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Central amygdala corticotropin-releasing factor neurons promote hyponeophagia but do not control alcohol drinking in mice

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Abstract

Corticotropin-releasing factor (CRF) signaling in the central nucleus of the amygdala (CeA) plays a critical role in rodent models of excessive alcohol drinking. However, the source of CRF acting in the CeA during alcohol withdrawal remains to be identified. In the present study, we hypothesized that CeA CRF interneurons may represent a behaviorally relevant source of CRF to the CeA increasing motivation for alcohol via negative reinforcement. We first observed that *Crh* mRNA expression in the anterior part of the mouse CeA correlates positively with alcohol intake in C57BL/6J males with a history of chronic binge drinking followed by abstinence and increases upon exposure to chronic intermittent ethanol (CIE) vapor inhalation. We then found that chemogenetic activation of CeA CRF neurons in *Crh*-IRES-Cre mouse brain slices increases gamma-aminobutyric acid (GABA) release in the medial CeA, in part via CRF1 receptor activation. While chemogenetic stimulation exacerbated novelty-induced feeding suppression (NSF) in alcohol-naïve mice, thereby mimicking the effect of withdrawal from CIE, it had no effect on voluntary alcohol consumption, following either acute or chronic manipulation. Furthermore, chemogenetic inhibition of CeA CRF neurons did not affect alcohol consumption or NSF in chronic alcohol drinkers exposed to air or CIE. Altogether, these findings indicate that

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Disclosure

The authors have no conflicts of interest to declare.

CeA CRF neurons produce local release of GABA and CRF and promote hyponeophagia in naïve mice, but do not drive alcohol intake escalation or negative affect in CIE-withdrawn mice. The latter result contrasts with previous findings in rats and demonstrates species specificity of CRF circuit engagement in alcohol dependence.

Introduction

Alcohol use disorders (AUDs) represent a spectrum of pathological patterns of alcoholic beverage consumption that affect 6.2% of the adult population in the United States of America¹ and more than 100 million people worldwide². AUDs are not only characterized by the intake of excessive amounts of alcohol but also by the emergence of a negative emotional state (e.g., anxiety, irritability) upon withdrawal. While the motivation to drink alcohol is initially driven by the desire to experience the pleasurable effects of intoxication (positive reinforcement), it becomes progressively driven by the need for relief from the negative emotional state associated with withdrawal (negative reinforcement)³. Excessive alcohol drinking (i.e., beyond intoxication threshold) and negative affect during withdrawal can be triggered in several mouse and rat models, which has enabled the dissection of underlying neurobiological mechanisms^{4–8}.

Multiple pharmacological and genetic studies have highlighted that recruitment of the extrahypothalamic corticotropin-releasing factor (CRF, encoded by the *Crh* gene) system plays a pivotal role in the emergence of negative reinforcement in alcohol-withdrawn animals, whereby activation of CRF type 1 receptors (CRF1) contributes to the excessive drinking, heightened anxiety, and stress sensitization characterizing alcohol dependence^{3, 9–12}. In particular, CRF is released in the central nucleus of the amygdala (CeA) during alcohol withdrawal¹³ and blockade of CRF1 signaling in the CeA reduces the anxiogenic-like effect of alcohol withdrawal^{14, 15}, dependence-induced increase in alcohol self-administration^{16, 17}, as well as heavy binge drinking¹⁸. CRF1 in the CeA also mediates the elevation of gamma-aminobutyric acid (GABA) dialysate induced by alcohol dependence¹⁹ and CRF1 overexpression in the CeA potentiates stress-induced reinstatement of alcohol seeking²⁰.

Despite this wealth of converging evidence, the specific neurons responsible for the release of CRF in the CeA during alcohol withdrawal have not been identified. In the present study, we tested the hypothesis that these neurons are intrinsic to the CeA rather than arising from an extrinsic source. This hypothesis was supported by evidence that *Crh* is upregulated in the CeA of alcohol-dependent and post-dependent rats^{19, 21, 22}, along with our own findings (reported here) that *Crh* expression in the anterior part of the CeA correlates with alcohol intake in mice with a history of chronic alcohol drinking followed by abstinence and increases upon exposure to chronic intermittent ethanol (CIE) vapor inhalation. Importantly, psychological stress also upregulates *Crh* in the CeA^{23–25} and CRF synthesis in the CeA plays a critical role in mediating anxiety-like behavior^{26, 27}. In addition, CRF1-expressing neurons in the CeA receive direct input from local interneurons²⁸ and CeA CRF neurons provide both local inhibitory GABA and excitatory CRF1-mediated signals to other CeA neurons in *Crh*-Cre rats²⁹, which supports the notion that CRF can be released from intrinsic

CeA neurons. Furthermore, studies in *Crh*-Cre rats have recently shown that chemogenetic stimulation of CeA CRF neurons elicits anxiety-like behavior^{30, 31}, while their optogenetic inhibition reduces escalated alcohol self-administration in alcohol-dependent rats³². In the mouse, CeA CRF neurons are more active than their non-CRF neighbors during repeated alcohol binge drinking³³.

According to the hypothesis that CeA CRF neurons can release CRF locally, we predicted that chemogenetic stimulation of mouse CeA CRF neurons would replicate cellular and behavioral hallmarks of alcohol dependence. While this manipulation produced local CRF1-mediated GABA release and elicited signs of negative affect resembling those seen during alcohol withdrawal, it did not increase voluntary alcohol consumption. Furthermore, their chemogenetic inhibition did not reverse ethanol intake escalation in alcohol-dependent mice. Accordingly, our data suggest that contrary to reports in rats, CeA CRF neurons are neither sufficient nor necessary to drive excessive alcohol drinking in mice.

Materials and Methods

Animals

C57BL/6J males were purchased from the Jackson Laboratories (stock #000664) or from Scripps Research rodent breeding colony. *Crh*-IRES-Cre breeders were obtained from The Jackson Laboratory (B6(Cg)Crh^{tm1(cre)Zjh}/J, stock # 012704,³⁴). All *Crh*-IRES-Cre mice used for experimentation were heterozygous males. Additional details are provided in the Supplement. All procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Scripps Research.

Viral vectors

Adeno-associated viral serotype 2 (AAV2) vectors encoding the hM3Dq (excitatory) or hM4Di (inhibitory) designer receptors³⁵ fused to the red fluorescent protein mCherry, under the control of the human synapsin promoter and in a Cre-dependent manner³⁶, were obtained from the Vector Core at the University of North Carolina (UNC) at Chapel Hill or from Addgene. Vectors were injected bilaterally into the anterior part of the CeA (AP -0.9 mm from bregma, ML \pm 2.8–3.0 from the midline, DV -4.5 mm from the skull). Additional details are provided in the Supplement.

Experimental cohorts

In situ hybridization data were collected from two cohorts of C57BL/6J mice subjected to chronic alcohol drinking and alcohol vapor inhalation, respectively. For chemogenetic stimulation, a first cohort of *Crh*-IRES-Cre mice was used for electrophysiological recordings. A second cohort was tested in the elevated plus-maze (EPM), social approach, and novelty-suppressed feeding (NSF) assays, with at least one week between tests; these mice were then single-housed and tested for alcohol drinking. A third cohort was tested for digging and marble burying, and their brains were used to quantify c-Fos induction one week later. A fourth cohort was used to confirm the phenotype observed in the NSF test and included negative control mice; these mice were also tested for locomotor activity

and fasting-refeeding in the home cage. For chemogenetic inhibition, electrophysiological validation was conducted in a first cohort of *Crh*-IRES-Cre mice and alcohol dependence was induced in a second cohort. The effect of CP376395 on alcohol drinking was tested in a separate cohort of C57BL/6J mice exposed to air or CIE.

Detailed methods for *in situ* hybridization, electrophysiology, immunohistochemistry, alcohol exposure, and behavioral testing are provided in the Supplement.

Statistical analysis

Data analysis was performed in Statistica 13.3 (TIBCO Software Inc.). The correlation of *Crh in situ* hybridization signal with ethanol intake was evaluated using Pearson's r . The effect of alcohol vapor inhalation on *Crh* signal was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's comparisons to the Air group. The effect of clozapine-N-oxide (CNO) on c-Fos counts, firing rate, and sIPSC parameters was analyzed using paired two-tailed Student's t -tests. The effect of CNO combined with R121919 was analyzed by repeated measures (RM) ANOVA and Pearson's correlation. CNO-responsive cells were identified by comparing the distribution of inter-event intervals at baseline and after CNO application using a Kolmogorov-Smirnov test. In hM3Dq-expressing mice, one-way RM-ANOVAs were used to analyze the effect of CNO on locomotor activity and ethanol intake (acute regimen). The effects of CNO on ethanol intake (chronic regimen), social approach, hyponeophagia, and refeeding were analyzed by two-way RM-ANOVA. Other behavioral endpoints were analyzed using unpaired two-tailed Student's t -tests. Behavioral measures obtained in hM4Di-expressing mice were analyzed by two-way RM-ANOVA. Tukey *posthoc* tests were conducted when relevant. The effect of CP376395 was analyzed by two-way RM-ANOVA followed by Dunnett's comparisons to vehicle. Data are shown as mean \pm s.e.m. on the graphs.

