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Dynorphin/Kappa Opioid Receptor Activity Within the Extended Amygdala Contributes to Stress-Enhanced Alcohol Drinking in Mice

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Abstract

Background: While there is high comorbidity of stress-related disorders and alcohol use disorder (AUD), few effective treatments are available and elucidating underlying neurobiological mechanisms has been hampered by a general lack of reliable animal models. Here we utilize a novel mouse model demonstrating robust and reproducible stress-enhanced alcohol drinking to examine the role of dynorphin/kappa opioid receptor (DYN/KOR) activity within the extended amygdala in mediating this stress-alcohol interaction.

Methods: Mice received repeated weekly cycles of chronic intermittent ethanol (CIE) exposure alternating with weekly drinking sessions ± forced swim stress (FSS) exposure. *Pdyn* mRNA expression was measured in the central amygdala (CeA) and DYN-containing CeA neurons (CeA^{DYN}) were then targeted for chemogenetic inhibition. Lastly, a KOR antagonist was microinjected into the CeA or bed nucleus stria terminalis (BNST) to examine the role of KOR signaling in promoting stress-enhanced drinking.

Results: Stress (FSS) selectively increased alcohol drinking in mice with a history of CIE exposure, and this was accompanied by elevated *Pdyn* mRNA levels in CeA. Targeted chemogenetic silencing of CeA^{DYN} neurons blocked stress-enhanced drinking and KOR antagonism in the CeA or BNST significantly reduced stress-induced elevated alcohol consumption without altering moderate intake in controls.

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DISCLOSURES

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Conclusions: Utilizing a novel and robust model of stress-enhanced alcohol drinking, a significant role for DYN/KOR activity within extended amygdala circuitry in mediating this effect was demonstrated, thereby providing further evidence that the DYN/KOR system may be a valuable target in the development of more effective treatments for individuals presenting with comorbidity of stress-related disorders and AUD.

Keywords

Stress; Alcohol; Dynorphin; Kappa opioid receptor; Amygdala; Bed nucleus stria terminalis

INTRODUCTION

Stress is commonly regarded as a potent trigger for relapse and a significant factor in promoting excessive alcohol (ethanol) drinking (1–3). There is a high comorbidity between stress-related disorders (including depression, anxiety disorders, and post-traumatic stress disorder; PTSD) and alcohol use disorder (AUD) (4–8). The prevalence and magnitude of the problem underscores the importance of understanding mechanisms underlying the influence of stress on alcohol use, which is essential for developing new and more effective treatment strategies for individuals suffering with co-existing stress and alcohol use disorders.

Stress and alcohol exposure influence overlapping systems and neural circuits in the brain, producing adaptations that compromise the ability of individuals to engage in behavioral flexibility that would enhance control over alcohol consumption, as well as appropriately respond to stressful events that may provoke return to excessive drinking (9,10). Despite significant advances in our understanding about how stress and alcohol alter brain function, the mechanisms and neurocircuits underlying the complex interactions between stress and alcohol consumption are not fully understood (11). Indeed, demonstrating reliable and consistent effects related to the interaction between stress and alcohol drinking in animal models has been challenging (12–14). The general lack of reliable preclinical models has, at least in part, impeded progress toward developing effective therapeutics that especially target stress-related excessive drinking.

To address this shortcoming, we developed a mouse model wherein repeated brief forced swim stress (FSS) exposure interacts with chronic intermittent ethanol (CIE) exposure to enhance alcohol drinking in dependent (CIE-exposed) mice but not alter more moderate stable intake in nondependent mice (15,16). Corroborating an earlier study in rats (17), this stress-enhanced drinking in CIE-exposed mice is robust and reproducible, having been demonstrated by other research groups as well (18–20). Increased drinking in mice with a history of both chronic alcohol exposure and stress experience provides an ideal opportunity to utilize this CIE-FSS Drinking model to probe mechanisms and potential targets relevant to the problem of stress-related excessive alcohol drinking.

Because both stress and chronic alcohol engage the dynorphin/kappa opioid receptor system, the role of this neuropeptide system in chronic alcohol-related dysphoria and elevated drinking has gained increasing attention (21–23). Dynorphins (DYN) are peptides derived from the precursor prodynorphin (Pdyn) that preferentially bind to kappa opioid

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receptors (KOR), producing physiological and behavioral effects via inhibitory G-protein (Gi) coupling and other signaling cascades (24–27). KOR activation has been shown to produce aversive/dysphoric effects as indicated by measures of conditioned avoidance, anxiety-like, and depression-like behavior (28,29). Stress exposure activates the DYN/KOR system, eliciting dysphoria- and anxiety-like behaviors (30) along with elevated DYN immunoreactivity in brain regions that are integral to reward and stress circuitries involved in alcohol/drug addiction (31).

Pharmacological manipulation of DYN/KOR activity has been shown to alter behavioral responses to stress and motivational effects of alcohol in a variety of experimental conditions (22,32). Systemic administration of KOR antagonists has been shown to reduce high levels of alcohol consumption associated with dependence and binge-drinking models in rats (33,34) and mice (20,35,36). Evidence points to involvement of the extended amygdala in mediating these effects. Interconnected brain structures comprising extended amygdala circuitry including the nucleus accumbens, central amygdala (CeA), and the bed nucleus of the stria terminalis (BNST) are rich in DYN and KOR and highly responsive to stress and chronic alcohol exposure (37–41). Direct infusion of the KOR antagonist norbinaltorphimine (norBNI) into these structures reduces excessive alcohol drinking in models of dependence (42–44) and binge-like drinking (35,45). While we recently demonstrated that systemic administration of a KOR antagonist can block the ability of stress to enhance alcohol consumption (36), the site of action mediating this effect is not known.

The present series of studies were designed to probe the role of DYN/KOR activity in two prominent structures within extended amygdala circuitry (CeA and BNST) as it relates to stress-enhanced alcohol drinking. Specifically, our CIE-FSS Drinking model was employed to: 1) examine how a history of CIE and FSS exposure, alone and in combination, affect expression of *Pdyn* mRNA within the CeA; 2) determine the effect of targeted chemogenetic silencing of DYN-expressing neurons within the CeA on stress-enhanced alcohol drinking; and 3) determine the contribution of KOR signaling within the CeA and BNST in mediating the ability of stress to further enhance elevated alcohol consumption associated with dependence. Results from these studies provide critical evidence indicating that a history of chronic alcohol exposure and stress engage the DYN/KOR system within the extended amygdala in mediating stress-enhanced drinking. As such, these findings support recent clinical efforts devoted to evaluating the therapeutic value of targeting this neuropeptide system in reducing heavy drinking in individuals comorbid with stress-related disorders and AUD (46).

MATERIALS and METHODS

A detailed description of all experimental procedures, including the CIE-FSS Drinking model, assays for Pdyn mRNA measurement, stereotaxic surgery, virus and drug infusions, histology, and drug preparations are provided in Supplemental Materials.

