Neuropsychopharmacology

ARTICLE

Endocannabinoid control of the insular-bed nucleus of the stria terminalis circuit regulates negative affective behavior associated with alcohol abstinence

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Negative affect is a core symptom domain associated with an array of neurological and psychiatric disorders and is only partially targeted by current therapies, highlighting the need for better, more targeted treatment options. This study focuses on negative affective symptoms associated with prolonged alcohol abstinence, one of the leading causes of relapse. Using a mouse model of chronic alcohol consumption followed by forced abstinence (CDFA), prolonged alcohol abstinence increased c-fos expression and spontaneous glutamatergic neurotransmission in the dorsal bed nucleus of the stria terminalis (dBNST), a region heavily implicated in negative affect in both humans and rodents. Further, pharmacologically enhancing endogenous cannabinoids (eCB) with JZL184 prevents abstinence-induced increases in dBNST neuronal activity, underscoring the therapeutic potential of drugs targeting the brain's eCB system. Next, we used a channelrhodopsin-assisted mapping strategy to identify excitatory inputs to the dBNST that could contribute to CDFA-induced negative affect. We identified the insular cortex (insula), a region involved in regulating interoception, as a dense, functional, eCB-sensitive input to the dBNST. Using a chemogenetic strategy to locally mimic eCB signaling, we demonstrate that the insula strongly influences the CDFA behavioral phenotype and dBNST neuronal activity. Lastly, we used an anterograde strategy for transynaptic targeting of Cre expression in combination with a G_q-DREADD to selectively recruit dBNST neurons receiving insula projections. Chemogenetic recruitment of these neurons mimicked behavioral and c-fos responses observed in CDFA. Collectively, this study supports a role for the insula-BNST neural circuit in negative affective disturbances and highlights the therapeutic potential of the eCB system for treating negative affective disorders.

Neuropsychopharmacology (2019) 44:526–537; https://doi.org/10.1038/s41386-018-0257-8

INTRODUCTION

Affective disorders, including depression and anxiety, are the most common psychiatric diseases. Close to 50 million adults in the US suffer from some form of depression and anxiety, yet only 40% are successfully treated [1]. While negative affective states are considered potent antecedents to many diseases, they have a particularly strong impact on drug addiction, including both the initiation and abstinence phases [2]. Alcohol use disorders (AUDs) are highly comorbid with psychiatric maladies such as generalized anxiety disorder and major depression [3–5]. Abstinent alcoholics commonly report stress and negative affect as potent triggers of cravings and relapse [3, 6], and the severity of negative affect is strongly correlated with relapse susceptibility [2, 7]. Treating these symptoms with traditional antidepressants has yielded inconsistent results, and in some cases these drugs can increase alcohol drinking [8, 9], underscoring the need to identify novel therapeutic targets through a greater understanding of the complex neurocircuitry regulating the disease state.

The endogenous cannabinoid (eCB) system negatively regulates anxiety, fear-learning, and stress-coping behaviors (for review see [10]), highlighting its therapeutic potential. The major components of the CNS eCB system are the presynaptic, G_i-coupled GPCR, cannabinoid receptor (CB_1R), the two endogenous ligands 2-arachadonylglycerol (2-AG) and *N*-arachidonylethanolamine (AEA), and their metabolic regulators, all of which are widely expressed throughout the brain [11, 12]. Pharmacological enhancement of 2-AG can prevent affective behaviors [13–16] and a wide body of literature supports a role for 2-AG in many phases of AUD [17–20] including abstinence-induced negative affective behaviors [21].

While the therapeutic potential of the eCB system for treating affective disorders is becoming increasingly apparent, the broad actions of the eCB system throughout the brain warrant a better understanding of the specific eCB-sensitive brain regions and neurocircuits regulating these effects. The dorsal bed nucleus of the stria terminalis (dBNST) is linked with modulating negative affective states and is a key center for integrating negative valence

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Received: 9 July 2018 Revised: 19 October 2018 Accepted: 21 October 2018
Published online: 2 November 2018