Results

Crh expression in the anterior part of the CeA correlates with ethanol intake and increases upon CIE

As described previously using CRF immunostaining and a GFP reporter strain, CRF expression varies along the rostral-caudal extent of the mouse CeA^{37, 38}. We confirmed this observation using chromogenic *in situ* hybridization to label *Crh* mRNA (Fig. 1A), as can also be seen in the Allen Mouse Brain Atlas (<https://mouse.brain-map.org>, experiment 292). At the most anterior level (≈ -0.8 mm from bregma), a V-shaped cluster of CRF neurons is found at the transition between the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and the CeA. At midlevel (≈ -1.2 mm from bregma), CRF neurons are more sparsely distributed, mostly within the lateral aspect of the CeA, and to a smaller extent within the medial CeA. At a more posterior level (≈ -1.6 mm from bregma), CRF neurons are tightly packed within the lateral CeA, with again a few cell bodies found in the medial CeA.

Semi-quantitative measures of *Crh* expression at these three antero-posterior levels were obtained in C57BL/6J males subjected to chronic alcohol binge drinking for nine weeks

and euthanized four weeks after their last drinking session. Average ethanol intake during the last drinking week ranged between 1.6 and 5.8 g/kg per 2-h session (Fig. 1B). *Crh* expression in the most anterior part of the CeA (bregma -0.8 mm, including IPAC) correlated positively with the average ethanol intake measured during the last week of drinking ($r=0.60$, $p=0.02$, Fig. 1C). No significant correlation was observed at more posterior levels of the CeA (bregma -1.2 mm: $r=0.13$, $p=0.65$; bregma -1.6 mm: $r=0.33$, $p=0.23$; Fig. 1C).

Additional analyses were conducted to determine whether *Crh* expression in the anterior CeA responded to alcohol intoxication or withdrawal. C57BL/6J males were exposed to a single bout (Acute) or eight bouts of 16 h of ethanol vapor inhalation. Mice subjected to eight bouts were euthanized either immediately after vapor exposure (Chronic) or following 72 h withdrawal (Withdrawal). *Crh* expression at bregma -0.8 mm was significantly higher in Chronic and Withdrawal mice (Fig. 1D; main effect of group: $F_{3,13}=4.5$, $p=0.022$; *posthoc* comparisons to Air group: $p=0.014$ and $p=0.035$, respectively).

Based on these results, the anterior level of the CeA was targeted for viral vector infusions in subsequent chemogenetic experiments.

Validation of chemogenetic approach to stimulate CeA CRF neurons

We used Cre-dependent expression of the excitatory designer receptor hM3Dq in *Crh*-IRES-Cre males to analyze the effects of CeA CRF neuron stimulation on CRF release, alcohol drinking and affective behaviors. We first assessed the specificity of Cre activity distribution in the CeA of *Crh*-IRES-Cre mice and verified that CNO activated hM3Dq-expressing cells both *in vivo* and in brain slices.

We analyzed the overlap between *Crh* and *tdTomato* mRNAs in CeA sections of *Crh*-IRES-Cre;Ai9 mice by double *in situ* hybridization (Fig. 2A). We found that 86.3 ± 4.9 % of *tdTomato*⁺ neurons expressed *Crh* (a measure of reporter fidelity), while 64.0 ± 5.4 % of *Crh*⁺ neurons expressed *tdTomato* (a measure of reporter penetrance). Consistent with these results, in *Crh*-IRES-Cre mice injected with an AAV2-hSyn-DIO-hM3Dq-mCherry vector in the CeA, mCherry-immunolabeled cell bodies showed the same distribution as *Crh* mRNA (Fig. 2B, compare with Fig. 1A left panel and Fig. 2A). Mice were perfused 90 min following i.p. administration of either vehicle or CNO (5 mg/kg) and their brains were processed for immunohistochemistry. mCherry immunoreactivity was used to label hM3Dq-expressing cells and c-Fos immunoreactivity was used as a marker of cellular activation (Fig. 2C–D). As expected, CNO increased the number of c-Fos⁺ cells selectively in the subpopulation expressing hM3Dq ($t_{11}=4.1$, $p=0.002$), but it did not affect the number of c-Fos⁺ cells among mCherry-negative cells ($t_{11}=-0.1$, $p=0.91$; Fig. 2D, left). Upon CNO administration, the proportion of active (c-Fos⁺) hM3Dq-expressing cells increased about 4-fold ($t_{11}=6.3$, $p<0.0001$; Fig. 2D, right).

In another cohort of *Crh*-IRES-Cre mice injected in the CeA with a Cre-dependent vector encoding hM3Dq-mCherry, action potentials were recorded from mCherry-positive (i.e., hM3Dq-expressing) CeA neurons (Fig. 3A–B). As expected, CNO (500 nM) application significantly increased their firing rate ($t_7=-6.5$, $p=0.0003$; Fig. 3B).

Stimulation of CeA CRF neurons increases local GABAergic transmission via activation of CRF1 receptors

We first evaluated the possibility that CeA CRF neurons release CRF in the medial CeA, where both CRF and ethanol are known to increase GABA release via the activation of CRF1 receptors³⁹. Voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSC) were obtained from mCherry-negative neurons (n=33 neurons from 11 mice) located in the medial CeA before and during application of CNO (500 nM). Overall, CNO produced a significant increase in sIPSC frequency (Fig. 3C–D, $t_{32}=-3.5$, $p=0.001$), but had no effect on amplitude (Fig. 3E, $t_{32}=-1.5$, $p=0.16$), rise time (Fig. 3F, $t_{32}=-0.8$, $p=0.45$) or decay time (Fig. 3G, $t_{32}=-0.2$, $p=0.83$). However, the response to CNO was variable between cells. Within-cell analysis of the cumulative distribution of inter-event intervals indicated that sIPSC frequency was significantly increased in 19 cells (blue bars in Fig. 3D–G), reduced in 3 cells, and unchanged in the remaining 11 cells ($p>0.05$). In the subset of neurons that responded with increased sIPSC frequency ($t_{18}=-7.6$, $p<0.0001$), there was still no effect of CNO on sIPSC amplitude ($t_{18}=-1.5$, $p=0.15$), rise time ($t_{18}=-0.6$, $p=0.53$) and decay time ($t_{18}=-0.7$, $p=0.52$).

In a subset of CNO-sensitive neurons (n=10), the CRF1 antagonist R121919 (1 μ M) was applied with CNO to determine whether the effect of CNO on sIPSC frequency was in part mediated by CRF1 activation (see Fig. 3A for schematic representation of tested hypothesis). There was a significant main effect of treatment (Fig. 3H, $F_{2,18}=6.1$, $p=0.01$) reflecting the increase in sIPSC frequency induced by CNO and partial reversal of the effect of CNO by R121919. *Posthoc* analysis revealed a significant difference between baseline and CNO ($p=0.008$). Furthermore, R121919 exerted a stronger inhibition in cells with higher sIPSC frequency, as indicated by a significant correlation between the reversal effect of R121919 (expressed as frequency change after addition of R121919) and baseline frequency (Fig. 3I, $r=-0.90$, $p<0.001$).

Altogether, these data indicate that chemogenetic stimulation of mouse CeA CRF neurons increases GABA release onto local medial CeA neurons and that this effect is at least partially mediated by CRF1 activation.

Stimulation of CeA CRF neurons does not affect alcohol drinking

These results, combined with the fact that CRF1 signaling in the CeA drives excessive alcohol intake in rodent models of binge-like drinking and dependence^{16–18}, led us to hypothesize that chemogenetic stimulation of CeA CRF neurons (Fig. 4A) may promote voluntary alcohol consumption. To test this hypothesis, the effect of CNO on ethanol intake during 2-h ethanol (15% v:v)/water two-bottle choice (2BC) drinking sessions was tested under different conditions (see Fig. 4B for experimental timeline).