Chronic Intermittent Ethanol (CIE) and Forced Swim Stress (FSS) Drinking Model

All studies involved use of adult male C57BL/6J mice and *Pdyn-IRES-Cre* mice (35,47) treated in the CIE-FSS Drinking model, as previously described (15,16,36). Briefly,

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after establishing stable 1-hr daily ethanol (15%; v/v) intake, mice were separated into four groups: control (CTL), CIE-alone, FSS-alone, and CIE+FSS. Mice received chronic intermittent ethanol (CIE) vapor or air exposure in inhalation chambers followed by test drinking sessions for 5 consecutive days. This pattern of weekly CIE (or air) exposure alternating with weekly test drinking sessions was repeated for 3 or 4 cycles (Figure 1). Mice in the FSS-alone and CIE+FSS groups experienced brief (10-min) FSS exposure 4-hr prior to each of the test drinking sessions. The remaining non-stressed mice (CTL and CIE groups) were left in their home cage undisturbed.

Study Procedures

Effects of CIE and FSS exposure on Pdyn mRNA expression in the CeA: C57BL/6J mice treated in the CIE-FSS Drinking model were sacrificed on the final day of Test 4 at 30-min, 4-hr, or 24-hr after FSS exposure (or at equivalent times for no-stress groups) (N= 6-10/group/time point). Collection of CeA samples, RNA extraction, and TaqMan quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were performed as previously described (48,49).

Effect of chemogenetic inhibition of Pdyn-expressing neurons in CeA on alcohol drinking in the CIE-FSS model: Adult male *Pdyn-IRES-Cre* mice received bilateral infusions of a Cre-dependent virus containing an inhibitory DREADD (AAV8-hSyn-DIO-hM4Di-mCherry) (N= 10-11/group) or control virus (AAV8-hSyn-DIO-mCherry) (N= 6-7/group) into the CeA to target Pdyn-expressing CeA neurons (CeA^{DYN}), as previously described (50). After at least 2 weeks of recovery, the Baseline phase of the study commenced. On Day-2 and Day-4 of Test 3, mice were injected (ip.) with vehicle (0.9% saline) or clozapine-*N*-oxide (CNO; 3 mg/kg) to activate the DREADD. The order of drug administration was counter-balanced for each group.

Effect of KOR antagonism in CeA or BNST on alcohol drinking in the CIE-FSS model: C57BL/6J mice received bilateral guide cannula positioned above the CeA (N= 7-9/group) or BNST (N= 8-10/group) (45,50). After at least 2 weeks recovery, Baseline drinking commenced. At 16-hr prior to the start of the drinking session on Day-3 of Test 3, separate groups of mice in each experimental condition received microinjection of the KOR antagonist norbinaltorphimine (norBNI; 2.5 μg/side) or vehicle (1X PBS) into the CeA or BNST.

Statistical Analysis

Alcohol intake (g/kg) was analyzed by ANOVA, with Group (CTL, CIE, FSS, CIE+FSS) as a between-subjects factor and Test Cycle (average weekly intake for Baseline and Tests 1-4) as a repeated measure. *Pdyn* mRNA expression in the CeA was expressed as a fold-change from the CTL condition and analyzed at each timepoint by ANOVA, with Group as the main factor. For the chemogenetic study, alcohol intake and change in intake relative to vehicle was analyzed by ANOVA, with Group and Virus as a between-subject variables and Drug (CNO vs. saline) as a repeated measure. For microinjection studies, ANOVAs included Group and Drug (norBNI vs. vehicle) as between-subject variables and Day as a repeated measure in analyses of alcohol consumption (average of Days 3 and 4) and

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difference in intake from respective vehicle group. When appropriate, significant main effects and interactions were further analyzed using Newman—Keuls post-hoc comparisons. In chemogenetic and pharmacological studies, only data from subjects in which targeted viral expression or placement of microinjectors were verified as correct were used in analyses.

RESULTS

Combined Chronic Intermittent Ethanol (CIE) and Stress (FSS) Exposure Increases Pdyn mRNA Expression in the CeA.

While alcohol consumption remained relatively stable over successive Test cycles in the in the FSS-alone and CTL groups, intake increased above baseline levels in CIE-exposed groups, with this effect most robust in the CIE+FSS group (Figures 2A, 2B). This was supported by a significant Group x Test Cycle interaction [$F(12,352)= 5.942$, $p< 0.001$], which revealed a modest increase in alcohol consumption in the CIE-alone group, with intake during Test 2 and Test 4 greater than Baseline ($ps< 0.05$). Alcohol intake in the CIE+FSS group was greater during all Test weeks compared to their Baseline level of intake ($ps< 0.001$), and drinking was greater during each Test week in the CIE+FSS condition compared to the CIE-alone group ($ps< 0.05$). Separate analysis of alcohol intake during the final test period prior to sacrifice (Test 4) revealed a main effect of Group [$F(3,88)= 13.025$, $p< 0.001$]. While alcohol intake for CIE-alone and FSS-alone groups did not significantly differ from the CTL group, CIE+FSS mice exhibited greater alcohol intake compared to all other groups ($ps< 0.001$) (Figure 2C). These results confirm our previous findings showing that stress (FSS) experience selectively enhances alcohol drinking in dependent (CIE-exposed) mice while not altering intake in nondependent (FSS-alone) mice (15,16,36).

Analysis of *Pdyn* mRNA expression in the CeA at the end of Test 4 revealed main effects of Group at 30-min [$F(3,30)= 7.055$, $p< 0.001$] and 4-hr [$F(3,23)= 3.33$, $p= 0.037$] following the last FSS exposure but not at 24-hr post-FSS [$F(3,27)= 0.415$, $p= 0.744$]. Post-hoc analyses showed that *Pdyn* mRNA expression was selectively elevated in the CIE+FSS group relative to the CTL group at 30-min and 4-hr after FSS exposure, with values normalizing at the 24-hr time point (Figure 2D). Neither CIE-alone nor FSS-alone treatments produced an increase in *Pdyn* mRNA levels in the CeA. Schematic depiction of bilateral tissue punches collected from the CeA is shown in Figure 2E.

Chemogenetic Inhibition of CeA^{DYN} Neurons Reduces Stress (FSS)-Enhanced Alcohol Drinking in CIE-Exposed Pdyn-IRES-Cre Mice.

Pdyn-IRES-Cre mice were used to target expression of an inhibitory (hM4Di) DREADD or control virus in CeA^{DYN} neurons (Figure 3A). Using this same strategy, we previously demonstrated fidelity of expression and functionality of this viral vector (35). In the present study, expression of the mCherry marker was confined to the CeA (Figure 3B).

Average weekly alcohol intake prior to administering CNO during Test 3 is shown in Table 1. ANOVA revealed a main effect of Group [$F(3,60)= 8.397$, $p< 0.001$] and Test Cycle [$F(2,120)= 22.632$, $p< 0.001$] but no effect of Virus [$F(1,60)= 1.895$, $p= 0.174$], indicating

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that viral expression did not influence alcohol drinking prior to testing. A Group x Test Cycle interaction [F(6,120)= 6.258, p< 0.001] was observed and post-hoc analyses revealed increased alcohol intake in the CIE-alone and CIE+FSS groups during Test 1 and Test 2 compared to their respective Baseline levels of intake (ps< 0.05).