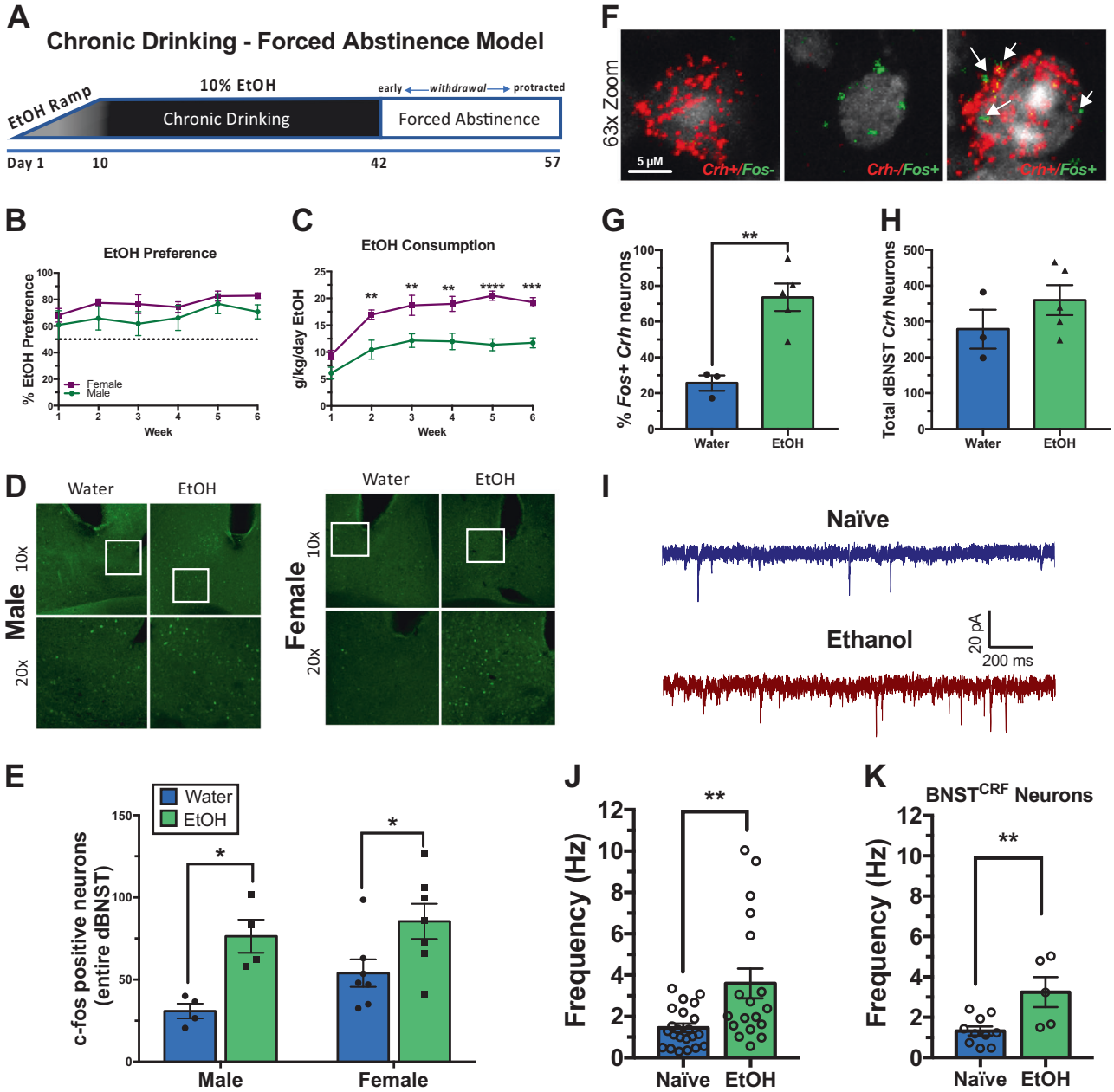


Fig. 1 Chronic-drinking followed by forced abstinence increases neuronal activity in the dBNST. **a** Chronic-drinking forced abstinence (CDFA) model. **b** Male and female C57BL/6J mice maintain a preference for ethanol over water throughout CDFA. **c** Female mice consume significantly more ethanol (g/kg/day) than male mice throughout CDFA (repeated measures two-way ANOVA, Sidak's post hoc $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; $n = 6$–11/group; data presented as mean ± SEM). **d** Representative image of c-fos protein staining in the dBNST in male and female mice. **e** Male and female mice have increased c-fos expression in the dBNST 15-days into forced abstinence (two-way ANOVA, Sidak's post hoc $^{*}p < 0.05$). Data presented as cells per 10× dBNST image per hemisphere and averaged per animal (presented as mean ± SEM). **f** Representative RNAScope® image showing DAPI nuclear stain (gray), *Fos* transcripts (green), and *Crh* transcripts (red). Left: *Crh+/Fos−* dBNST neuron. Middle: *Crh−/Fos−* dBNST neuron. Right: *Crh+/Fos+* dBNST neuron. White arrows represent *Fos* transcripts localized on $BNST^{CRF}$ neurons. **g** The percentage of $BNST^{CRF}$ neurons that express the *Fos* transcript is increased in CDFA mice 15-days into forced abstinence (Tukey's multiple comparison post hoc $^{**}p < 0.01$). Data presented as cells per 3 paneled 63× BNST images per hemisphere in one slice and averaged per animal (presented as individual data points and mean ± SEM). **h** The total number of $BNST^{CRF}$ neurons is similar in both the water and ethanol groups. **i** Representative electrophysiology trace in ethanol-naive mice and CDFA-mice 15-days into forced abstinence. **j** sEPSC frequency in all BNST neurons recorded from. BNST cells from ethanol-exposed mice have a significantly higher sEPSC frequency relative to controls (Tukey's multiple comparison post hoc $^{**}p < 0.01$). Data presented individual data points with mean ± SEM. **k** sEPSC frequency in $BNST^{CRF}$ neurons. CRF cells were identified from CRF-*tomato* mice. $BNST^{CRF}$ cells from ethanol-exposed mice have a significantly higher sEPSC frequency relative to controls (Tukey's multiple comparison post hoc $^{**}p < 0.01$). Data presented individual data points with mean ± SEM

or anxiety-like states driven by cortical, subcortical, midbrain, and hindbrain inputs [22, 23]. The dBNST heavily expresses the CB_1R, which has been shown to regulate excitatory and inhibitory drive from the amygdala and extended amygdala [12, 24–26]. Moreover, the dBNST is widely regarded as a critical node for stress-related psychopathologies such as anxiety and depression (for review see [23]) and contains a dense population of neurons expressing corticotropin-releasing factor (CRF, *Crh*). dBNST CRF-

expressing neurons have been shown to negatively regulate affective behaviors [27, 28], and positively regulate alcohol-seeking behavior [29–31] and stress-induced drug relapse [32]. CRF signaling in the dBNST is involved in many facets of AUD including binge drinking [33], withdrawal [34], and reinstatement of ethanol-seeking [35, 36], however compounds directly targeting CRF signaling have thus far been unsuccessful at treating AUD in humans [37]. Strong preclinical evidence links extended amygdala CRF signaling to depression and stress disorders [38–40].

Here, we build upon the previously established role of ethanol abstinence in producing negative affect in female mice [21, 41–43] by demonstrating that these behaviors involve eCB-sensitive increased circuit activity in the dBNST. Using a chronic drinking-forced abstinence model (CDFA), enhancing endogenous 2-AG with the monoacylglycerol (MAG) lipase inhibitor JZL184 prevents ethanol abstinence-induced increases in neuronal activity specifically in dBNST-CRF cells (dBNST^{CRF}). We implicate 2-AG, through its actions on dBNST neuronal signaling, as a potential effective alternative therapeutic target for treating negative affect. Next, we elucidate a component of the neural circuitry governing abstinence-induced negative affect. Using channelrhodopsin-assisted neuronal mapping with whole-cell electrophysiology, we identify dense, eCB-sensitive projection from the caudal-anterior insular cortex (insula) to dBNST^{CRF} neurons. The insula is critically involved in regulating interoception and affective behaviors and has recently been implicated in addiction neurocircuitry [44–46]. Chemogenetically manipulating the insula confirmed a significant role for the insula in producing affective disturbances, and control of dBNST cells in forced abstinence. Finally, using an anterograde strategy for transsynaptic targeting of Cre [47] in combination with Cre-dependent G_q-DREADD delivery in dBNST, we recruited dBNST neurons receiving projections from the insula (dBNST^{insula}) and demonstrated that their recruitment mimics CDFA-related behavioral changes. Together, we establish the insula-dBNST circuit as a promising target for regulating affective disorders including those associated with prolonged ethanol abstinence. eCB-based pharmacotherapies targeting affective circuitry could represent a compelling safer, more effective alternative to the current treatment options for AUD and affective disorders.

METHODS AND MATERIALS

See Supplementary Information for detailed methods.

Singly-housed C57BL/6J mice (both sexes) were purchased from The Jackson Laboratory (Bar Harbor, ME) and CRF-*tomato* mice were bred as previously described [29, 48]. All procedures were conducted with approval of the Institutional Animal Care and Use Committee at Vanderbilt University, and were within the guidelines set forth by the Care and Use of Mammals in Neuroscience and Behavioral Research (2003).

Two-bottle choice ethanol drinking was conducted as described in Fig. 1a and as previously described [21]. All adeno-associated viral (AAV) vectors were purchased from the Addgene Viral Vector Core and used as received. CRF-*tomato* mice or C57BL/6J mice were stereotaxically injected with 300 nL (50 nL/min) of AAV into the caudal anterior insular cortex or dBNST as indicated in the results. All mice were allowed to recover for at least 1 week before further experimentation. The concentrations and routes of administration used for compounds in this study are as follows: JZL (i.p. 8 mg/kg; ex vivo: 1 μM; Cayman Chemical), Clozapine-N-oxide (i.p. 3 mg/kg; ex vivo: 10 μM; MilliporeSigma), and Rimonabant (5 μM; APIChem), WIN55,212-2 (4 μM; MilliporeSigma). Mice were handled and behavioral studies performed as previously described [21, 49, 50].

All data were analyzed using GraphPad Prism 7. Data that included more than two groups or factors were analyzed using a one-way or two-way ANOVA, respectively. When appropriate, Tukey's or Sidak's multiple comparison post hoc test was performed and corrected *p*-values are presented in the text. For comparison of two groups, a paired or unpaired two-tailed Student's *t*-test was used.

RESULTS

Chronic drinking followed by forced abstinence increases c-fos expression in the dBNST