Acute CNO (1, 5 and 10 mg/kg) administration (30 min prior to drinking session) did not affect ethanol intake (Fig. 4C, $F_{3,39}=1.6$, $p=0.21$). We then attempted to replicate the pattern of CRF release in the CeA elicited by chronic intermittent ethanol (CIE) exposure. In the CIE-2BC model, weeks of 2BC (Mon-Fri) are alternated with weeks of CIE consisting of four 16-h periods of ethanol vapor inhalation separated by 8-h periods of air inhalation

(Mon-Fri)⁴. Extracellular CRF levels are known to gradually increase in the amygdala of rats withdrawn from chronic alcohol exposure starting about 6 h after the onset of withdrawal¹³. Accordingly, to replicate the predicted pattern of CRF release experienced by CIE-exposed mice, we injected CNO (5 mg/kg) once per day (Tue-Fri) and conducted 2BC sessions on alternate weeks (Fig. 4B). In the CIE-2BC model, CIE-exposed mice typically start increasing their voluntary ethanol intake after 1–3 weeks of CIE exposure^{4, 40}. However, alternating weeks of repeated CNO administration with weeks of 2BC for three rounds did not affect ethanol intake compared to mice repeatedly injected with vehicle (Fig. 4D). A two-way ANOVA of weekly average intakes revealed no effect of time ($F_{3,36}=1.4$, $p=0.26$), no effect of treatment ($F_{1,36}=0.01$, $p=0.91$) and no time \times treatment interaction ($F_{3,36}=0.3$, $p=0.86$). We examined whether repeated CNO administration may produce a delayed effect but there was again no effect of treatment on ethanol intake following 3 weeks of deprivation (Fig. 4E, $t_{12}=-1.0$, $p=0.33$). Finally, we exposed the mice to a single week of CIE, which was not sufficient to alter ethanol intake in subsequent 2BC sessions (Tue-Fri). We then tested the ability of acute CNO (5 mg/kg) administration to sensitize the mice and increase their post-vapor ethanol intake. There was again no significant effect of CNO (Fig. 4F, $t_6=-0.2$, $p=0.87$).

In conclusion, chemogenetic stimulation of mouse CeA CRF neurons is not sufficient to increase ethanol drinking following acute or chronic administration, or in combination with CIE exposure.

Stimulation of CeA CRF neurons exacerbates hyponeophagia

We then examined whether chemogenetic stimulation of CeA CRF neurons (Fig. 5A) may elicit anxiety-like behavior. CNO (1 or 5 mg/kg) did not affect locomotor activity in a familiar environment (Fig. 5B, $F_{2,16}=0.9$, $p=0.44$). The dose of 5 mg/kg was used in all other tests. In the digging assay (Fig. 5C), CNO tended to increase the total duration of digging ($t_{10}=-2.0$, $p=0.07$), but had no effect on marble burying ($t_{10}=-0.5$, $p=0.62$). In the EPM (Fig. 5D), CNO had no effect on the total distance traveled ($t_{10}=0.1$, $p=0.94$), the number of entries on open arms ($t_{10}=0.4$, $p=0.73$) or the time spent on open arms ($t_{10}=-1.5$, $p=0.16$). In the social approach test (Fig. 5E), there was a significant main effect of compartment ($F_{2,24}=29.9$, $p<0.0001$), reflecting a preference for the chamber containing a stranger mouse compared to both the center and empty cup compartments ($p<0.0001$ for both *posthoc* comparisons). CNO did not alter this preference, as reflected by a lack of treatment effect ($F_{1,12}=1.5$, $p=0.24$) and compartment \times treatment interaction ($F_{2,24}=0.2$, $p=0.80$). There was also no effect of CNO on the number of transitions between compartments ($t_{12}=0.6$, $p=0.56$), in agreement with the lack of effect of CNO on locomotor activity.

In the NSF test (Fig. 5F), it took longer for the food-deprived mice to start eating in the arena than in their home cage (main effect of environment: $F_{1,12}=57.1$, $p<0.0001$) and CNO selectively prolonged the arena latency (environment \times treatment interaction: $F_{1,12}=8.3$, $p=0.01$; *posthoc* comparison Arena-Vehicle vs Arena-CNO: $p=0.0006$; Home cage-Vehicle vs Home cage-CNO: $p=1.00$). We replicated this finding in an independent cohort, which also included control mice (Cre-dependent tdTomato expression, no hM3Dq). In this second replicate (Fig. 5G), CNO again increased the latency to start eating the pellet in the arena

but not the home cage in hM3Dq-expressing mice (environment \times treatment interaction: $F_{1,6}=6.9$, $p=0.04$; *posthoc* comparison Arena-Vehicle vs Arena-CNO: $p=0.007$; Home cage-Vehicle vs Home cage-CNO: $p=1.00$), and it had no effect in Control mice (main effect of environment: $F_{1,6}=32.1$, $p=0.001$; main effect of treatment: $F_{1,6}=1.0$, $p=0.36$; environment \times treatment interaction: $F_{1,6}=0.5$, $p=0.50$). To verify that the anxiogenic-like effect of CNO in the NSF test was not related to reduced appetite, we further examined the effect of CNO on feeding in a home cage setting. CNO had no effect on food consumption after 24-h deprivation at any of the time points examined (Fig. 5H, $F_{1,7}=0.09$, $p=0.77$).

In summary, chemogenetic stimulation of mouse CeA CRF neurons promotes hyponeophagia without altering appetite. The anxiogenic-like effect of this manipulation is not detected in other assays of anxiety-like behavior.

Chemogenetic inhibition of CeA CRF neurons does not reverse alcohol dependence phenotypes

Although stimulating CeA CRF neurons was not *sufficient* to increase alcohol drinking, this population might still be *necessary* to ethanol intake escalation in the CIE-2BC model. We employed chemogenetic inhibition to test this hypothesis. *Crt*-IRES-Cre mice were injected in the CeA with a Cre-dependent vector encoding hM4Di-mCherry (Fig. 6A). CNO reduced the frequency of events recorded in loose cell attached configuration selectively in hM4Di-expressing cells, thereby validating our approach (Fig. 6B, mCherry+: $t_5=-6.3$, $p=0.003$; mCherry-: $t_4=0.5$, $p=0.68$). The effect of CNO on CIE-induced behaviors was then tested (see Fig. 6C for experimental timeline). As expected, withdrawal from CIE increased voluntary alcohol consumption (Fig. 6D, main effect of CIE: $F_{1,18}=18.2$, $p<0.001$), digging activity (Fig. 6E, $F_{1,16}=4.1$, $p=0.06$), and hyponeophagia (Fig. 6F, $F_{1,14}=9.6$, $p=0.008$). However, there was no significant effect of CNO (ethanol intake: $F_{2,36}=0.8$, $p=0.46$; digging duration: $F_{1,16}=2.4$, $p=0.14$; arena latency: $F_{1,16}=0.001$, $p=0.97$) or CIE \times CNO interaction (ethanol intake: $F_{2,36}=1.7$, $p=0.19$; digging duration: $F_{1,16}=0.8$, $p=0.39$; arena latency: $F_{1,16}=1.0$, $p=0.35$) on either measure.

Given the lack of effect of chemogenetic stimulation or inhibition of CeA CRF neurons, we sought to verify the involvement of CRF1 signaling in the control of alcohol drinking under our experimental conditions. As expected, systemic administration of the CRF1 antagonist CP376395 dose-dependently reduced ethanol intake during 2-h 2BC sessions in both air- and CIE-exposed C57BL/6J mice (Fig. 6G; main effect of dose: $F_{2,30}=20.5$, $p=0.000002$; main effect of CIE: $F_{1,30}=17.3$, $p=0.0008$; dose \times CIE interaction: $F_{2,30}=0.17$, $p=0.85$). *Posthoc* analysis indicated a significant effect of the 15 mg/kg dose ($p=0.00001$), but not 5 mg/kg ($p=0.69$).

In conclusion, chemogenetic inhibition of mouse CeA CRF neurons does not reverse behavioral hallmarks of alcohol dependence in mice, and it does not reduce alcohol drinking or hyponeophagia in moderate drinkers despite pharmacological evidence of a significant role of CRF1 signaling in alcohol intake.

Discussion

Our study first shows that chronic binge-like drinking and repeated alcohol vapor inhalation both elevate *Crh* expression at the most anterior level of the mouse CeA, suggesting that anterior CeA CRF neurons may be functionally involved in the behavioral consequences of chronic intermittent alcohol exposure. However, these neurons are neither necessary nor sufficient to drive ethanol intake escalation in mice. Their chemogenetic stimulation increases GABA release onto medial CeA neurons and exacerbates hyponeophagia but does not affect voluntary alcohol consumption. Furthermore, their chemogenetic inhibition does not alter ethanol intake or hyponeophagia in alcohol-dependent or non-dependent mice. The latter finding is at odds with the effect of optogenetic manipulation in rats³², which highlights species differences in the function of CeA CRF circuits. The reduction in alcohol intake elicited by CP376395 is consistent with the effects of systemic CRF1 blockade on binge-like drinking previously reported in mice and confirms that CRF1 signaling promotes alcohol consumption under our experimental conditions^{12, 18, 41–43}.