Analysis of alcohol intake during Test 3 indicated a marginally significant Group x Virus x Drug interaction [F(3,60)= 2.340, p= 0.082]. Separate analysis of alcohol intake in mice expressing hM4Di in CeA^{DYN} neurons revealed a significant Group x Drug interaction [F(3,38)= 8.521, p< 0.001]. Vehicle (saline) administration in mice with a history of CIE-alone and CIE+FSS consumed more alcohol than CTL mice that received vehicle (ps< 0.05). Further, alcohol intake was significantly greater in the CIE+FSS group compared to the CIE-alone condition (p< 0.005) whereas FSS-alone did not significantly alter alcohol consumption Selective silencing of CeA^{DYN} neurons following CNO injection resulted in significant reduction in alcohol intake in the CIE+FSS group (p< 0.001). CNO injection produced a marginal reduction in drinking in the CIE-alone group (p= 0.082) but did not alter alcohol intake in the FSS-alone or CTL groups (Figure 3C). Similar analysis of alcohol consumption in mice treated with control virus indicated a main effect of Group [F(3,22)= 16.14, p< 0.001], but no Group x Drug interaction [F(3,22)= 0.128, p= 0.381]. As expected, alcohol intake was significantly greater in CIE-alone mice compared to CTL mice (p< 0.05) and stress further enhanced this elevated drinking (CIE+FSS > CIE-alone) (p< 0.01). Alcohol intake in the FSS-alone group did not differ from the CTL group. CNO injection in mice that harbored the control virus did not alter alcohol drinking in any of the groups (Figure 3D).

To further examine the apparent “selective” effect of CNO in mice treated with the active (hM4Di-containing) virus, data expressed as a change from the vehicle (saline) condition for each subject was analyzed (Figure 3E). Post-hoc analysis of the Group x Virus interaction [F3,60)= 2.34, p< 0.05] indicated that CNO injection significantly reduced alcohol intake (relative to vehicle) only in CIE+FSS mice that harbored active vs. control virus (p< 0.001) and this reduction was greater in CIE+FSS mice compared to all other groups that were treated with active virus (ps< 0.01). Thus, inactivation of CeA^{DYN} neurons following CNO injection significantly attenuated the ability of stress (FSS) to further enhance alcohol consumption in CIE-exposed mice, and this effect was only observed in mice treated with the inhibitory (hM4Di) DREADD-containing virus.

Intra-CeA Injection of a KOR Antagonist Blocks Stress Enhancement of Alcohol Drinking in CIE-Exposed Mice.

Alcohol intake prior to microinjection of the KOR antagonist during Test 3 is shown in Table 2. ANOVA indicated significant main effects of Group [F(3,64)= 14.823, p< 0.001] and Test Cycle [F(2,128)= 75.339, p< 0.001], and a Group x Test Cycle interaction [F(6,128)= 12.882, p< 0.001]. Post-hoc analyses indicated that alcohol intake in the CIE-alone and CIE+FSS groups during Test 1 and Test 2 was greater than their respective Baseline levels of intake (ps< 0.05). In contrast, FSS-alone did not alter alcohol drinking in nondependent mice.

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Microinjection of norBNI into the CeA during Test 3 (Figures 4A, 4B) selectively attenuated elevated drinking in mice with a history of CIE-alone and CIE+FSS without affecting more moderate levels of consumption in FSS-alone and CTL groups (Figure 4C). This finding is supported by analysis of average alcohol intake during the two days following vehicle or norBNI microinjection, which revealed a significant Group x Drug interaction [$F(3,60)= 6.742$; $p< 0.001$]. Post-hoc analyses showed that vehicle-treated mice with a history of CIE-alone and CIE+FSS exposure consumed more alcohol than CTL mice ($ps< 0.005$) and, replicating our earlier finding, alcohol intake was significantly greater in CIE+FSS compared to CIE-alone mice ($p< 0.05$). Microinjection of norBNI reversed elevated alcohol intake in both CIE-alone and CIE+FSS groups ($ps< 0.01$). Analysis of daily alcohol intake during Test 3 revealed a similar profile of results (Figures S2A and S2B). To evaluate the relative magnitude of the norBNI effect across all groups, data were also analyzed as a difference from the average vehicle intake for each group. ANOVA revealed a significant Group x Drug interaction [$F(3,60)= 6.742$, $p< 0.001$], and post-hoc analysis indicated that while a trend was apparent in the CIE-alone group ($p= 0.125$), norBNI significantly reduced alcohol intake relative to the respective vehicle condition only in the CIE+FSS group ($p< 0.001$). Further, the reduction in alcohol drinking following intra-CeA norBNI injection (relative to vehicle) was significantly greater in the CIE+FSS group compared to all other groups, which did not differ from each other ($ps< 0.01$) (Figure 4D). Collectively, these results suggest that blocking KOR signaling in the CeA was especially effective in reducing stress-enhanced drinking in the model. Schematic representation of injection sites for mice that received vehicle or norBNI into the CeA is shown in Figures S3A and S3B, respectively.

Intra-BNST Injection of a KOR Antagonist Blocks Stress Enhancement of Alcohol Drinking in CIE-Exposed Mice.

Given our previous work showing that KOR antagonism in the BNST reduced heavy (binge-like) alcohol drinking (45) and that dynorphinergic neurons in the CeA project to the BNST (40,51), this study was conducted to examine whether blocking KOR signaling in the BNST attenuates the ability of stress to further enhance elevated drinking in CIE-exposed mice. As in previous experiments, alcohol consumption increased over Baseline levels during Test 1 and Test 2 in CIE-alone and CIE+FSS groups while intake remained relatively stable for FSS-alone and CTL groups (Table 3). This was supported by significant main effects of Group [$F(3,68)= 13.527$, $p< 0.001$] and Test Cycle [$F(2,136)= 21.644$, $p< 0.001$], and the Group x Test Cycle interaction [$F(6,136)= 9.009$, $p< 0.001$].

Intra-BNST norBNI administration during Test 3 (Figures 5A, 5B) reduced elevated alcohol intake in CIE-alone and CIE+FSS groups without altering moderate intake in FSS-alone and CTL groups (Figure 5C). Analysis of average intake during the two days after microinjection revealed a Group x Drug interaction [$F(3,64)= 3.975$, $p= 0.012$]. Post-hoc analyses showed that alcohol intake following vehicle administration was significantly greater in CIE+FSS mice compared to the CIE-alone group ($p< 0.01$), and both groups consumed more alcohol than FSS-alone and CTL mice ($ps< 0.001$), which did not significantly differ from each other. Microinjection of norBNI into the BNST significantly reduced elevated alcohol consumption in the CIE-alone ($p< 0.005$) and CIE+FSS ($p< 0.001$) groups. Analysis of daily alcohol intake during Test 3 produced a similar profile of results

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(Figures S4A and S4B). Analysis of the difference in alcohol intake following intra-BNST norBNI injection relative to the appropriate average vehicle intake for each group revealed a significant Group x Drug interaction [$F(3,64)= 3.975$, $p< 0.01$]. While intra-BNST norBNI reduced alcohol drinking (relative to vehicle levels) in the CIE-alone group ($p= 0.024$) and CIE+FSS mice ($p< 0.001$), this effect was significantly more robust in the CIE+FSS group compared to all other groups, including CIE-alone mice ($ps< 0.05$) (Figure 5D). In contrast, norBNI injection into the BNST did not alter alcohol intake in FSS-alone or CTL groups. Schematic representation of injection sites for mice that received vehicle or norBNI into the BNST is shown in Figure S5A and S5B, respectively.