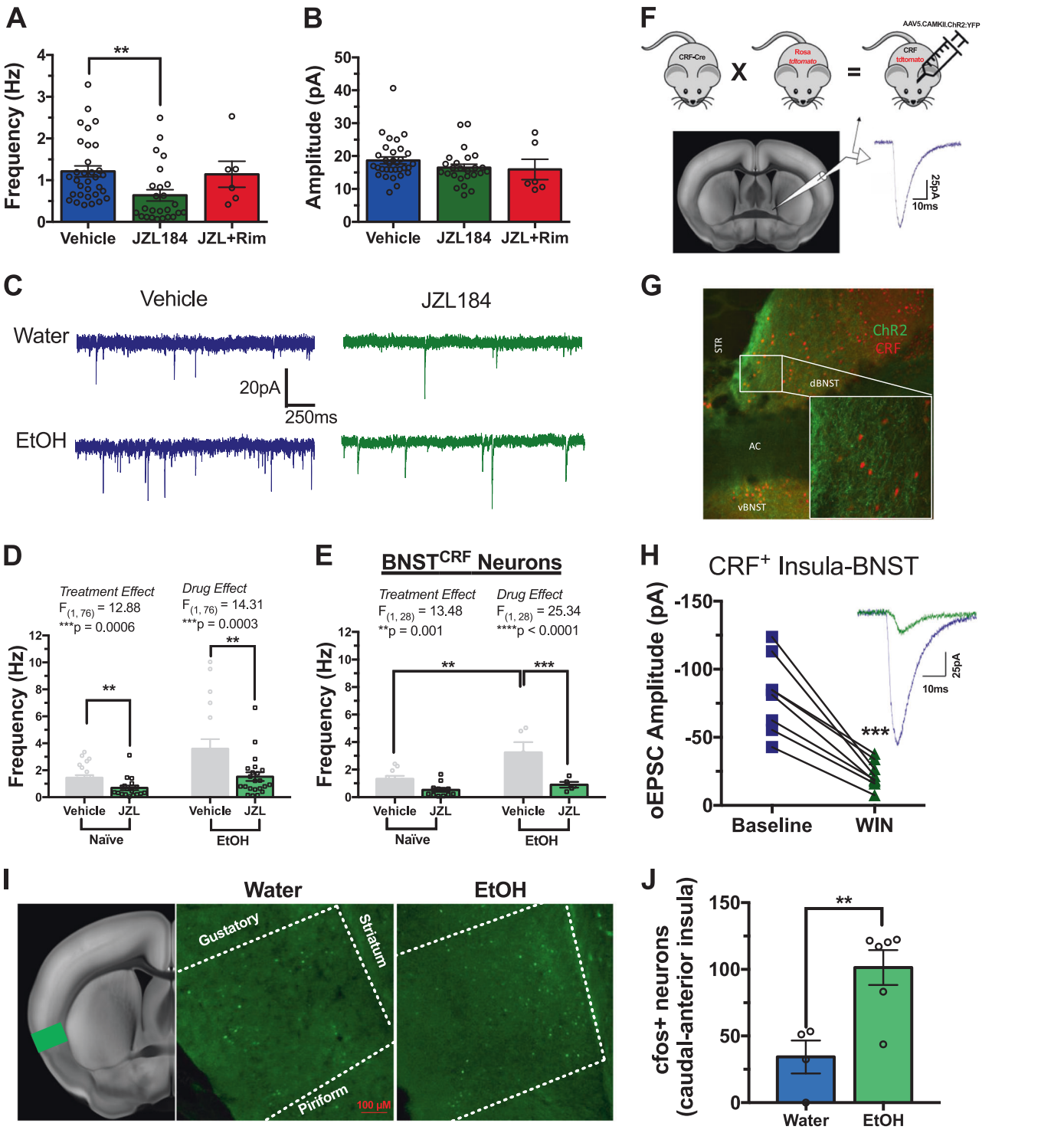
C57BL/6J mice were given 24 h access to ethanol and water for 6 weeks (Fig. 1a). Both male and female mice established a similar preference for ethanol (Fig. 1b), although female mice consumed significantly more than males throughout CDFA (repeated measures two-way ANOVA $F_{(1,15)} = 37.9$, $p < 0.0001$; Sidak's multiple comparison test: week 1, $p = 0.381$; week 2, $p = 0.005$; week 3, $p = 0.005$; week 4, $p = 0.002$; week 5, $p < 0.0001$; week 6, $p = 0.001$; Fig. 1c). Fifteen days into forced abstinence, mice were sacrificed and tissue sections containing the dBNST were processed to examine expression of the immediate early gene, c-fos, an indirect marker of neuronal activity. Two-way ANOVA revealed a significant effect of ethanol treatment on c-fos+ dBNST neurons/animal ($F_{(1,18)} = 14.7$, $p = 0.001$) and Sidak's post hoc confirmed that both male and female mice showed an increase in dBNST c-fos expression in abstinence (male: 30.9 ± 4.5 c-fos+ dBNST neurons/animal water versus 76.4 ± 10.1 c-fos+ dBNST neurons/animal ethanol, $p = 0.022$, female: 53.9 ± 8.4 c-fos+ dBNST neurons/animal water versus 85.4 ± 10.7 c-fos+ dBNST neurons/animal ethanol, $p = 0.036$; Fig. 1d, e). Of note, there was no observed sex difference in BNST c-fos expression ($F_{(1,18)} = 2.6$, $p = 0.127$). Because of the increased female consumption, previous studies from our lab and others demonstrating that ethanol abstinence leads to robust affective disturbances in females [21, 43, 50], and the higher prevalence of depression among the human female population [51], we focused the remainder of this study on female mice.

CDFA results in cell-type specific increases in dBNST Fos expression

CRF cells in the dBNST participate in behavioral responses to stressors [28, 32], regulate alcohol seeking [33–36], and negative affect [27]. Therefore, we also examined activity specifically within this population following CDFA. Fluorescent in situ hybridization determined that the percentage of BNST^{CRF} cells expressing the *Fos* transcript was significantly increased 15 days into abstinence in female mice (25.6 ± 4.3% water versus 73.6 ± 7.7% ethanol; $p = 0.003$; Fig. 1f, g), while total number of CRF cells in each group was not different (278.7 ± 54.2 cells per dBNST water versus 359.6 ± 41.6 cells per dBNST ethanol; $p = 0.28$; Fig. 1h). Of note, the percentage of *Fos*+ dBNST^{CRF-} cells was also increased in abstinence (8.8 ± 1.3% water versus 54.8 ± 11.2% ethanol; $p = 0.013$; Supplemental Figure 2A), suggesting CRF cells may only represent one type of dBNST neuron impacted by abstinence. Using acute brain slice whole-cell patch clamp electrophysiology, we assessed glutamatergic signaling onto dBNST neurons and dBNST^{CRF} neurons at the 15-day abstinence timepoint. AMPA-mediated spontaneous EPSC (sEPSC) frequency was significantly increased in unidentified dBNST neurons (1.4 ± 0.2 Hz naive versus 3.6 ± 0.7 Hz ethanol; $p = 0.001$; Fig. 1i, j), and in dBNST^{CRF} cells identified using CRF-*tomato* mice [29, 48] (1.3 ± 0.2 Hz naive versus 3.3 ± 0.7 Hz ethanol; $p = 0.001$; Fig. 1k). CDFA had no effect on sEPSC amplitude in either experiment (Supplemental Figure 1B–C).

JZL184 decreases spontaneous glutamate release in the BNST through increased 2-AG levels

Next, we determined the eCB sensitivity of excitatory drive on dBNST neurons. Acute slices from female mice were pretreated for



at least 1 h with vehicle (0.1% (v/v) DMSO), JZL184 (1 µM), or JZL184+rimonabant (CB_1R antagonist; 5 µM), and sEPSC frequency and amplitude were measured. There was a significant effect of treatment ($F_{(2,60)} = 4.7$, $p = 0.013$; Fig. 2a, b) and post hoc testing reported a significant decrease in sEPSC frequency of dBNST slices pretreated with JZL184 (1.2 ± 0.1 Hz vehicle versus 0.6 ± 0.1 Hz; $p = 0.011$), but no difference in JZL184+rimonabant (1.1 ± 0.3 Hz; $p = 0.975$). Neither JZL184 nor JZL184+rimonabant influenced sEPSC amplitude (Fig. 2b) suggesting the JZL184-induced increase in 2-AG is acting in a CB_1R-dependent mechanism to modulate presynaptic glutamate release. To determine the effect of systemic JZL184 on 2-AG levels in the BNST, female mice were given an i.p. injection of JZL184 (10 mg/kg) and lipidomic 2-AG analysis was conducted in BNST tissue 2 h later. JZL184 significantly increased BNST 2 AG levels (Supplemental Figure 2A), and decreased arachidonic acid levels (Supplemental Figure 2B), while having no effect on anandamide levels (Supplemental Figure 2C), suggesting systemic administrations of this compounds alters dBNST eCB metabolite levels.

