induced hyperexcitability of these BNST-VTA projections increases CRF/GABA release, and in a feed-forward fashion, the binding of CRF to the CRF-1R promotes further CRF/GABA release in the VTA and drives further binge drinking. When a CRF-1R antagonist is applied to this circuit, CRF is freed to bind to the lower affinity CRF-2R, also located on these BNST to VTA projections, resulting in decreased CRF/GABA release and decreased alcohol consumption. Similarly, applying exogenous CRF-2R agonists potentiates this braking mechanism, reducing alcohol consumption.