DISCUSSION

The present studies validate the CIE-FSS Drinking paradigm as a framework for modeling stress-enhanced alcohol drinking and demonstrate the significant contribution of DYN/KOR activity within the extended amygdala in mediating this behavior. A history of repeated FSS in CIE-exposed mice resulted in escalation of voluntary alcohol consumption and this was accompanied by elevated *Pdyn* mRNA expression in the CeA. Targeted chemogenetic silencing of DYN-containing neurons in the CeA completely blocked the ability of stress to enhance alcohol drinking in the model. Further, pharmacological blockade of KOR within the CeA or BNST normalized drinking in mice with a history of both stress and CIE exposure. Together, these data suggest that CeA^{DYN} neurons are uniquely responsive to a history of chronic alcohol exposure and stress, and KOR signaling within the CeA and BNST plays an important role in mediating stress-enhanced alcohol drinking.

While chronic alcohol exposure and stress are known to produce dynamic alterations in brain gene expression (52–56), only recently have genomic changes in relation to stress-alcohol interactions been explored (18). This latter study revealed both unique transitory and long-lasting changes in gene expression in prefrontal cortex associated with stress-enhanced alcohol consumption in the CIE-FSS Drinking model. In the present study, we show that *Pdyn* mRNA levels in the CeA are elevated at 30-min and 4-hr after FSS exposure only in mice with a history of both FSS and CIE exposure. This change was not observed in other groups (CIE-alone and FSS-alone conditions) that did not exhibit increased alcohol intake relative to the CTL condition. Studies in rats have shown an upregulation in *Pdyn* mRNA expression in CeA following chronic alcohol drinking (57) and acute withdrawal from CIE exposure (43). Forced swim stress experience also has been reported to increase *Pdyn* mRNA levels in extended amygdala structures (58,59). Together these findings indicate that the CeA is highly responsive to stress and chronic alcohol exposure and changes in *Pdyn* transcriptional activity may contribute to enhanced motivation to drink following combined stress and chronic alcohol exposure.

The CeA is a key structure within extended amygdala circuitry with rich expression of both DYN and KORs (38–40). In the present study, using a validated transgenic mouse model (47) along with a validated DREADD-containing viral construct (35), targeted chemogenetic inactivation of CeA^{DYN} neurons blocked stress-enhanced alcohol drinking. Vehicle injections in mice expressing the inhibitory (hM4Di) DREADD did not alter alcohol drinking and reduced alcohol intake in mice injected with CNO is not likely to be attributed

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to off-target effects of CNO since the ligand did not alter alcohol consumption in mice that received control virus treatment. Using a similar experimental strategy, silencing CeA^{DYN} neurons was shown to significantly reduce alcohol consumption in a binge-drinking model (35). Likewise, genetic deletion of Pdyn in the CeA reduced alcohol drinking in models of high intake (38). Together, these data indicate that dynorphinergic activity in the CeA plays a significant role in regulating alcohol consumption, including elevated drinking associated with stress.

CeA^{DYN} neurons produce effects through signaling at KOR both locally within the CeA as well as in several projection regions. Studies have shown KOR antagonism within the CeA and other extended amygdala structures (e.g., BNST) reduces alcohol drinking (35,42,43,45), and this is congruent with findings showing that systemic administration of KOR antagonists reduce alcohol consumption in a variety of models (20,22,33–36). Results from the present study indicate that direct injection of the KOR antagonist norBNI into the CeA or BNST blocked the ability of stress to enhance voluntary alcohol drinking in the CIE+FSS group. Thus, these are the first data to directly implicate a role for DYN/KOR activity within extended amygdala circuitry in contributing to stress-induced excessive drinking. While there is evidence for dynorphinergic projections from the CeA to the BNST (40,51), the extent to which these results are mediated by KOR signaling within the CeA and other projection sites, including the BNST, will require more direct circuitry-based examination.

DYN-containing neurons in the CeA are primarily GABAergic and known to co-express other neuropeptides that influence alcohol drinking, such as corticotropin release factor, and neurotensin (60,61). Thus, co-release of GABA and other peptides may contribute to stress-enhanced drinking in this model, and this possibility cannot be ruled out. However, results from our chemogenetic and pharmacological studies strongly implicate a significant role for engagement of DYN/KOR activity within extended amygdala circuitry in the ability of stress to further elevate alcohol consumption in subjects with a history of chronic alcohol exposure.

While stress-enhanced drinking in the CIE-FSS model has been demonstrated in both female and male mice (62,63), one limitation of this study is that only males were used to examine the role of DYN/KOR activity. Sex-related differences in sensitivity to DYN/KOR function have been noted, especially regarding reward and aversion/dysphoria related behaviors (64–66). Thus, it will be important in future studies to examine whether manipulation of DYN/KOR activity within extended amygdala circuitry produces sex-related differences in stress-enhanced alcohol drinking. This is especially relevant given the high prevalence of co-occurring stress-related disorders and alcohol use disorder in women (5,8).

In summary, despite stress being a significant contributing factor in heavy drinking, as reflected by high comorbidity of stress-related disorders and AUD, few effective treatments are available and the lack of preclinical models that reliably demonstrate stress-enhanced drinking has hindered efforts to address the problem. We have established a model demonstrating robust and highly reproducible stress-induced elevation of alcohol consumption. Using chemogenetic and pharmacological approaches, we show that