Fig. 2 Insula inputs onto dBNST^{CRF} neurons are endocannabinoid sensitive. **a** sEPSC frequency in dBNST neurons in the presence of vehicle (0.1% (v/v) DMSO), JZL184 (1 μM), or JZL184+rimonabant (5 μM). Pretreating slices with JZL184 for at least 1 h significantly decreased sEPSC frequency (one-way ANOVA treatment effect, $*p < 0.05$; Tukey's multiple comparison post hoc $*p < 0.05$) and JZL184+rimonabant pretreatment had no effect on sEPSC frequency (Tukey's multiple comparison post hoc $p = 0.975$). Data presented individual data points with mean ± SEM. **b** Neither JZL184 alone nor JZL184+rimonabant had an effect on sEPSC amplitude (one-way ANOVA; $p = 0.284$). Data presented individual data points with mean ± SEM. **c** Representative electrophysiology trace from ethanol naive mice and CDFA mice 15 days into forced abstinence. Slices were treated with either vehicle (0.1% (v/v) DMSO) or JZL184 (1 μM). **d** Pretreating BNST slices for at least 1 h in JZL184 decreases sEPSC frequency in ethanol-naive mice (two-way ANOVA treatment effect, $***p < 0.001$; Tukey's multiple comparison post hoc, $**p < 0.01$) and prevents CDFA-induced increase in sEPSC frequency (drug effect, $***p < 0.001$; Tukey's multiple comparison post hoc $**p < 0.01$). Data in gray is presented and described in Fig. 1. Data presented individual data points with mean ± SEM. **e** Pretreating BNST slices from CRF-*tomato* mice in JZL184 decreases sEPSC frequency in ethanol-naive mice (two-way ANOVA treatment effect, $***p < 0.001$; Tukey's multiple comparison post hoc, $**p < 0.001$) and prevents CDFA-induced increase in sEPSC frequency (dark green line; drug effect, $****p < 0.0001$, Tukey's multiple comparison post hoc $***p < 0.001$). Data in gray is presented and described in Fig. 1. Data presented individual data points with mean ± SEM. **f** Schematic for CRF-*tomato* mouse breeding, ChR2 injection in the insula, and ChR2-evoked optical EPSCs in the BNST. Atlas image from Allen Brain Mouse Atlas [73]. **g** Representative image of CRF cells in the dBNST (red) and ChR2-labeled insula fibers in the dBNST (green). **h** Blue light stimulation evoked an optical EPSC in dBNST^{CRF} neurons that was significantly decreased after 10-min bath application of WIN55,212-2 (4 μM; Student's paired *t*-test, $***p < 0.001$). Data presented as individual cells before and after 10 min of WIN55,212-2. **i** Representative 10× image of c-fos protein staining in the caudal-anterior insula from water mice and ethanol mice 15-days into abstinence. Atlas image from Allen Brain Mouse Atlas [73]. **j** Mice 15-days into abstinence exhibit increased caudal-posterior insula c-fos expression relative to water control mice (Student's unpaired *t*-test, $**p < 0.01$). Data presented as cells per 10× caudal-posterior insula image per hemisphere and averaged per animal (presented as individual data points and mean ± SEM)

JZL184 prevents CDFA-induced increases in dBNST spontaneous glutamatergic neurotransmission

Next, we determined whether JZL184 could prevent the CDFA-induced increase in glutamate release onto dBNST neurons. Acute slices containing dBNST from female mice 15 days into abstinence were pretreated with vehicle or JZL184 and sEPSCs were measured. JZL184 pretreatment prevented the abstinence-induced increase in sEPSC frequency in unidentified dBNST neurons (treatment effect $F_{(1,76)} = 12.9$, $p < 0.001$; drug effect $F_{(1,76)} = 14.3$, $p < 0.001$; 3.6 ± 0.7 Hz ethanol-vehicle versus 1.5 ± 0.3 Hz ethanol-JZL184, $p = 0.002$; Fig. 2c, d) and specifically in dBNST^{CRF} cells (treatment effect $F_{(1,28)} = 13.5$, $p = 0.001$; drug effect $F_{(1,28)} = 25.3$, $p < 0.0001$; 3.3 ± 0.7 Hz ethanol-vehicle versus 0.9 ± 0.2 Hz ethanol-JZL184, $p < 0.001$; Fig. 2e). CDFA had no effect on sEPSC amplitude in either experiment (Supplemental Figure 1A-B) suggesting postsynaptic glutamate receptors are not directly altered in abstinence.

Insula inputs onto dBNST^{CRF} neurons are suppressed by CB_1R activation

The abstinence-induced increase in *Fos* expression coupled with an increase in sEPSC frequency suggests a hyperactive, eCB-sensitive glutamatergic input to the dBNST drives abstinence-induced alterations in BNST microcircuitry. The insula is highly involved in interoception and affective behaviors and plays a key role in addiction-related negative affect [45, 52, 53]. The insula sends dense glutamatergic input to the dBNST [54], therefore the functionality of this input specifically onto dBNST^{CRF} cells was assessed. AAV5.CaMKII.ChR2 (ChR2) was stereotaxically injected into the caudal anterior insula of female *CRF-tomato* mice (Fig. 2f–h). Four to six weeks later, whole-cell patch clamp recordings were conducted in dBNST^{CRF} cells. Blue-light stimulation of the ChR2-expressing insula afferents in the dBNST produced an optically-evoked EPSC (oEPSC) in 80% of dBNST^{CRF} cells suggesting a high functional insula input to dBNST^{CRF} cells. To determine whether this input is eCB-sensitive, the CB_1R agonist WIN55,212-2 (4 μM) was bath applied to dBNST^{CRF} cells that produced stable oEPSCs for 10 min. Following 10 min of WIN55,212-2, oEPSC amplitude was significantly reduced (−81.1 ± 9.8 pA baseline versus −22.6 ± 3.5 pA WIN55,212-2; $t_{(7)} = 7.0$, $p < 0.001$; Fig. 2f–h). Taken together, insula afferents onto dBNST^{CRF} cells are eCB-sensitive and in turn, a possible source of the observed abstinence-induced increase in sEPSC frequency. To evaluate neuronal activity in the insula, c-fos expression was measured on day 15 of abstinence. Caudal-anterior insula c-fos was significantly increased 15-days into abstinence in female mice ($t_{(8)} = 3.53$, $p = 0.008$; 34.3 ± 12.4 c-fos+ caudal-anterior insula neurons/animal water versus 101.3 ± 13.0 c-fos+ caudal-anterior insula neurons/animal ethanol; Fig. 2i, j).

Activating insula hM4Di blocks CDFA-induced hyperactivity in dBNST^{CRF} cells

Given the functional connectivity between the insula and dBNST^{CRF} cells and the ability of JZL184 to mitigate abstinence-induced increases in dBNST sEPSC frequency, and the abstinence-induced increase in insula c-fos expression, the next experiment tested the role of the insula in modulating neuronal hyperactivity in the dBNST. Presynaptic CB_1R are widely expressed on glutamatergic terminals in the dBNST [12, 24, 26], originating from a variety of dBNST inputs [25]. CB_1R act through G_i-coupled GPCR signaling pathways. To isolate insula inputs onto dBNST neurons, we stereotaxically injected the G_i-coupled DREADD, hM4Di bilaterally into the insula of female mice 1 week prior to CDFA (Fig. 3a), thus modeling G_i-coupled CB_1R-expressing insula terminals in the dBNST. Neither the stereotaxic surgery, nor the presence of hM4Di, had an effect on ethanol preference (Fig. 3b) or consumption (Fig. 3c). Fifteen days into forced abstinence, mice were treated with CNO (3 mg/kg) and processed for in situ hybridization 2 h later. In agreement with Fig. 1d, e, the number of *Fos*+ dBNST neurons was significantly increased in abstinence ($F_{(2,10)} = 11.4$, $p = 0.003$; 148.5 ± 37.9 dBNST neurons/animal water-saline versus 411.7 ± 47.9 dBNST neurons/animal ethanol-saline $p = 0.001$; Fig. 3d). CNO-injected CDFA mice had fewer dBNST *Fos*+ neurons relative to ethanol-saline treatment (247.2 ± 20.5 dBNST neurons/animal; $p = 0.021$; Fig. 3d). Further cell-type specific analysis confirmed that the effect can be attributed, in part, to a decrease in percentage of *Fos*-expressing dBNST^{CRF} cells ($F_{(2,10)} = 12.4$, $p = 0.002$; 73.6 ± 7.7% ethanol-saline versus 40.6 ± 6.1% ethanol-CNO $p = 0.01$; Fig. 3e). The effect of CNO on dBNST^{CRF-} neurons trended towards a significant decrease (54.8 ± 11.2% ethanol-saline versus 26.6 ± 5.3% ethanol-CNO $p = 0.053$; Supplemental Figure 3) suggesting that while dBNST^{CRF} cells represent a specific population of dBNST neurons impacted by CDFA, other cell types may be involved.