While multiple studies had previously demonstrated *Crh* upregulation in the CeA of animals withdrawn from chronic alcohol exposure^{19, 21, 22, 44}, most of them did not consider anteroposterior level in their analysis. In rats withdrawn from CIE for 2 h, CRF immunoreactivity selectively increased in the most posterior area of the CeA¹⁷. In contrast, we found that the impact of chronic binge drinking on *Crh* expression was specific to the most anterior level of the CeA – the area that shows the highest density of CRF-producing neurons in mice while most CRF-expressing neurons are located at middle and posterior levels in rats³⁷. Furthermore, CIE also increased *Crh* expression at this level and the effect persisted after 72 h withdrawal. These observations indicate that the anatomical organization of the CeA CRF system as it relates to alcohol sensitivity differs between rats and mice.

The penetrance of Cre activity in CeA CRF neurons was lower in *Crh*-IRES-Cre mice (64%) than in *Crh*-Cre rats (99%), which may also explain differential experimental outcomes in the two species. However, our quantification by *in situ* hybridization may have been less sensitive than the method used in rats (immunohistochemistry, confocal imaging at 63x and 3D reconstruction)²⁹. Previous characterization of the distribution of Cre activity in the amygdala of *Crh*-IRES-Cre;Ai14 mice by immunohistochemistry concluded that the pattern “largely recapitulated” the distribution of CRF, although no quantification was presented⁴⁵. We found that the fidelity of Cre activity for CeA CRF neurons was reasonably high (86% - which may again be an underestimate), confirming that the cellular and behavioral effects of CNO reported in the present study result, for the most part, from the activation of CeA CRF neurons.

There was no effect of CeA CRF neuron stimulation on the overall activity of other CeA neurons (as indexed by c-Fos expression in mCherry-negative CeA neurons). This observation contrasts with the effect of the same manipulation in *Crh*-Cre rats, where robust c-Fos induction was also detected in non-CRF neurons of the medial and lateral CeA and was mediated by CRF1 activation²⁹. This discrepancy could relate to the differential anatomy of the CRF system, with the dense cluster of CRF neurons at the junction of the IPAC and anterior CeA being unique to mice and possibly exerting a stronger inhibitory

control over the remainder of the CeA than the more posterior CRF neurons. Consistent with this hypothesis, the connectivity of CeA CRF neurons is species-specific, as local synapses are predominant in mice⁴⁶, while long-range projections to the BNST, lateral hypothalamus and parabrachial nucleus are robust in rats^{30, 32}. Alternatively, the discrepancy may stem from a differential excitatory/inhibitory signaling balance between the two species, whereby a higher ratio of GABA to CRF may be released in mice compared to rats, inhibitory neuromodulators may be co-released to a larger extent in mice, or inhibitory signaling may override excitatory signaling via mouse-specific postsynaptic mechanisms. Of note, the GABA release induced by CNO likely reduced the activity of neurons directly contacted by hM3Dq-expressing neurons but c-Fos immunolabeling is not well suited to detect such inhibition owing to low baseline expression of this immediate early gene.

Chemogenetic stimulation of CeA CRF neurons triggered GABA release onto a subset of medial CeA neurons, as reflected by increased sIPSC frequency in response to CNO. This result is consistent with the ability of optogenetic stimulation of CeA CRF terminals to evoke IPSCs in a subset of CeA neurons in *Crh*-Cre rats and mice^{29, 46}. The CNO insensitivity of some medial CeA mCherry-negative ties in with the notion that all medial CeA cells may not be contacted by CRF (i.e., hM3Dq-expressing) neurons. Combined with the lack of activation of mCherry-negative CeA cell bodies we observed with c-Fos labeling, our data indicate that mouse CeA CRF neurons release GABA from their own terminals (given that CeA CRF neurons are GABAergic⁴⁷) or possibly stimulate GABA release from extrinsic afferents, but not from local non-CRF interneurons as may be the case in rats (see discussion above). The ability of R121919 to reverse this increase in GABA release in cells with high baseline release is consistent with previous work showing that exogenous CRF acts at presynaptic CRF1 receptors to increase GABA release in the mouse CeA³⁹. We chose to focus on inhibitory transmission as it provides a consistent readout of CRF1 signaling in both the rat and mouse CeA, while CRF effects on excitatory transmission are more complex, at least in rats⁴⁸⁻⁵⁰. Relevant to the present study, although ethanol can increase glutamate release in the mouse and rat CeA and this action requires activation of CRF receptors in both species, CeA CRF neurons do not mediate this effect in the mouse^{50, 51}.

The effect of chemogenetic stimulation of CeA CRF neurons on anxiety-like behavior appears to be species- and assay-dependent. While this manipulation did not affect mouse EPM behavior in the present study, it reduces open arm exploration in rats^{30, 31}. Previous studies in *Crh*-IRES-Cre mice also showed that chemogenetic activation of CeA CRF neurons increases immobility in the EPM (although this may not necessarily translate into reduced time spent on open arms) and optogenetic activation of the CeA CRF projection to the locus coeruleus reduced exploration of open sections of an elevated zero-maze^{52, 53}. We therefore anticipated that activating CeA CRF neurons would reduce open arm exploration in the EPM, but it had no effect. It is possible that other projections of CeA CRF neurons oppose the anxiogenic-like effect of the coerulear projection, resulting in a lack of EPM phenotype in the present study.

It is important to note, however, that mice and rats can manifest negative affect in different ways, even when undergoing a similar aversive experience. For instance, while it has been repeatedly shown that rats withdrawn from chronic alcohol exposure exhibit anxiety-like

behavior in the EPM^{54–61}, a similar phenotype has been difficult to detect in mice using the same assay⁶². However, negative affect can be captured in alcohol-withdrawn mice using alternative assays. Most relevant to the present study, C57BL/6J males display increased digging activity and exacerbated hyponeophagia, but unaltered light-dark box exploration and social interaction, when tested 3–10 days into withdrawal from CIE^{8, 63–67}. The latter data are consistent with our observation that chemogenetic stimulation of CeA CRF neurons in mice exacerbates hyponeophagia and tends to increase digging activity but has no effect in the EPM and social approach tests. However, chemogenetic inhibition of CeA CRF neurons failed to reverse the exacerbated digging and hyponeophagia of CIE-withdrawn mice, discounting a functional implication of these neurons in withdrawal-induced negative affect. Altogether, our results indicate that mouse CeA CRF neurons have the capacity to drive hyponeophagia but do not contribute to the hyponeophagic phenotype associated with CIE withdrawal. Based on the molecular dissection of the anxiety-like behavior driven by CeA CRF neurons in rats, the NSF phenotype we observed in alcohol-naïve mice may result from the combined release of CRF and dynorphin³¹. Furthermore, although our electrophysiological results demonstrated local GABA and CRF release upon activation of CeA CRF neurons, it is possible that the effect of CNO in the NSF assay is driven by CeA CRF neurons projecting outside of the CeA. There is, to the best of our knowledge, no experimental approach currently available to target CRF neurons intrinsic to the CeA while sparing CRF neurons projecting to other regions.

We had hypothesized that CeA CRF neurons would not only drive negative affect but also increase alcohol drinking by mimicking negative reinforcement-driven escalation of alcohol self-administration elicited by CIE⁶⁸. However, neither acute nor chronic chemogenetic stimulation of CeA CRF neurons altered alcohol intake in mice given limited access to alcohol. Furthermore, chemogenetic inhibition failed to reduce ethanol intake in alcohol-dependent or non-dependent mice. This observation contrasts with the ability of optogenetic inhibition of the same neuronal population to selectively reduce escalated alcohol self-administration in CIE-exposed rats³². This discrepancy demonstrates a differential involvement of CeA CRF neurons in the control of ethanol intake in mice vs. rats. Our results, combined with the prior demonstration that intra-CeA CRF1 antagonism reduces alcohol consumption in binge drinking and CIE-exposed mice^{16, 18}, indicate that an extrinsic source of CRF to the CeA promotes alcohol drinking in mice. Future work will aim to elucidate the identity of behaviorally relevant CRF-containing projections to the CeA and their recruitment by CIE exposure.

A recent study demonstrated that a subset of CeA CRF neurons is activated prior to alcohol licks in binge-drinking mice³³. The inability of chemogenetic inhibition to reduce the alcohol intake of Air or CIE mice indicates that these neurons may encode the anticipation of drinking but are not required for moderate or excessive consumption. Alternatively, there may be another subset of CeA CRF neurons exerting an opposing influence on alcohol consumption, such that concomitant inhibition of both subsets in our study may have neutralized the role of the pre-lick activated subset.