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DYN/KOR activity within extended amygdala circuitry plays a significant role in mediating the ability of stress to increase drinking in mice with a history of chronic alcohol exposure. These findings align with other preclinical studies showing that long-acting (20,33–35) and short-acting (36,67,68) KOR antagonists reduce high levels of alcohol consumption and relapse-like behavior provoked by stress. Together, these results support clinical studies that target the DYN/KOR system in the development of more effective treatments for individuals presenting with comorbidity of stress-related disorders and AUD (46,69).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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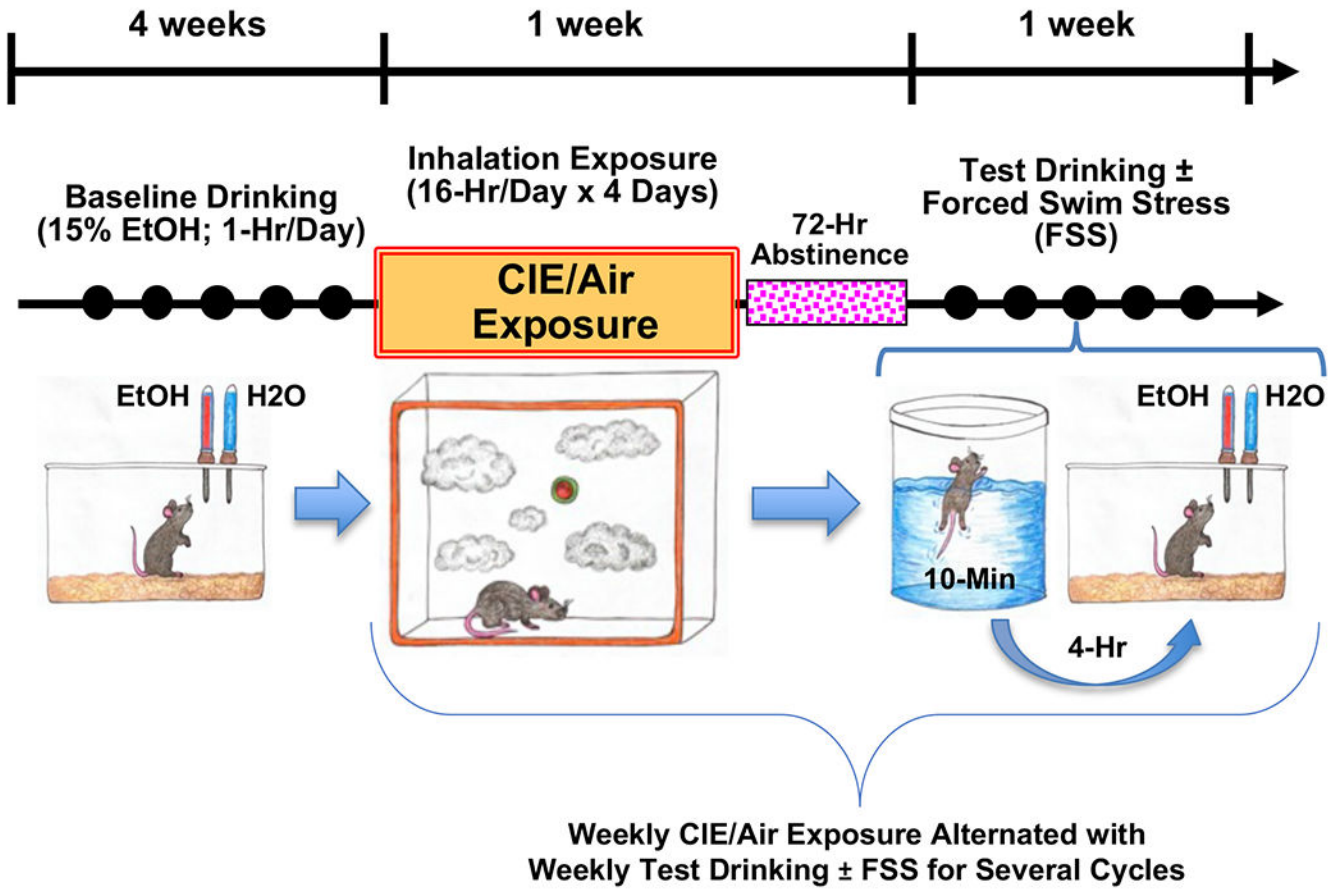

Figure 1: CIE-FSS Drinking Model.

Mice were treated in the CIE-FSS Drinking model involving weekly CIE or Air exposure cycles alternating with weekly Test drinking sessions (1-hr) with or without FSS exposure (10-min; 4-hr prior to each drinking session). Solid circles represent daily 1-hr drinking sessions. After establishing stable Baseline drinking over 4-weeks, mice were separated into 4 groups: CTL, CIE-alone, FSS-alone, CIE+FSS. Weekly CIE/Air exposures alternated with weekly (5-Day) Test drinking sessions for several cycles.

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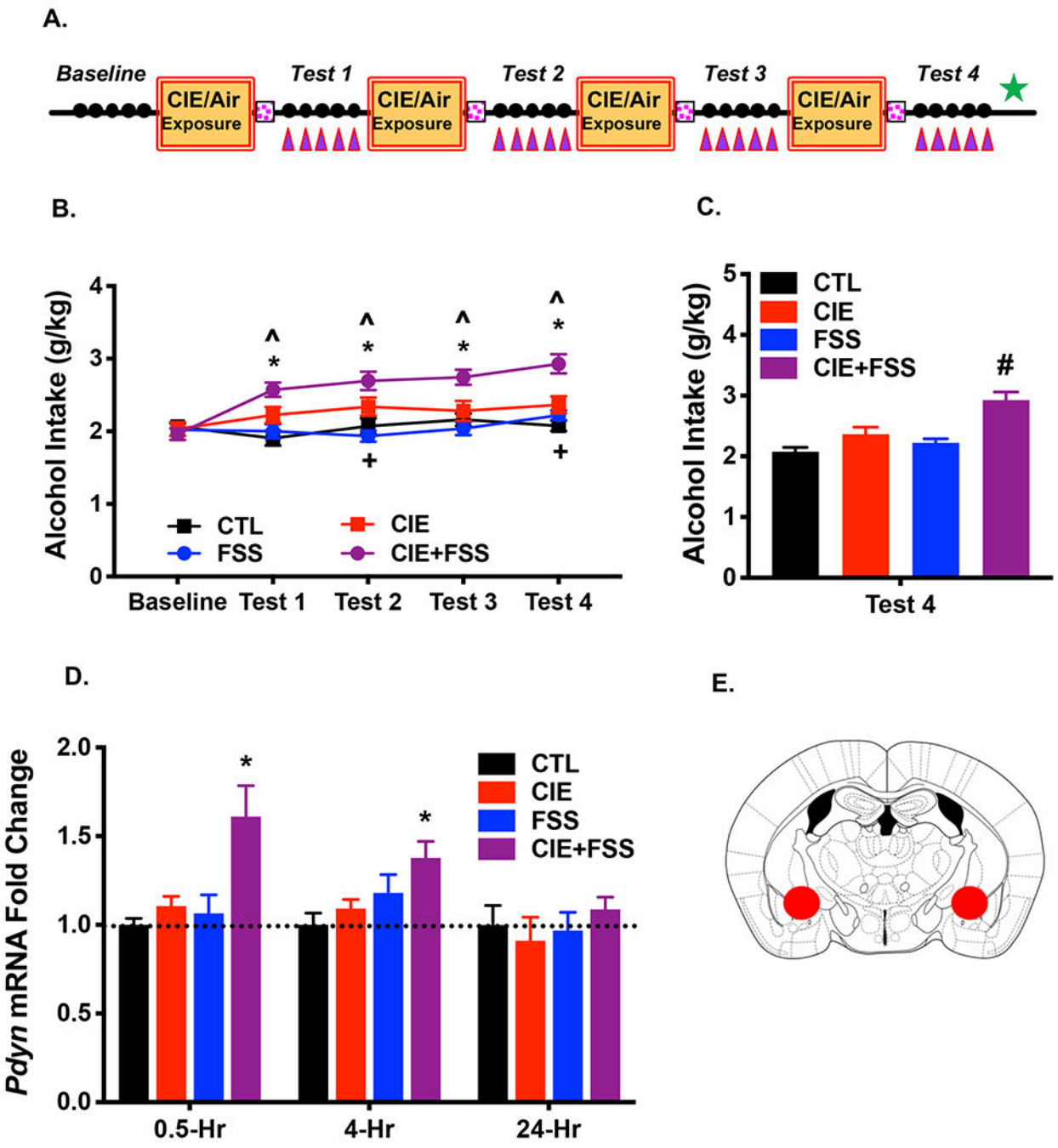

Figure 2: A *History of CIE+FSS Exposure Increases Pdyn mRNA Expression in the CeA*.