Activating insula hM4Di reduces abstinence-induced increase in dBNST glutamatergic signaling

The next experiment used the same chemogenetic strategy as above to determine the effect of insula hM4Di on dBNST synaptic physiology. hM4Di was injected bilaterally into the insula of female mice prior to CDFA. Whole-cell patch clamp recordings

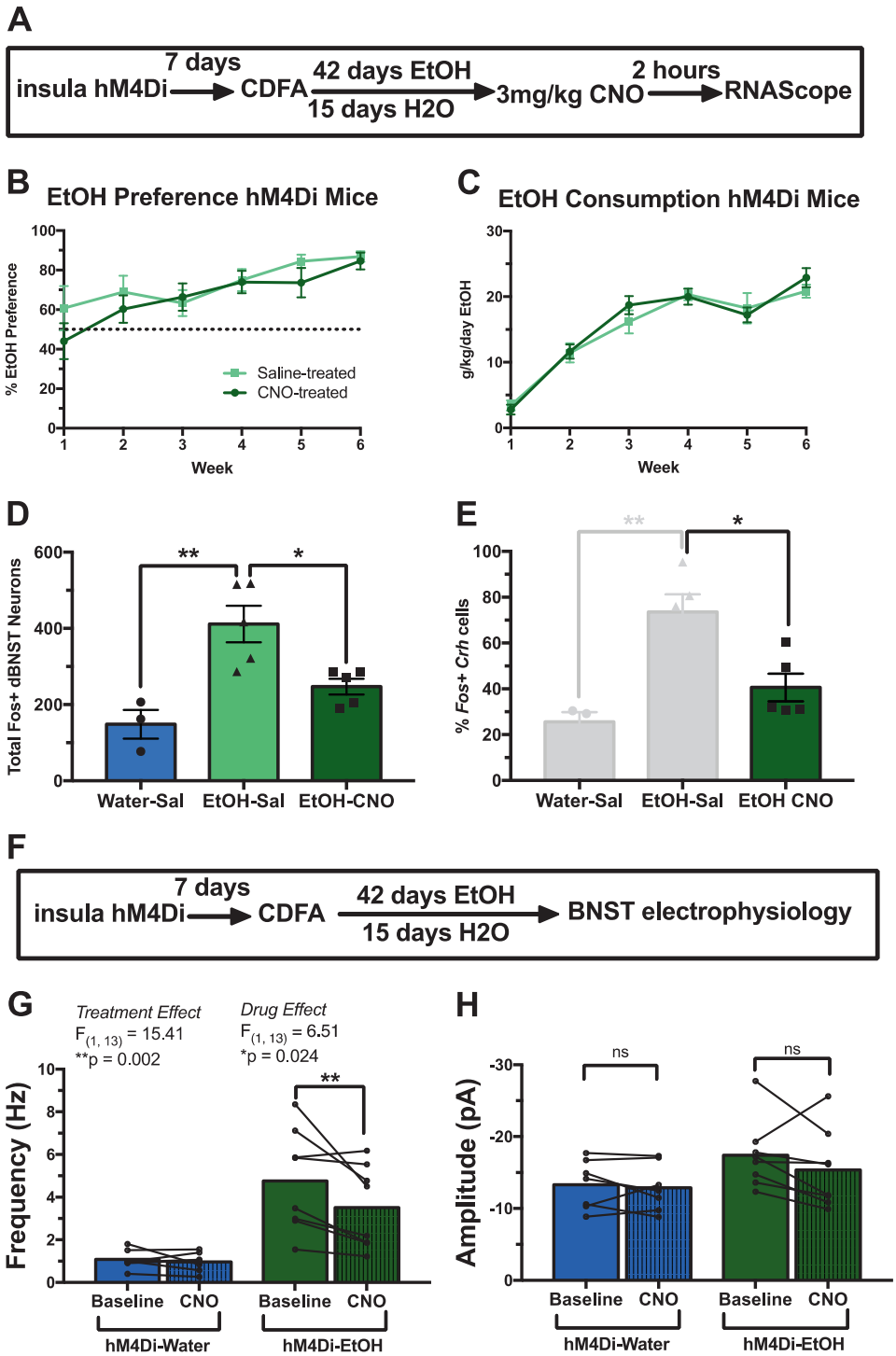


Fig. 3 Chemogenetic inhibition of insula neurons reduces CDFA-induced increase in neuronal activity. **a** Timeline for insula hM4Di RNAScope® experiment. **b** Ethanol preference for all insula hM4Di mice treated with either saline (light green) or CNO (dark green). **c** Ethanol consumption (g/kg/day) for all insula hM4Di mice treated with either saline (light green) or CNO (dark green). **d** Total number of *Fos*+ BNST neurons. CDFA significantly increases *Fos* expression in the BNST 15-days into abstinence (one-way ANOVA, $^{**}p < 0.01$; Tukey's multiple comparison post hoc, $^{**}p < 0.01$). CNO (3 mg/kg) activation of the insula hM4Di significantly reduced *Fos* expression in the BNST 15-days into ethanol abstinence (Tukey's multiple comparison post hoc, $^{**}p < 0.01$). **e** CNO activation of the hM4Di in the insula significantly reduced the percentage of *Fos*+ $BNST^{CRF}$ neurons 15-days into ethanol abstinence (one-way ANOVA, $^{*}p < 0.05$; Tukey's multiple comparison post hoc, $^{*}p < 0.05$). Data in gray is presented and described in Fig. 1. **f** Schematic for electrophysiology experimental timeline. hM4Di was injected into both caudal-anterior insulae prior to CDFA. sEPSC were recorded in BNST neurons before and after bath application of CNO (10 µM). **g** sEPSC frequency is significantly increased 15-days into EtOH abstinence. Bath application of CNO significantly reduced sEPSC frequency in ethanol abstinent mice but not in water mice (two-way ANOVA; treatment effect $^{**}p < 0.01$; drug effect $^{*}p < 0.05$; Sidak's multiple comparison post hoc, $^{**}p < 0.01$). Data presented as individual data points before and after drug application. **h** CDFA had no effect on sEPSC amplitude in BNST neurons. CNO did not alter sEPSC amplitude