Aside from fear learning and anxiety, CeA neurons have also been implicated in feeding control. Specifically, lateral CeA PKC δ -positive neurons suppress food intake⁶⁹. In the NSF

test, we found that chemogenetic stimulation of CeA CRF neurons increased the latency of food-deprived mice to start eating in an anxiogenic arena but did not affect their home cage feeding. This observation is consistent with the minimal overlap between mouse CeA populations expressing PKC δ versus CRF^{46, 70}. Interestingly, CRF suppresses feeding when administered in the brain and CRF signaling mediates the anorexigenic effect of several stressors (see⁷¹ for review). While CeA CRF neurons can be recruited by stress (^{72, 73}, but see⁷⁴), our findings indicate that their activity is not sufficient to inhibit feeding.

A general limitation of our study is that mice from each cohort were subjected to multiple behavioral tests and CNO injections. We cannot rule out that potential effects of CNO may have been occluded by prior testing, despite the 1-week interval between tests. However, this concern is mitigated by the robust effect of CNO on hyponeophagia in hM3Dq-expressing mice, which was observed after multiple injections. It is also possible that significant effects of CNO would have been detected in hM4Di-expressing CIE-withdrawn mice if we had used a higher dose of CNO.

Altogether, our results indicate that excitation of CeA CRF neurons is sufficient to produce negative affect but not to increase the motivation to consume alcohol in mice. Furthermore, although CeA neurons are known to be hyperactive during CIE withdrawal in both rats and mice^{32, 75–77}, we found that the CRF-expressing subset does not contribute to the behavioral phenotypes of CIE withdrawal in mice. Their lack of involvement in CIE-induced ethanol intake escalation contrasts with their role in rats and indicates that observations made in one species may not translate to another species. This consideration is relevant to the targeting of CRF signaling for the treatment of alcohol use disorders in humans, as the differential engagement of central amygdala CRF neurons in the control of alcohol consumption in two phylogenetically close species (rats and mice) likely extends to a more distantly related species (humans) and provides a possible explanation for the disappointing results of clinical trials that were motivated in large part by the promises of preclinical studies conducted in rats^{78, 79}. Relevant to the latter point, available evidence points to substantial discrepancies in the anatomical localization and molecular identity of amygdala CRF neurons between humans and rodents. Notably, in the human amygdala, *CRH* transcripts are enriched in a subpopulation of inhibitory neurons that expresses high levels of *CALB2* (calretinin), *CCK*, and *VIP* transcripts⁸⁰. Both CRF- and calretinin-immunoreactive cells are scarce in the human central nucleus^{81, 82}. Moreover, *CCK/Cck* and *VIP/Vip* do not co-localize with CRF/*Crh* in the rodent amygdala^{37, 83, 84}.

Altogether, our findings, combined with the ability of intra-CeA CRF1 antagonism to reduce ethanol intake in C57BL/6J mice, suggest that CRF acting in the mouse CeA to promote alcohol drinking arises from an extrinsic source^{16, 18}. Future work will be needed to identify other mechanisms contributing to alcohol drinking escalation in CIE-exposed mice, including additional neural circuits releasing CRF in the CeA, additional projection targets of CeA CRF neurons, as well as non-CRF signaling pathways that may potentiate the influence of CRF transmission on alcohol drinking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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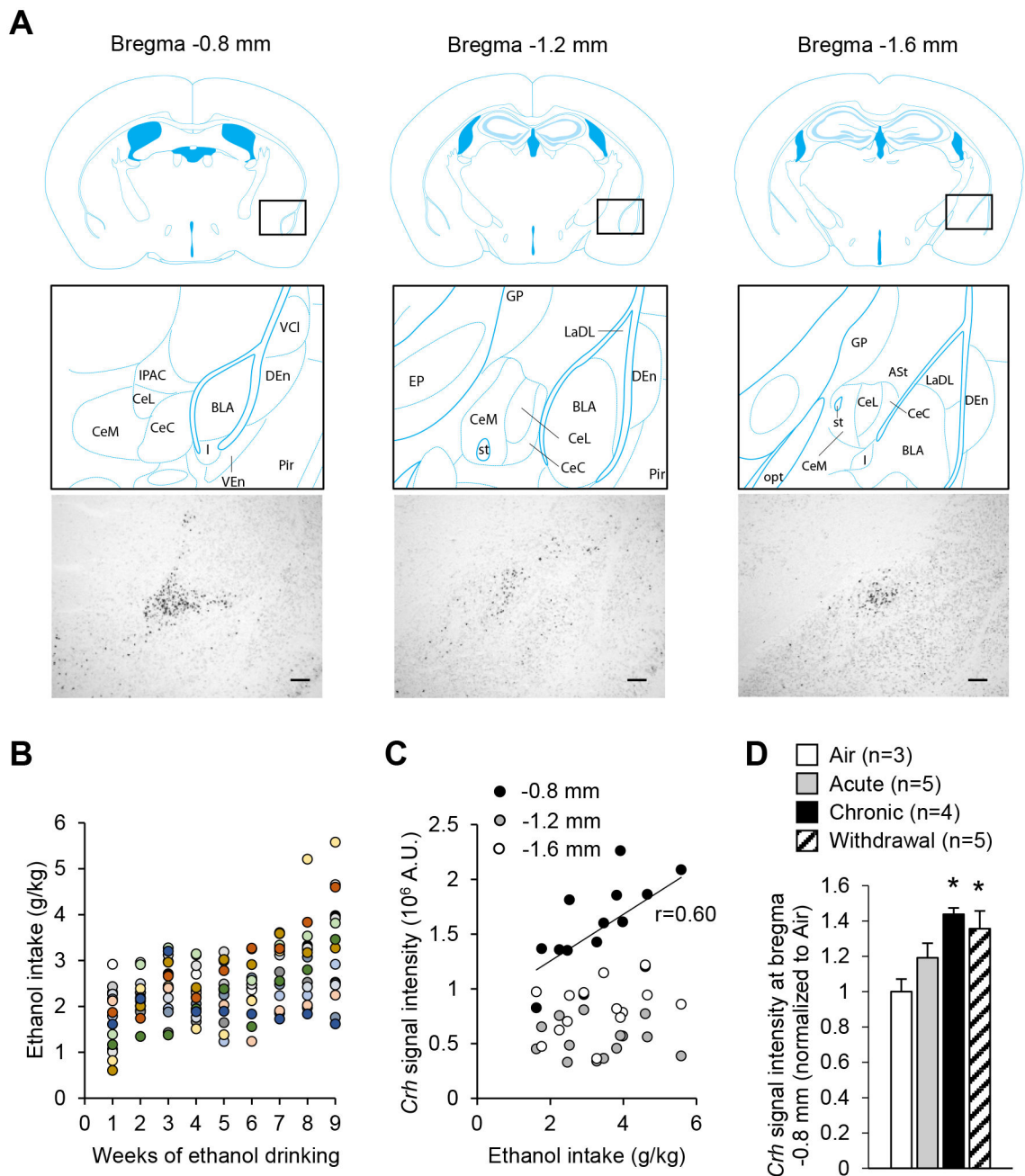


Figure 1. *Crh* mRNA levels in the anterior CeA increase upon chronic intermittent alcohol exposure.

A. Representative images of *Crh* mRNA distribution at three antero-posterior levels of the mouse CeA (scale bars = 200 μ m) and corresponding brain atlas diagrams highlighting a V-shaped cluster of CRF neurons at the junction of the anterior CeL and IPAC (left panels), and scattered CRF neurons at more posterior levels of the CeL (middle and right panels). Brain atlas diagrams were reproduced from⁸⁵. BLA, basolateral amygdaloid nucleus; CeC, capsular part of the CeA; CeL, lateral division of the CeA; CeM, medial division of the CeA; DEn, dorsal endopiriform claustrum; EP, entopeduncular nucleus; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; GP, globus pallidus; I, intercalated

nuclei of the amygdala; LaDL, lateral amygdaloid nucleus, dorsolateral part; opt, optic tract; Pir, piriform cortex; st, stria terminalis; VCI, ventral part of claustrum; VEn, ventral endopiriform claustrum. **B.** Average weekly ethanol intake in a cohort of mice subjected to 2-h two-bottle choice (ethanol 15% v:v vs water) sessions five days per week for nine weeks. Each color represents an individual mouse. **C.** *Crh* chromogenic *in situ* hybridization signal density at three antero-posterior levels of the CeA as a function of ethanol intake during the last week. There was a significant correlation at the most anterior level (\approx bregma -0.8 mm, $p < 0.05$), but not at more posterior levels. **D.** *Crh* chromogenic *in situ* hybridization signal density at the most anterior level of the CeA in mice exposed to a single 16-h bout of alcohol vapor inhalation (Acute), eight bouts (Chronic), or eight bouts followed by three days of withdrawal (Withdrawal). Data are shown as mean \pm s.e.m. of *Crh* signal normalized to Air controls. Data were analyzed by one-way ANOVA; *, $p < 0.05$ vs. Air, Dunnett's *posthoc* test.