(A) Mice were sacrificed, and brain tissue was collected at 30-min, 4-hr, or 24-hr following the last FSS exposure during Test 4 (denoted by green star) to assess *Pdyn* mRNA expression in CeA (purple triangles denote FSS exposures) (N= 6-10/group/time point). **(B)** Average weekly alcohol consumption across each phase of the study. Alcohol intake increased compared to Baseline in the CIE-alone group during Test 2 and Test 4 (+ ps< 0.05); Alcohol drinking in the CIE+FSS group was significantly greater during each of the Test cycles compared to Baseline (* ps< 0.05) and compared to the CIE-alone group (^ ps<

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0.05); Alcohol intake in FSS-alone and CTL groups during Test cycles did not differ from Baseline levels. **(C)** Average alcohol consumption during Test 4. Alcohol intake was greater in the CIE+FSS group compared to all other groups which did not significantly differ from each other (# ps< 0.001). **(D)** *Pdyn* mRNA expression in the CeA was significantly elevated in CIE+FSS compared to CTL mice at 30-min (* p< 0.01) and 4-hr (* p< 0.05) post-FSS exposure at the end of Test 4, returning to CTL levels at 24-hr. All values are mean ± s.e.m. **(E)** Schematic representation of tissue punches collected from the CeA.

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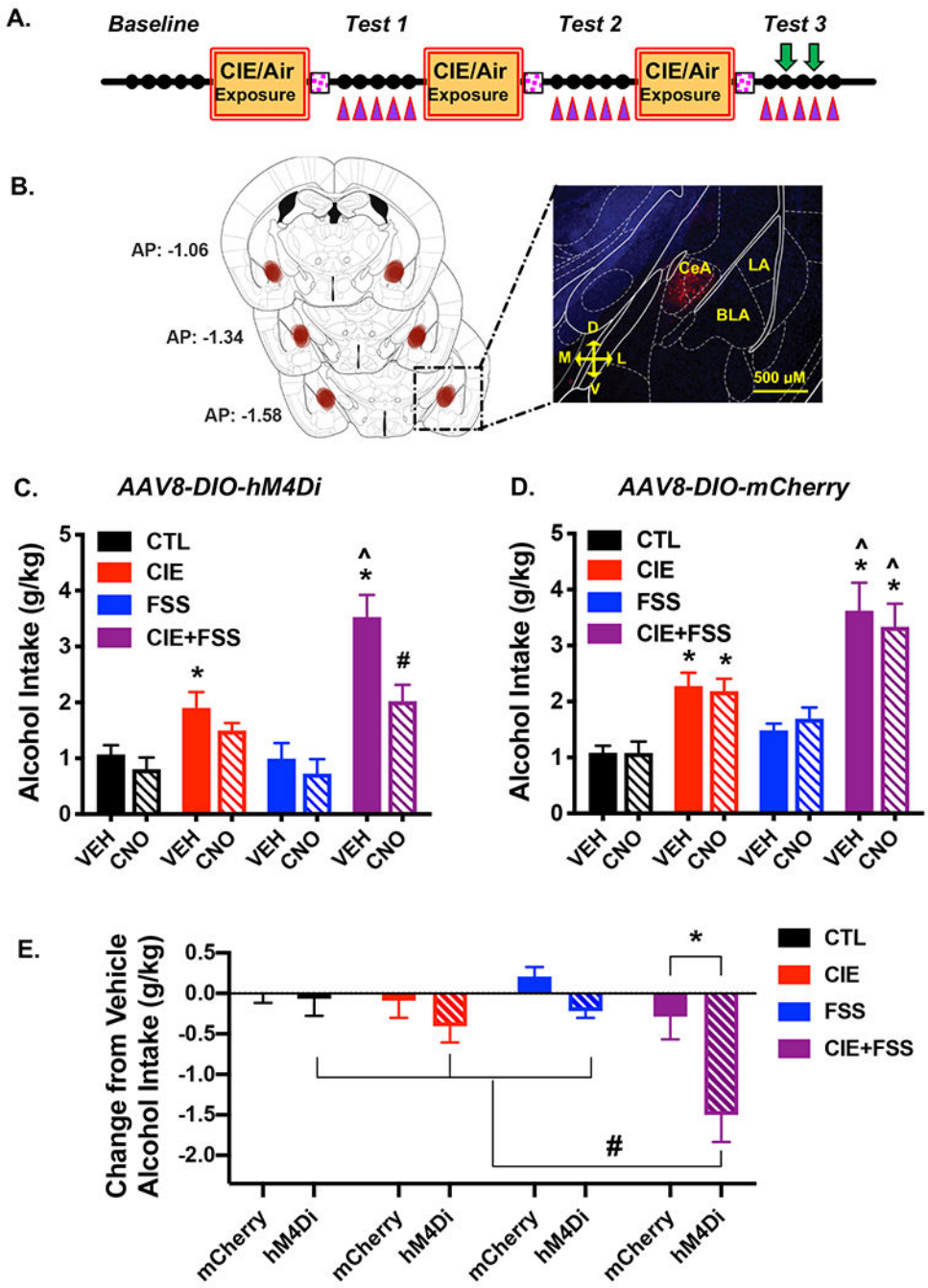

Figure 3: Chemogenetic Inhibition of CeA^{DYN} Attenuates Stress-Enhanced Alcohol Drinking.

(A) For targeted expression of an inhibitory DREADD, *Pdyn-IRES-Cre* mice were bilaterally infused with AAV8-hSyn-DIO-hM4Di-mCherry or AAV8-hSyn-DIO-mCherry into the CeA 2-weeks prior to start of Baseline drinking; vehicle or CNO (3 mg/kg) was injected 30-min prior to the 2nd and 4th drinking sessions during Test 3 in a balanced cross-over design (denoted by green arrows). (**B**) Viral expression was localized to the CeA as indicated by visualization of mCherry fluorescence. **(C)** Average alcohol intake in mice expressing hM4Di-contaning virus (N= 10-11/group). In vehicle-treated mice, alcohol

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consumption was greater in CIE-alone and CIE+FSS groups compared to CTL and FSS-alone groups which did not differ (* ps< 0.05). Also, intake following vehicle injection was greater in CIE+FSS mice compared to CIE-alone mice (^ p< 0.005). Activation of the inhibitory DREADD expressed in CeA^{DYN} neurons via CNO injection resulted in a significant reduction in alcohol intake in the CIE+FSS group compared to those mice receiving vehicle (# p< 0.001). **(D)** Average alcohol intake in mice treated with control virus (N= 6-7/group). Vehicle injection resulted in the expected greater alcohol intake in CIE-alone and CIE+FSS groups compared to the other groups (* ps< 0.05), and intake was greater in the CIE+FSS mice compared to CIE-alone mice (^ p< 0.005). CNO injection did not alter alcohol drinking in any group relative to when those mice received vehicle injection. (**E**) Change from respective vehicle alcohol intake across treatment and virus groups. CNO injection significantly reduced alcohol intake (relative to vehicle) in CIE+FSS mice that harbored active vs. control virus (* p< 0.001) and this reduction was greater in CIE+FSS mice compared to when CNO was injected in CIE-alone, FSS-alone, and CTL groups that were treated with active virus (# ps< 0.01). All values are mean ± s.e.m.