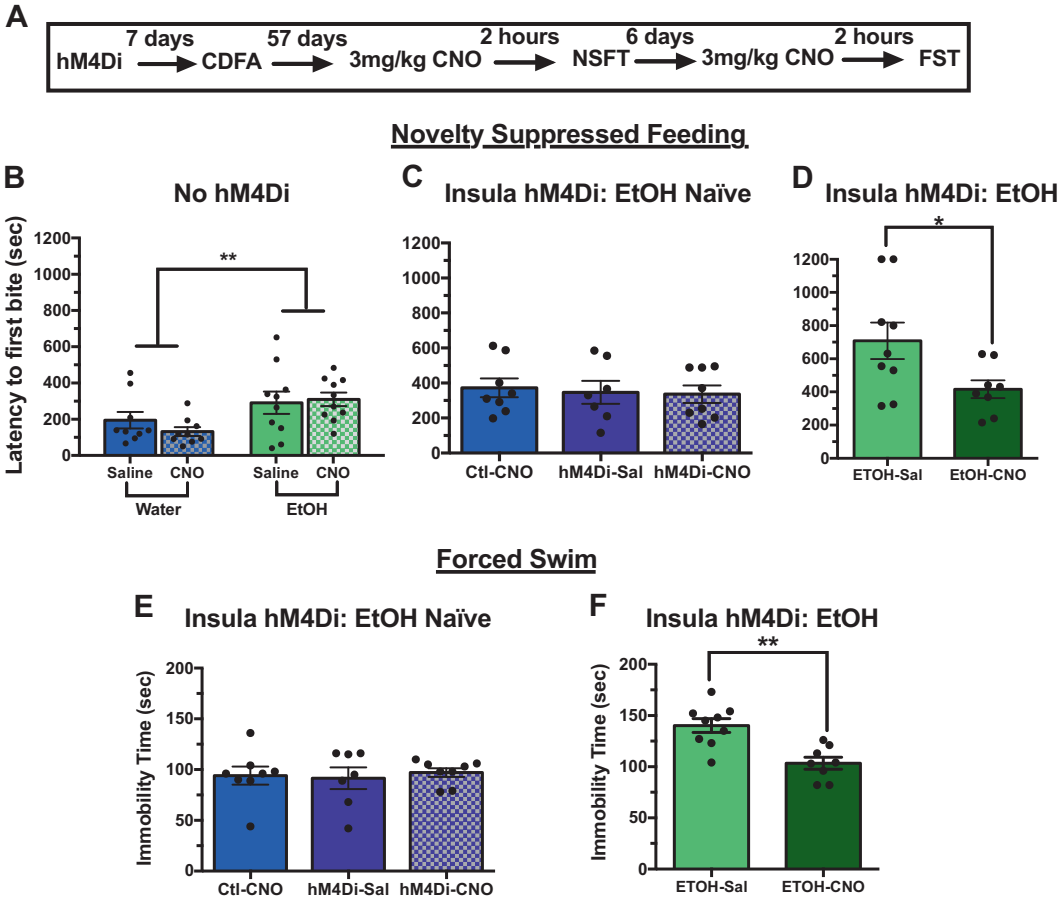


Fig. 4 Chemogenetic inhibition of insula neurons decreases abstinence-induced negative affect. **a** Experimental timeline for CDFA behavioral experiments in (**d**) and (**f**). **b** CNO (3 mg/kg) in the absence of hM4Di has no effect on CDFA-induced increase in latency to first bite on NSFT (two-way ANOVA treatment effect $^{**}p < 0.01$, Tukey's multiple comparison post hoc water-sal versus water-CNO, $p = 0.773$; ethanol-sal versus ethanol-CNO, $p = 0.991$). **c** Latency to feed on NSFT is not different between ethanol-naive mice, No-hM4Di, CNO-treated (3 mg/kg) mice (Ctl-CNO), ethanol-naive insula hM4Di, saline-treated mice (hM4Di-Sal), and ethanol-naive insula hM4Di, CNO-treated (3 mg/kg) mice (hM4Di-CNO). **d** CNO administered to insula hM4Di mice 15-days into the abstinence phase of CDFA results in a significant reduction in latency to first bite on NSFT (Student's unpaired *t*-test, $^{*}p < 0.05$). **e** Forced swim test immobility time is not different between ethanol-naive mice, No-hM4Di, CNO-treated mice (Ctl-CNO), ethanol-naive insula hM4Di, saline-treated mice (hM4Di-Sal), and ethanol-naive insula hM4Di, CNO-treated mice (hM4Di-CNO). **f** Immobility time during FST was significantly decreased in CNO-treated insula hM4Di mice relative to saline-treated mice (Student's unpaired *t*-test, $^{*}p < 0.05$). All data presented as individual data points with mean ± SEM

from dBNST neurons were conducted 15-days into abstinence. In agreement with Fig. 1j, sEPSC frequency was significantly increased in ethanol-abstinent mice (treatment effect $F_{(1,13)} = 15.4$, $p = 0.002$; Fig. 3g). Bath application of CNO (10 µM) significantly decreased sEPSC frequency in the ethanol-hM4Di group (4.8 ± 0.8 Hz baseline versus 3.5 ± 0.7 Hz CNO; $p = 0.009$; Fig. 3g) but not the water-hM4Di group (1.1 ± 0.2 Hz baseline versus 1.0 ± 0.2 Hz CNO; $p = 0.946$; Fig. 3g). CNO did not have an effect on sEPSC amplitude in either the water or the ethanol group (Fig. 3h). Additionally, bath application of CNO had no effect on sEPSC frequency or amplitude in naive or ethanol mice without insula hM4Di (Supplemental Figure 4A–B).

Activating insula hM4Di prevents abstinence-induced negative affective symptoms

We and others have previously demonstrated that CDFA reliably produces enhanced negative affective behavior in female mice in prolonged abstinence [21, 43, 50] that can be prevented by acute JZL184 treatment [21]. To assess the role of CB_1R-like signaling in insular neurons in abstinence-induced negative affect, hM4Di was injected into the insula of female C57BL/6J prior to CDFA. Beginning at abstinence day 15, negative affective symptoms were assessed using the novelty-suppressed feeding task (NSFT) [21, 43, 50]. Mice were treated with CNO (3 mg/kg) 2 h before testing. In the absence of hM4Di, two-way ANOVA revealed a significant effect of ethanol versus water treatment ($F_{(1,34)} = 9.1$, $p = 0.005$) but no effect of CNO on the latency to feed ($F_{(1,34)} = 0.2$, $p = 0.625$; Fig. 4b) or consumption 10 min after NSFT (one-way ANOVA, $F_{(3,14)} = 2.5$, $p = 0.103$; Supplemental Figure 5A). In hM4Di ethanol-naive mice, latency to feed was not significantly different between groups ($F_{(2,20)} = 0.11$, $p = 0.894$; No-hM4Di-CNO 372.3 ± 54.1 s versus hM4Di-Sal 346.6 ± 65.4 s versus hM4Di-CNO 336.4 ± 49.6 s; Fig. 4c). In CDFA mice, latency to feed was significantly decreased in CNO-treated mice relative to saline (708.6 ± 109.8 s ethanol-saline versus 416.9 ± 53.9 s ethanol-CNO; $t_{(15)} = 2.29$, $p = 0.037$; Fig. 4d). Consumption 10 min post-NSFT was not different between any groups in any of the experiments (Supplemental Figure 5). Next, mice were tested on an additional behavioral assessment for negative affect, the forced swim test (FST) [55, 56]. CNO had no effect on immobility time in insula hM4Di, ethanol-naive mice ($F_{(2,20)} = 0.11$, $p = 0.894$; no-hM4Di-CNO 94.1 ± 8.9 s versus hM4Di-Sal 91.6 ± 10.7 s versus hM4Di-CNO 97.1 ± 4.3 s; Fig. 4e). CDFA insula-hM4Di mice were treated with CNO 6 days after NSFT (abstinence day 21) showed significantly