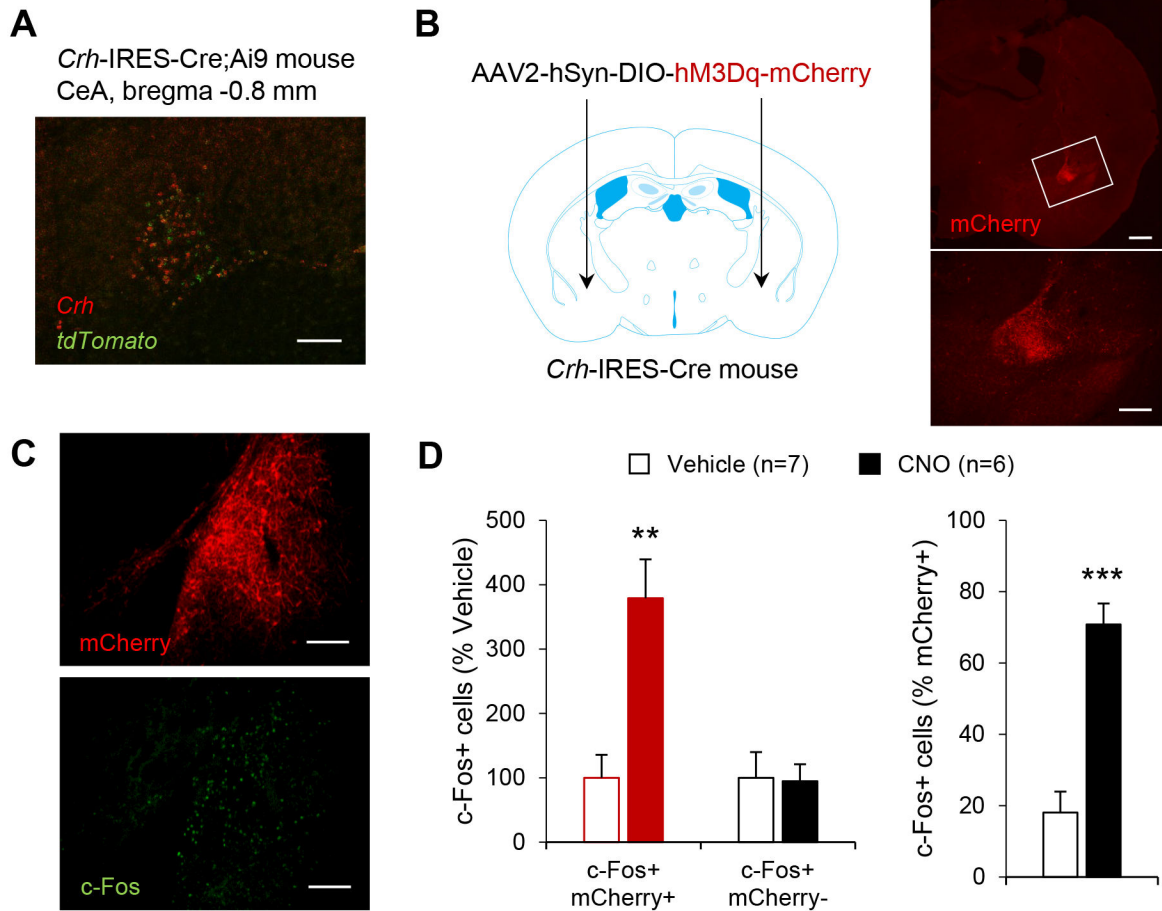


Figure 2. Validation of Cre activity and chemogenetic stimulation in CeA CRF neurons of *Crh*-IRES-Cre mice.

A. Distribution of *Crh* (red) and *tdTomato* (green) mRNAs in the CeA of *Crh*-IRES-Cre;Ai9 mice, as visualized by double fluorescent *in situ* hybridization (scale bar = 100 μ m). **B.** mCherry immunolabeling recapitulates the V-shaped pattern of *Crh* expression in the CeA of *Crh*-IRES-Cre mice injected with a Cre-dependent AAV vector encoding hM3Dq-mCherry. The area framed in the top picture (scale bar = 500 μ m) is shown at higher magnification in the bottom picture (scale bar = 100 μ m). **C.** Double immunostaining of mCherry (red) and c-Fos (green) was used to evaluate neuronal activation in the CeA following i.p. injection of vehicle (n=7) or CNO 5 mg/kg (n=6) in *Crh*-IRES-Cre mice injected with AAV2-hSyn-DIO-hM3Dq-mCherry in the CeA (scale bars = 100 μ m). **D.** Chemogenetic stimulation of CeA CRF neurons increased c-Fos expression selectively in mCherry-positive CeA neurons. Data are shown as mean \pm s.e.m. of the number of c-Fos+ cells expressed as percentage of Vehicle values (left graph) or percentage of mCherry+ cells (right graph). Data were analyzed by unpaired t-test: **, p<0.01; ***, p<0.001.

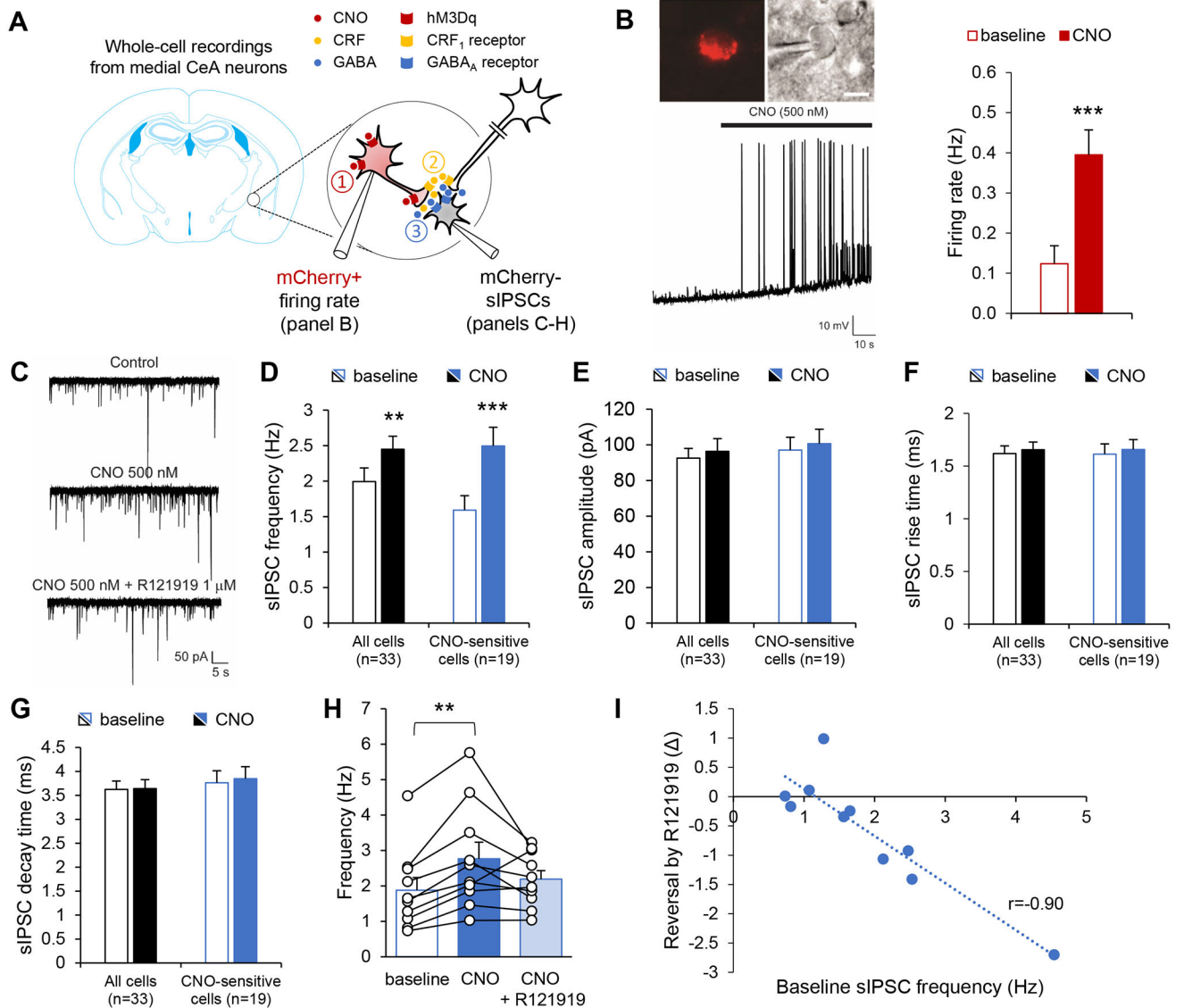


Figure 3. Chemogenetic stimulation of CeA CRF neurons increases GABA release onto medial CeA neurons in a CRF1-dependent manner.