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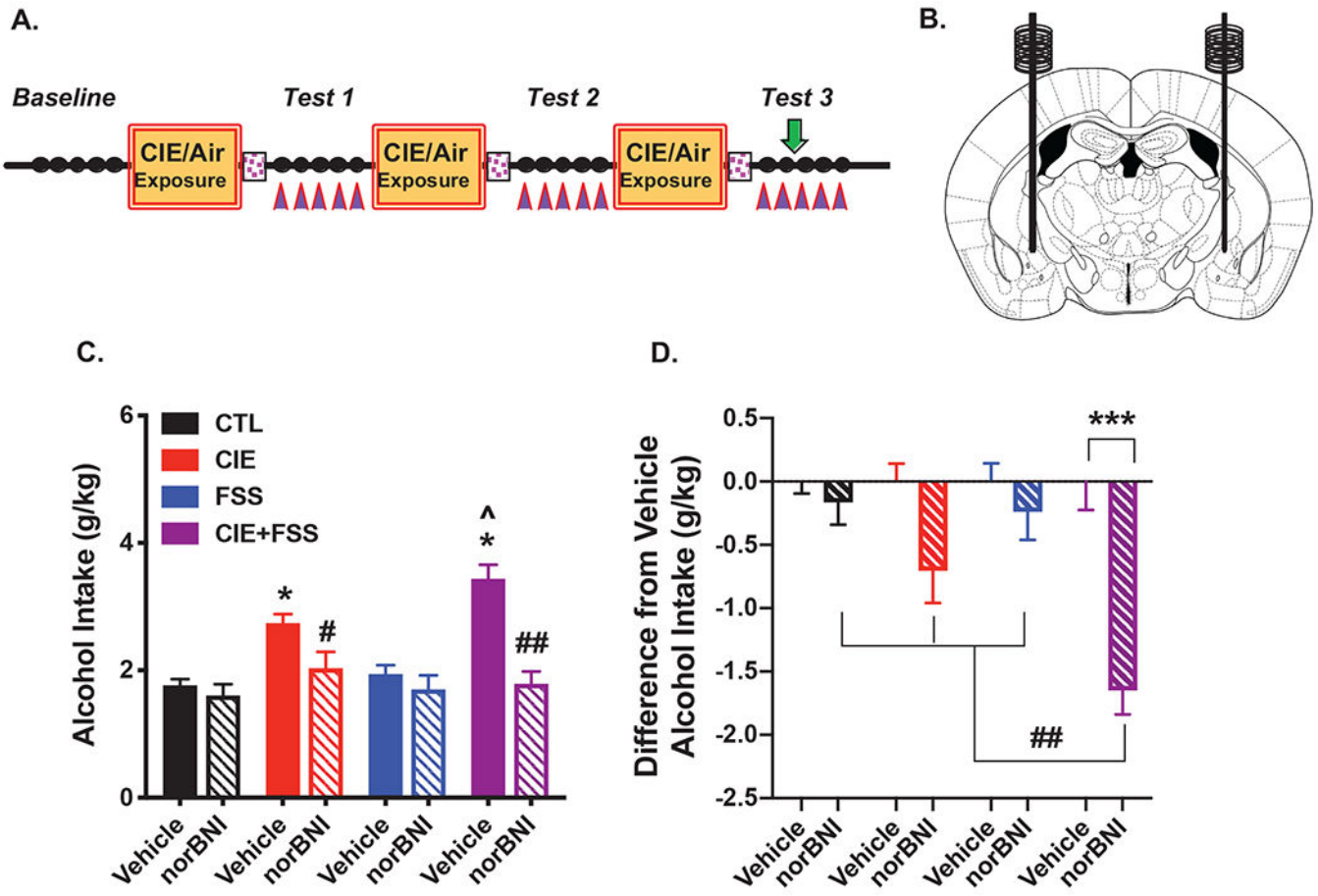

Figure 4: Microinjection of norBNI into the CeA Attenuates Stress-Enhanced Alcohol Drinking.

(A) Guide cannulae were implanted over the CeA 2-weeks prior to the start of Baseline drinking; vehicle or norBNI (2.5 μg/side) was infused 16-hr prior to the 3rd drinking session during Test 3 (denoted by green arrow). (**B**) Schematic representation of stereotaxic bilateral placement of guide cannulae over the CeA. **(C)** Average alcohol intake (over Day 3 and Day 4) in mice receiving vehicle (N= 7-9/group) or norBNI (N= 8-9/group) injection in the CeA. Vehicle-treated CIE-alone and CIE+FSS groups consumed significantly more alcohol than CTL and FSS-alone groups (which did not differ) (* ps< 0.05) and alcohol intake was greater in CIE+FSS compared to CIE-alone mice (^ p< 0.05); norBNI treatment blocked elevated drinking in CIE-alone (# p< 0.01) and CIE+FSS (## p< 0.001) groups without altering intake in the FSS-alone or CTL groups. **(D)** Difference from respective vehicle alcohol intake across treatment groups. Intra-CeA norBNI injection significantly reduced alcohol intake (relative to vehicle) only in the CIE+FSS group (*** p< 0.001), and this effect was significantly greater in the CIE+FSS group compared to all other groups, which did not differ from each other (## ps< 0.01).

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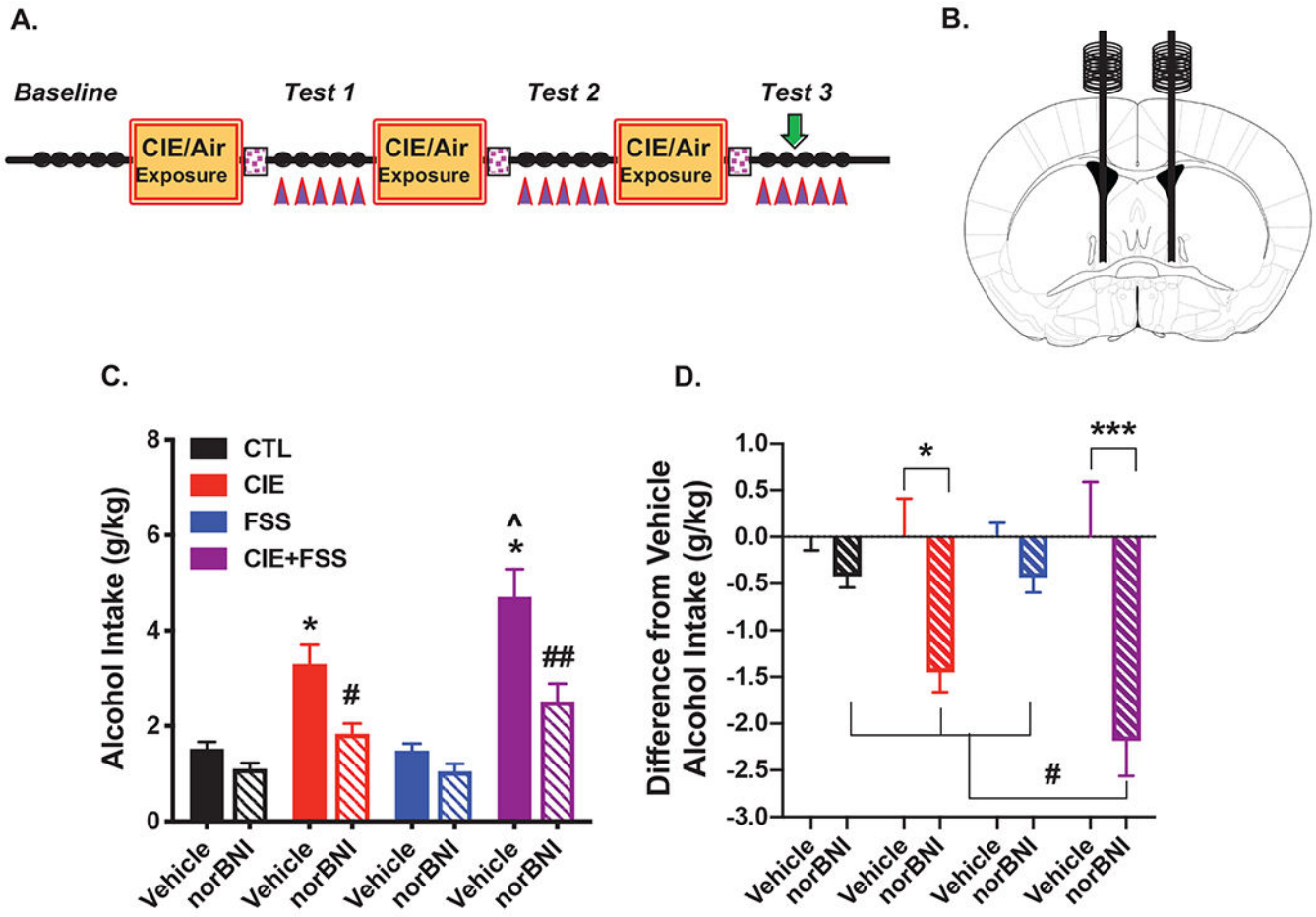