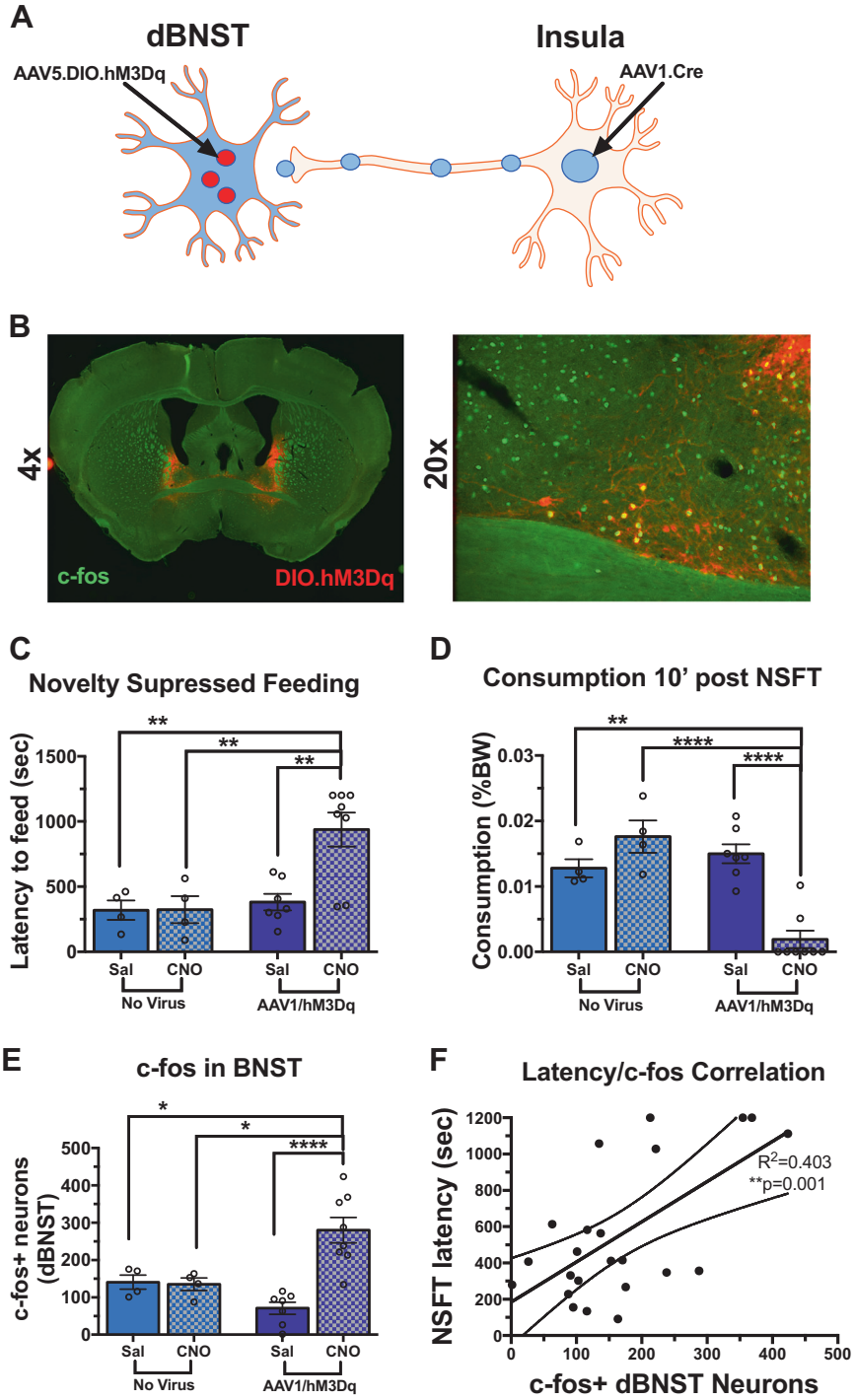


Fig. 5 Chemogenetic activation of dBNST neurons receiving projections from the insula produces a negative affect phenotype. **a** Anterograde transsynaptic Cre (AAV1.Cre) was injected into both caudal-anterior insulae. Cre-dependent hM3Dq (AAV5.DIO.hM3Dq) was injected into both dBNST. **b** Representative 4× (left) and 20× image of DIO.hM3Dq (red) and c-fos (green) expression in the dBNST. **c** CNO (3 mg/kg) significantly increased latency to first bite in the AAV1.Cre/hM3Dq mice relative to the no-virus control groups and AAV1.Cre/hM3Dq-saline group (Tukey's multiple comparison post hoc, $^{**}p < 0.01$). **d** CNO (3 mg/kg) significantly decreased 10′ post-NSFT home cage consumption in AAV1.Cre/hM3Dq mice relative to the no-virus control groups and AAV1.Cre/hM3Dq-saline group (Tukey's multiple comparison post hoc, $^{**}p < 0.01$, $^{****}p < 0.0001$). **e** CNO (3 mg/kg) significantly increased BNST c-fos+ neurons in the AAV1.Cre/hM3Dq mice relative to the no-virus control groups and AAV1.Cre/hM3Dq saline group (Tukey's multiple comparison post hoc, $^{**}p < 0.01$). Data presented as cells per 10× dBNST image per hemisphere and averaged per animal (presented as individual data points and mean ± SEM). **f** Latency to first bite on NSFT positively correlates with number of c-fos+ dBNST neurons ($R^2 = 0.403$, $^{**}p = 0.001$). Data presented as linear regression best fit line with 95% confidence interval bands

reduced immobility time (140.1 ± 6.7 s ethanol-saline versus 103.4 ± 5.8 s ethanol-CNO; $t_{(15)} = 4.1$, $p = 0.001$; Fig. 4f). Collectively, activation of CB_1R-like signaling specifically within insula neurons decreased prolonged abstinence-induced negative affective behaviors.

Chemogenetically activating insula-controlled BNST neurons produces a negative affective phenotype

The data thus far suggest a role for the insula in mediating abstinence-induced negative affect. The next set of experiments sought to directly assess the role of dBNST neurons that receive insula inputs ($dBNST^{insula}$) in negative affective behaviors. To isolate $dBNST^{insula}$ neurons, we used a novel viral anterograde transsynaptic gene transfer strategy [47]. AAV1.Cre was injected bilaterally into the insula, and a Cre-dependent, DIO.hM3Dq virus was injected into the dBNST of female mice, thereby activating only those cells in the dBNST that receive projections from the insula (Fig. 5a). This strategy produced robust expression of hM3Dq in BNST, particularly in dorsal anterolateral portions. In the absence of insula AAV1.Cre, we did not observe any DIO.hM3Dq expression (Supplemental Figure 6), highlighting the fidelity of this approach. The role of $dBNST^{insula}$ neurons in negative affective behaviors was assessed using NSFT. As in Fig. 4, CNO (3 mg/kg) or saline was administered systemically 2 h prior to NSFT. AAV1/hM3Dq CNO mice exhibited a robust increase in latency to first bite relative to the control groups ($F_{(3,19)} = 8.8$, $p = 0.001$; AAV1/hM3Dq-CNO 937.5 ± 130 s versus no-virus-saline 319.5 ± 74.5 s, $p = 0.006$, AAV1/hM3Dq-CNO versus no-virus-CNO 323.3 ± 103.4 s, $p = 0.006$, AAV1/hM3Dq-CNO versus AAV1/hM3Dq-saline 381.3 ± 62.7 s, $p = 0.003$; Fig. 5b, c). AAV1/hM3Dq mice also consumed significantly less food in the home cage 10 min immediately after NSFT ($F_{(3,19)} = 21.1$, $p < 0.0001$; AAV1/hM3Dq-CNO 0.002 ± 0.001% b.w. versus no-virus saline 0.013 ± 0.001% b.w. $p = 0.001$, AAV1/hM3Dq-CNO versus no-virus-CNO 0.018 ± 0.002% b.w., $p < 0.0001$, AAV1/hM3Dq-CNO versus AAV1/hM3Dq-saline 0.015 ± 0.001% b.w., $p < 0.0001$; Fig. 5b–d).

Immediately following the 10-min post-NSFT consumption period, mice were sacrificed and brain tissue was processed for c-fos immunohistochemistry. In agreement with the behavioral phenotype, the number of c-fos+ dBNST neurons was significantly increased in the AAV1/hM3Dq-CNO group relative to the control groups ($F_{(3,19)} = 13.1$, $p = 0.014$; AAV1/hM3Dq-CNO 280.1 ± 34.0 c-fos+ dBNST neurons/animal versus no-virus-saline 140.8 ± 18.9 c-fos+ dBNST neurons/animal $p = 0.010$, AAV1/hM3Dq-CNO versus no-virus-CNO 135.3 ± 16.8 c-fos+ dBNST neurons/animal, $p < 0.0001$, AAV1/hM3Dq-CNO versus AAV1/hM3Dq-saline 70.9 ± 16.1 c-fos+ dBNST neurons/animal, $p < 0.0001$; Fig. 5b, e). Linear regression analysis determined a significant correlation between the latency to feed on NSFT versus c-fos+ dBNST neurons/animal (Goodness of Fit $R^2 = 0.403$, $p = 0.001$; Fig. 5f) providing evidence to support the involvement of the dBNST in regulating negative affective behaviors using the NSFT.

DISCUSSION

Protracted abstinence is arguably the most challenging period to treat a recovering addict. While the initial withdrawal phase is marked by distinct stages that persist for a relatively finite period of time (approximately 5–7 days [57]), the abstinence phase can vary greatly between individuals, and the cravings and drive to relapse do not necessarily decrease over time [2, 58]. The inability to cope with stress and negative affective symptoms are commonly listed as triggers of cravings and relapse, and indeed, abstinence itself is often described as a persistent chronic stressor. In the present study, we address this problem by identifying insula-BNST circuitry as key responders to prolonged ethanol abstinence. While novel therapeutics such as NMDA receptor antagonists (e.g., ketamine) and opioids have shown recent promise for treating negative affect [21, 43, 59], therapeutics targeting the eCB system may represent a safer alternative treatment with low abuse liability [60]. Administering the MAG lipase inhibitor JZL184 15 days into ethanol abstinence alleviated hyperactive neuronal activity in the dBNST consistent with the emerging literature on the large therapeutic potential of the eCB system for treating affective disorders (for review see [61, 62]). Using a chemogenetic approach to anatomically limit eCB-like signaling, hM4Di activation in insula neurons was sufficient to prevent ethanol abstinence-induced hyperactivity of dBNST cells, similar to systemic JZL184. Moreover, hM3Dq activation specifically in $dBNST^{insula}$ neurons produced a robust negative affect phenotype and a strong dBNST c-fos response. These results not only highlight a critical role for the insula in ethanol abstinence, but also identify a strong, functional, eCB sensitive input to dBNST cells that regulates dBNST neuronal activity.