A. *Crh*-IRES-Cre mice were injected in the anterior CeA with a Cre-dependent AAV vector encoding hM3Dq-mCherry. Whole-cell recordings were then obtained from mCherry+ neurons (panel B) or mCherry- neurons (panels C-I) to test the hypothesis that chemogenetic stimulation of CeA CRF neurons (*red*, ①) triggers the release of GABA (*blue*) and CRF (*yellow*) in the medial CeA (②), which may in turn stimulate GABA release (③) onto non-CRF neurons (*grey*) via the activation of CRF1 receptors located on intrinsic or extrinsic GABAergic presynaptic terminals³⁹. **B.** Firing rates were recorded from CeA mCherry+ neurons. Top: red fluorescence and differential interference contrast images of patched CeA neuron. Bottom: representative current-clamp trace before and during CNO application (500 nM). Right: Firing rates are shown as mean ± s.e.m. (n=8 neurons). Data were analyzed by paired t-test: ***, p<0.001. **C-I.** Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded from medial CeA mCherry- neurons. **C.** Representative

traces before and during CNO application (500 nM) followed by subsequent R121919 co-application (1 μ M). CNO increased sIPSC frequency (**D**), but did not affect sIPSC amplitude (**E**), rise time (**F**) or decay time (**G**). Data are shown as mean \pm s.e.m. for the whole set of recorded neurons (n=33, black bars), as well as for the subset of neurons whose sIPSC frequency was significantly increased by CNO (n=19, blue bars). Data were analyzed by paired t-test: **, p<0.01; ***, p<0.001. **H-I**. In a subset of CNO-sensitive cells (n=10), the CRF1 antagonist R121919 was co-applied following CNO alone. **H**. sIPSC frequencies are shown as mean \pm s.e.m. and were analyzed by one-way ANOVA; **, p<0.01, Tukey *posthoc* test. **I**. The effect of R121919 (expressed as sIPSC frequency difference before and after addition of R121919) was correlated with baseline sIPSC frequency; p<0.001.

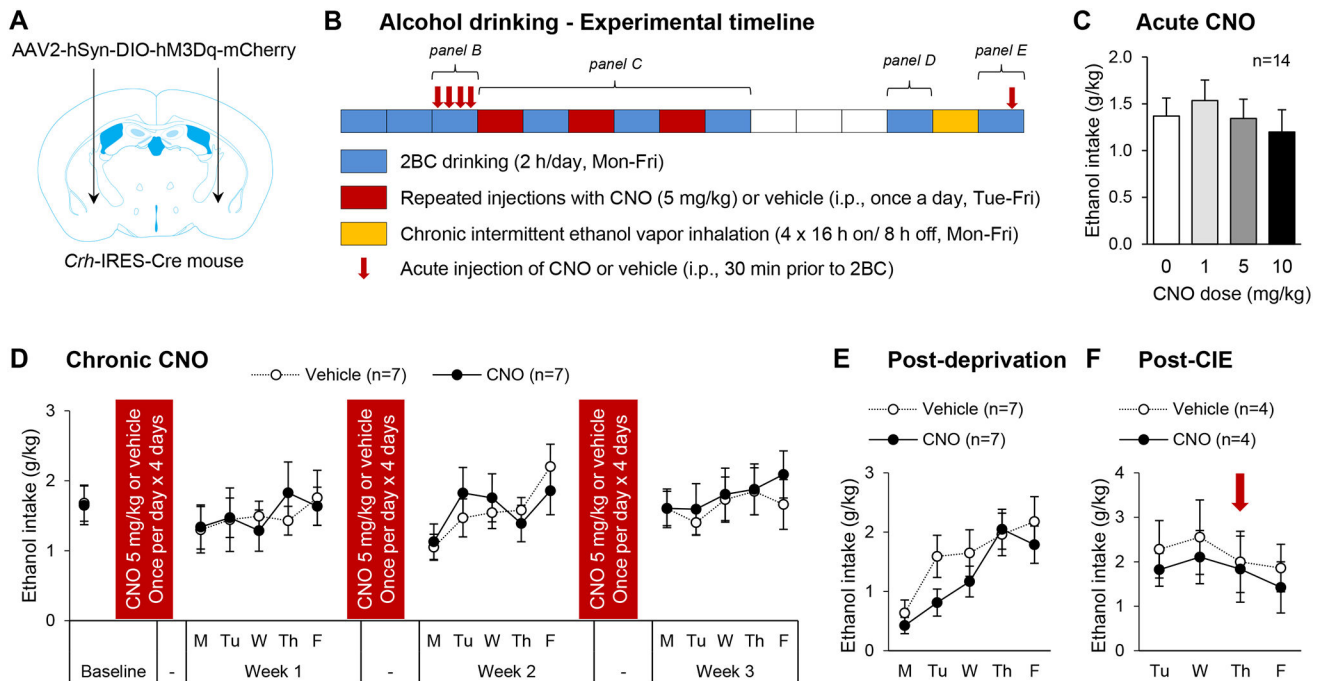


Figure 4. Chemogenetic stimulation of CeA CRF neurons does not affect alcohol drinking.

A. *Crh-IRES-Cre* male mice were injected with a Cre-dependent hM3Dq-encoding vector in the anterior CeA and were tested for voluntary ethanol intake under different experimental conditions. **B.** Experimental timeline. Each box represents one week, the color code indicates the experimental procedure conducted during that week. Two-bottle choice (2BC) drinking sessions were conducted Mon-Fri, starting at the beginning of the dark phase and lasting 2 h (blue boxes). Mice were given ten baselining sessions prior to testing the acute effect of CNO (0, 1, 5 and 10 mg/kg, i.p., 30-min pretreatment) according to a within-subject Latin-square design over four consecutive days (red arrows; data shown in panel C). An additional 2BC session without pretreatment was conducted and mice were then split in two groups exhibiting equivalent baseline ethanol intake, which were repeatedly injected with either CNO (5 mg/kg) or vehicle. Weeks of CNO or vehicle administration (once per day, Tue-Fri; red boxes) were alternated with weeks of 2BC drinking sessions (Mon-Fri, as described above; blue boxes; data shown in panel D) for a total of 3 rounds. Mice were then given a 3-week ethanol deprivation period (white boxes), after which 2BC sessions were resumed for a week (blue box; data shown in panel E). Next, the mice were exposed to four cycles of chronic intermittent ethanol exposure (16-h ethanol vapor inhalation followed by 8-h air inhalation, Mon-Fri; yellow box). The mice were then returned to their home cages and 2BC sessions resumed four days later (Tue-Fri; blue box; data shown in panel F). On the third session (Thu), CNO (5 mg/kg) or vehicle was administered 30 min prior to the session (red arrow). **C-F.** Ethanol intake is expressed in g ethanol per kg body weight in 2-h session. Data are shown as mean \pm s.e.m. Number of mice per group is shown in the legend of each graph.