Figure 5: Microinjection of norBNI into the BNST Attenuates Stress-Enhanced Alcohol Drinking.

(A) Guide cannulae were implanted over the BNST 2-weeks prior to the start of Baseline drinking; vehicle or norBNI (2.5 μg/side) was infused 16-hr prior to the 3rd drinking session during Test 3 (denoted by green arrow). **(B)** Schematic representation of stereotaxic bilateral placement of guide cannulae over the BNST. **(C)** Average alcohol intake (over Day 3 and Day 4) in mice receiving vehicle (N= 8-10/group) or norBNI (N= 8-10/group) injection in the BNST. Following vehicle injection, CIE+FSS mice consumed significantly more alcohol than the CIE-alone group (^ $p < 0.05$), and both CIE+FSS and CIE-alone groups consumed more alcohol than CTL and FSS-alone groups (which did not differ) (* ps< 0.05); norBNI treatment blocked elevated drinking in CIE-alone (# $p < 0.005$) and CIE+FSS (## $p < 0.001$) groups without altering intake in the FSS-alone or CTL groups. **(D)** Difference from respective vehicle alcohol intake across treatment groups. Intra-BNST norBNI injection significantly reduced alcohol intake (relative to vehicle) in the CIE-alone group (* $p < 0.05$) and the CIE+FSS group (*** $p < 0.001$), and this effect was significantly greater in the CIE+FSS group compared to all other groups, which did not differ from each other (# ps< 0.05).

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Table 1:

Average Weekly Alcohol Intake Prior to Chemogenetic Inhibition of CeA^{DYN} Neurons.

Alcohol intake (g/kg) in mice expressing hM4Di or mCherry in CeA^{DYN} neurons during Baseline and Test weeks prior to challenge with vehicle or CNO (3 mg/kg) during Test 3. Values are mean ± s.e.m.

AAV	Group	Baseline	Test 1	Test 2
hM4Di	CTL	1.48 ± 0.12	1.44 ± 0.14	1.49 ± 0.16
	FSS	1.57 ± 0.13	1.78 ± 0.12	1.94 ± 0.17
	CIE	1.49 ± 0.18	1.95 ± 0.26 *	1.84 ± 0.20 *
	CIE+FSS	1.54 ± 0.15	2.13 ± 0.25 *	2.54 ± 0.22 *
mCherry	CTL	1.62 ± 0.17	1.72 ± 0.17	1.33 ± 0.13
	FSS	1.49 ± 0.11	1.56 ± 0.19	1.65 ± 0.06
	CIE	1.61 ± 0.11	2.53 ± 0.19 *	2.37 ± 0.20 *
	CIE+FSS	1.73 ± 0.13	2.83 ± 0.34 *	2.57 ± 0.32 *

* differs from respective Baseline levels ($p < 0.05$).

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Table 2:

Average Weekly Alcohol Intake Prior to Injection of KOR Antagonist into CeA.

Alcohol intake (g/kg) in mice during Baseline and Test weeks prior to bilateral microinjection of vehicle of norBNI (2.5 μg/side) into the CeA during Test 3. Values are mean ± s.e.m.

Group	Baseline	Test 1	Test 2
CTL	1.88 ± 0.07	2.19 ± 0.09	1.89 ± 0.08
FSS	1.90 ± 0.08	2.37 ± 0.15	2.03 ± 0.12
CIE	1.88 ± 0.08	2.82 ± 0.11 *	2.94 ± 0.16 *
CIE+FSS	1.82 ± 0.08	3.04 ± 0.14 *	3.08 ± 0.14 *

* differs from respective Baseline levels ($p < 0.05$).

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Table 3:
Average Weekly Alcohol Intake Prior to Injection of KOR Antagonist into BNST.

Alcohol intake (g/kg) in mice during Baseline and Test weeks prior to bilateral microinjection of vehicle of norBNI (2.5 μg/side) into the BNST during Test 3. Values are mean ± s.e.m.

Group	Baseline	Test 1	Test 2
CTL	1.97 ± 0.12	1.85 ± 0.12	1.84 ± 0.15
FSS	2.02 ± 0.19	2.18 ± 0.12	2.02 ± 0.16
CIE	2.13 ± 0.13	2.84 ± 0.13 *	2.99 ± 0.16 *
CIE+FSS	1.99 ± 0.12	2.88 ± 0.12 *	2.93 ± 0.15 *

* differs from respective Baseline levels ($p < 0.05$).

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KEY RESOURCES TABLE

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Add additional rows as needed for each resource type	Include species and sex when applicable.	Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use “this paper” if new.	Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources.	Include any additional information or notes if necessary.
Antibody	Rat, anti-mCherry	Millipore	# M11217	
Antibody	Goat anti-Rat conjugated AlexaFluor 555	Invitrogen	# P36961	
Bacterial or Viral Strain	AAV8-hSyn-DIO-hM4Di-mCherry	Addgene	# 50459-AAV8	
Bacterial or Viral Strain	AAV8-hSyn-DIO-mCherry	Addgene	# 44362-AAV8	
Biological Sample	Mouse brain	This study	N/A	
Cell line	N/A			
Chemical Compound or Drug	clozapine-N-oxide hydrochloride	Tocris	# 6329	
Chemical Compound or Drug	nor-binaltorphiminie dihydrochloride	Tocris	#0347	
Commercial Assay Or Kit	mirVana miRNA Extraction kit	Invitrogen	# AM1560	
Commercial Assay Or Kit	QuantiTect Reverse Transcription kit	Quagen	# 205314	
Deposited Data; Public Database	N/A			
Genetic Reagent	N/A			
Organism/Strain	Mouse, C57BL/6J, male	Jackson Laboratories	#000664	
Organism/Strain	Mouse, Pdyn-IRES-Cre, male	PMID:30555162; PMID: 24487620	N/A	
Peptide, Recombinant Protein	N/A			
Recombinant DNA	N/A			
Sequence-Based Reagent	TaqMan qRT-PCR primers (Pdyn, Ppia)	ThermoFisher Scientific	Pdyn (# Mm00457573_ml) Ppia (# Mm02342430_gl)	
Software; Algorithm	N/A			
Transfected Construct	N/A			
Other				