Using c-fos as proxy for neuronal activity in abstinence

The immediate early gene c-fos can be used as a crude surrogate marker of in vivo neuronal activity brought about by a discrete stimulus [63, 64]. In the CDFA model, significant c-fos protein expression was detected in the dBNST and insula 15 days into abstinence without a discrete stimulus (Figs. 1d, e and 2i, j), fitting with the hypothesis that prolonged abstinence is a persistent stressor. This finding is not without precedent, as studies from Harris and Aston-Jones reported increased dBNST c-fos expression in prolonged abstinence following chronic morphine [65, 66]. We extend this observation to show this specifically within $dBNST^{CRF}$ cells and demonstrate a means of modulating this activity. Interestingly, a similar trend for CDFA-induced increase in c-fos was also observed in $dBNST^{CRF-}$ cells, suggesting that while $dBNST^{CRF}$ cells may not be the sole contributor to the CDFA-induced phenotype in the dBNST, they represent a specific cell type affected by CDFA. Whether these $dBNST^{CRF-}$ cells are separate distinguishable population of dBNST neurons or the product of microcircuit regulation from $dBNST^{CRF}$ cells should be addressed in future studies.

Potential role for eCB signaling in the dBNST in negative affective behaviors

The CDFA-induced increase in dBNST c-fos expression, coupled with an increase in sEPSC frequency suggests dBNST hyperactivity 15 days into prolonged ethanol abstinence. eCBs are synthesized as a result of a postsynaptic response to neurotransmitter release and act presynaptically at G_i-coupled CB_1R to quell further neurotransmitter release [67]. Compounds acting directly at CB_1R are accompanied by a variety of off-target effects, therefore in this study, we used JZL184, an inhibitor of the 2-AG degradation enzyme MAG lipase, which enhances levels of the endogenous ligand for CB_1R [15, 68, 69], and prevents affective behaviors [13, 14, 16]. We recently reported that JZL184 prevents CDFA-induced negative affective behaviors [21], and the data presented here suggest the compound is acting, at least in part, through actions in the dBNST, as the CDFA-induced increase in sEPSC frequency was prevented by JZL184 (Fig. 2).

Insula-BNST circuitry in negative affect

While the dBNST is highly interconnected with multiple nodes of affective circuitry, the insula input onto $dBNST^{CRF}$ neurons was targeted for several reasons. The insula provides interoceptive cues through neuronal projections to cortical and subcortical brain regions, including dense projections to the dBNST [54]. It functions to guide future actions by incorporating internal and external states to predict the outcome of a potential behavior. The observed eCB-sensitivity of this input to the dBNST provides further evidence linking this circuit to negative affective behaviors. The insula could accomplish this through two potentially related

processes. A hyperactive insula suggests a magnification of incentive representation and heightened internal awareness which could lead to a negative affective state, while a hypoactive insula may signify a discounting of risk representation. Either outcome would plague individuals afflicted with an AUD and contribute to an impaired ability to control ethanol seeking despite facing a high probability of deleterious ramifications. While the observed increases in insula and dBNST *Fos* and dBNST sEPSC frequency suggest increased insula-BNST circuit activity, whether this is the result of increased excitatory glutamate drive or a downregulation of inhibitory GABA neurotransmission is unknown. In future studies, it will be important to assess the functional state of the insular neurons that project to the dBNST.

The use of chemogenetics to mimic CB_1R actions in insula-BNST neural circuit

CB_1R are presynaptically-localized and widely expressed throughout the brain, making it difficult to isolate the role of eCB signaling in a specific pathway. To overcome this, we utilized chemogenetics to mimic insula neuron CB_1R. CNO-facilitated hM4Di inhibition of insula neurons prevented abstinence-induced increases in $dBNST^{CRF}$ neurons expressing *Fos* (Fig. 3). Accordingly, activating hM4Di in insula terminals in the dBNST reduced the CDFA-induced increase in sEPSC frequency, closely resembling the observed effect using JZL184 (Fig. 2). To our knowledge, this is the first functional evidence for insula modulation of dBNST neuronal activity and suggests a key role for insula-guided afferents expressing G_i-coupled receptors in the dBNST.

The role of the insula in producing CDFA-induced negative affect was also assessed. CNO significantly reduced negative affective behaviors assessed using NSFT and FST, thus highlighting an important role for the insula in mediating affective behaviors. This data closely mirrors previously reported data showing JZL184 mitigates abstinence-induced negative affect [21], collectively suggesting the negative affect-relieving effect of JZL184 may be acting at insula afferents in the dBNST. It is important to note that off-target effects of CNO (converted to clozapine) have previously been observed [70, 71] and were carefully considered in this study, as we observed no effects of CNO in the absence of hM4Di on sEPSC frequency (Supplemental Figure 4A), or the observed behavioral phenotype (Fig. 4b, c, e).

Using viral-mediated gene transfer and chemogenetics to isolate $BNST^{insula}$ neurons

Recent advances in viral-mediated gene transfer have allowed for precise isolation of discrete populations of neurons [47]. Here we used an anterograde transsynaptic Cre virus, to isolate $dBNST^{insula}$ neurons. Injecting Cre-dependent DIO.hM3Dq into the dBNST allowed for exclusive activation of this neuronal population. In doing so, CNO facilitated a robust increase in latency to feed on NSFT (Fig. 5c), in essence modeling the negative affective phenotype consistently observed in abstinence following CDFA. Interestingly, home cage food consumption after NSFT was also significantly decreased in the AAV1/hM3Dq-CNO group (Fig. 5d). While we interpret this phenotype as heightened anxiety-induced appetite suppression, the possibility of general, anxiety independent appetite suppression driving this phenotype cannot be completely ruled out. Mazzone et al., recently demonstrated that activating hM3Dq in BNST-GABA neurons produces an anxiolytic phenotype, supporting our interpretation and suggesting the $dBNST^{insula}$ neurons may be a subclass of BNST-GABA neurons [72]. In addition, a strong correlation between dBNST c-fos and NSFT latency highlights an integral role for the dBNST in this task. (Fig. 5f). Taken together, this experiment provides foundational evidence for a role for $dBNST^{insula}$ neurons in negative affect and future studies will aim to further characterize these distinct population neurons.

CONCLUSION

We characterize a distinct role for the dBNST in mediating negative affect in prolonged ethanol abstinence. Our data provide the framework to implement eCB-based pharmacotherapies as a reliable alternative treatment option for affective disorders, as enhancing 2-AG was sufficient to prevent abstinence-induced hyperactivity in the dBNST, a brain area historically linked to affective disturbances. Further, we provide evidence for the involvement of the insula in negative affective behaviors. Activating hM4Di in insula neurons effectively prevented the CDFA phenotype in the dBNST and prevented prolonged abstinence-induced negative affective behaviors. Lastly, we directly test the importance of the insula-BNST synapse in negative affective behaviors using a novel viral genetic strategy. Activating $dBNST^{insula}$ neurons with DIO.hM3Dq modeled both the CDFA-induced negative affect and dBNST c-fos phenotype, directly implicating these neurons in mediating negative affect. The results presented here pave the way for future studies to further tease apart the complete affective circuitry involving the insula-BNST pathway, and will facilitate novel targeted treatment strategies for affective disorders.

FUNDING AND DISCLOSURE

The authors declare no financial interests or protentional conflict of interest. This work was supported by National Institutes of Health Grants R37AA019455 (DGW) and R01AA026186 (SP). Imaging and image data analyses were performed in part through the use of the Vanderbilt University School of Medicine Cell Imaging Shared Resource (supported by NIH Grants CA68485, DK20593, DK58404, DK59637, and EY08126). Behavioral studies were carried out at the Vanderbilt Neurobehavioral Core Facility Research (supported by the EKS NICHD of the NIH under Award U54HD083211). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We thank Elana Milano for technical assistance.

ADDITIONAL INFORMATION

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41386-018-0257-8).

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