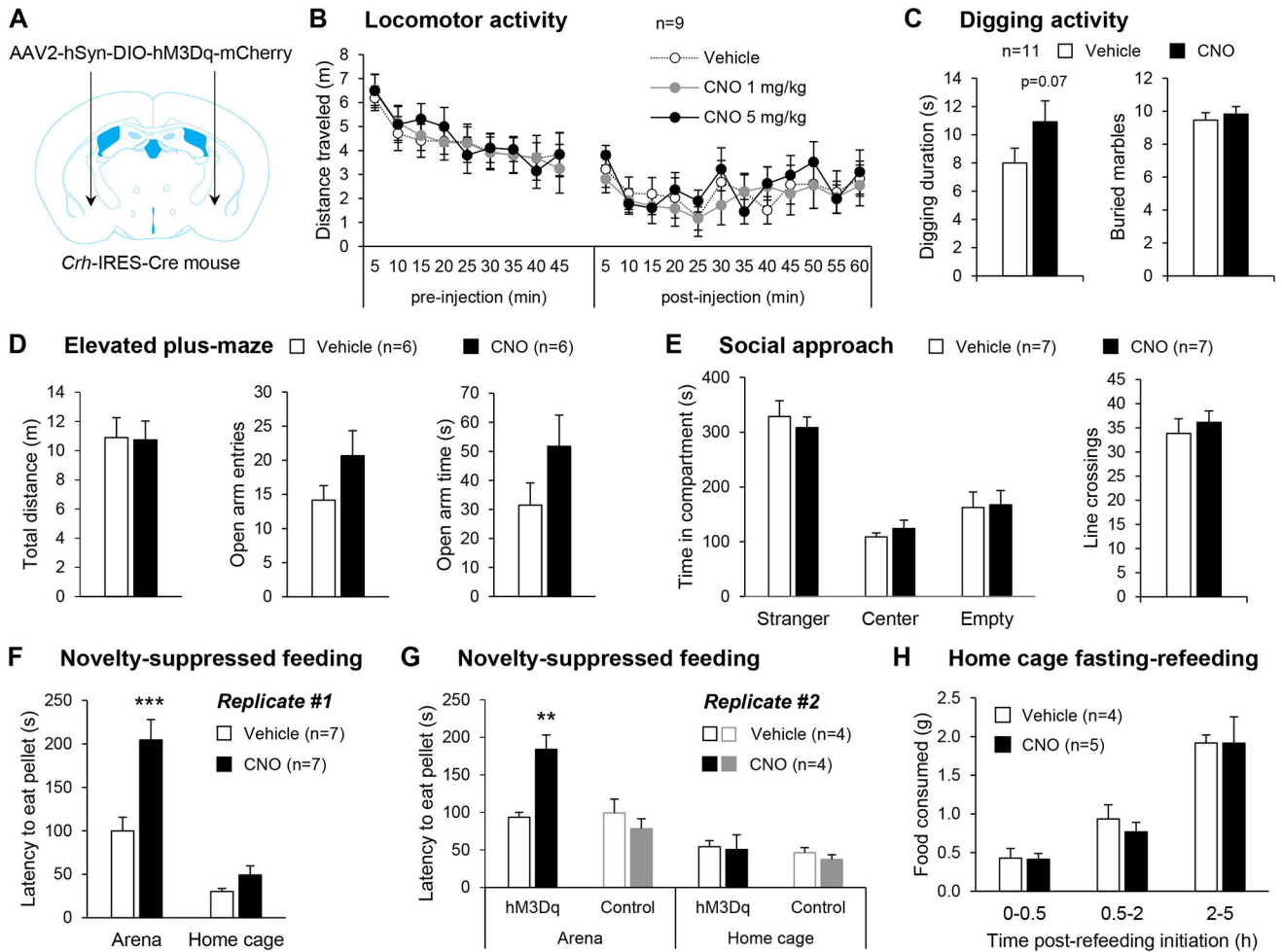


Figure 5. Chemogenetic stimulation of CeA CRF neurons exacerbates hyponeophagia without altering other affective responses nor appetite.

A. *Crh*-IRES-Cre male mice were injected with a Cre-dependent hM3Dq-encoding vector in the anterior CeA. **B.** Locomotor activity 45 min prior and 60 min following i.p. injection of vehicle or CNO. **C-H.** Mice were tested in assays probing affective behavior 30 min after i.p. injection of saline or CNO (5 mg/kg). **C.** CNO tended to increase digging duration but did not affect marble burying. **D.** CNO did not affect open arm exploration in the elevated plus maze. **E.** CNO did not affect the preference for social interaction. **F-G.** CNO increased the latency to start feeding in an anxiogenic arena, but not in the home cage. This effect of CNO was replicated in an independent cohort and was not observed in control mice with no hM3Dq expression. **, p<0.01; ***, p<0.001, Tukey *posthoc* test. **H.** CNO did not alter the amount of food consumed following 24-h food deprivation. Data are shown as mean ± s.e.m. Number of mice per group is shown in the legend of each graph.

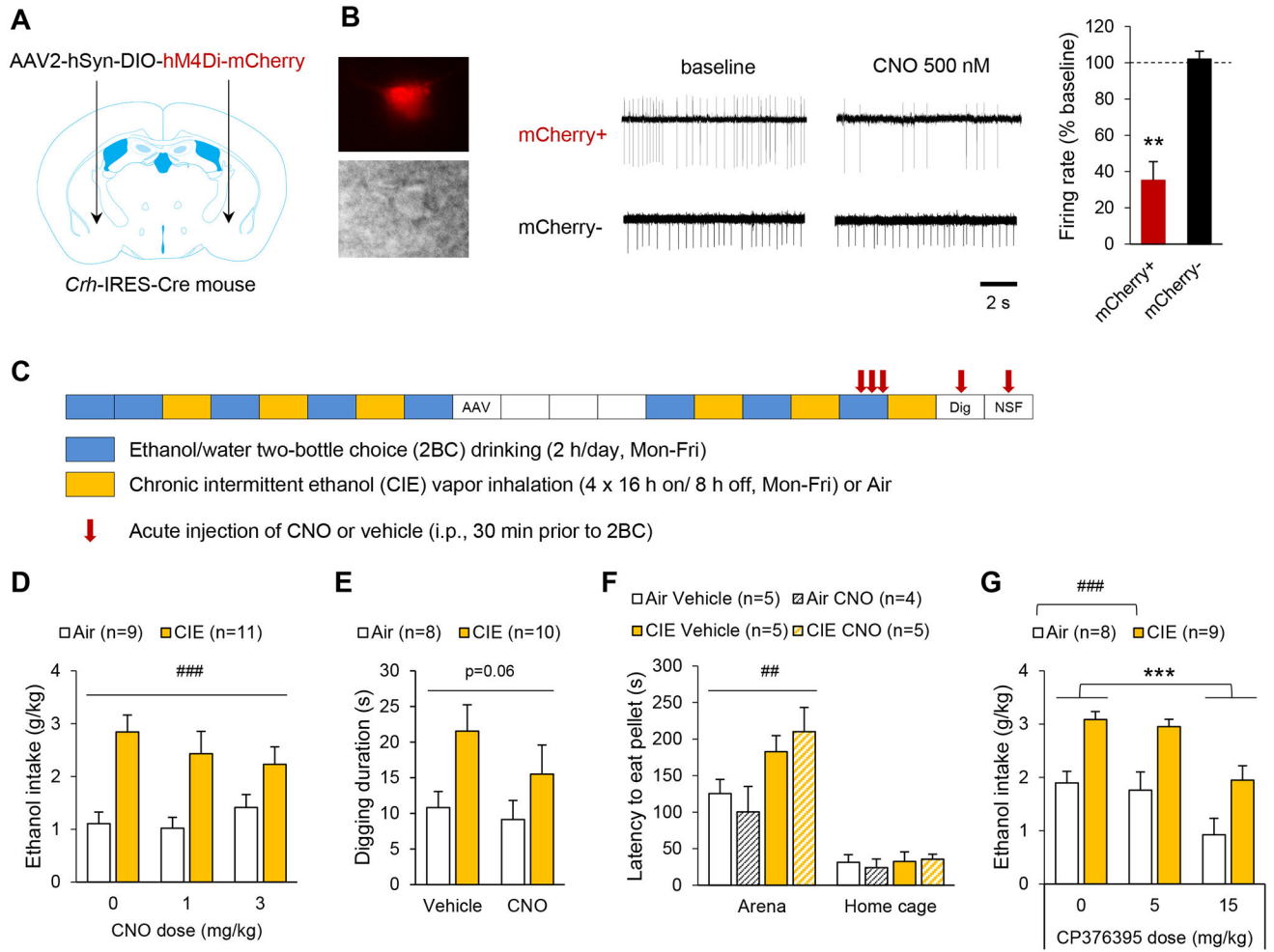


Figure 6. Chemogenetic inhibition of CeA CRF neurons does not reverse ethanol intake escalation or affective disturbance in alcohol-dependent mice.

A-F. *Crh*-IRES-Cre male mice were injected with a Cre-dependent hM4Di-encoding vector in the anterior CeA. **B.** Firing rates were recorded from CeA mCherry+ and mCherry- neurons. Left: red fluorescence and differential interference contrast images of patched CeA neuron. Middle: representative traces before and during CNO application (500 nM). Right: CNO-induced change in firing rate (mCherry+, n=5; mCherry-, n=4). **, p<0.01, one-sample t-test. **C.** Experimental timeline for behavioral testing. Each box represents one week. AAV designates the timepoint at which the viral vector was injected, mice recovered for 3 weeks before resuming alcohol drinking sessions. The digging test (Dig) was performed 7 and 10 days after last vapor exposure (within-subject design). The novelty-suppressed feeding (NSF) test was conducted 14 days after last vapor exposure (between-subject design). **D-F.** Exposure to CIE increased ethanol intake (**D**), digging activity (**E**), and hyponeophagia (**F**). Acute CNO administration had no significant effect on these measures. Data were analyzed by two-way ANOVA (main effect of CIE: ##, p<0.01; ###, p<0.001). **G.** C57BL/6J mice subjected to the CIE-2BC procedure were injected with the CRF1 antagonist CP376395 30 min prior to 2BC. CP376395 reduced ethanol intake in both Air and CIE mice. Data were analyzed by two-way repeated-measures ANOVA (###, p<0.001,

main effect of CIE; ***, $p < 0.001$, Dunnett's *posthoc* test). Data are shown as mean \pm s.e.m. Number of mice per group is shown in the legend of each graph.

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