Title: Chemogenetic inhibition of a monosynaptic projection from the basolateral amygdala to the ventral hippocampus reduces appetitive and consummatory alcohol drinking behaviors

Abbreviated Title: Amygdala-hippocampal circuitry in alcohol drinking

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HIGHLIGHTS

- The basolateral amygdala sends a monosynaptic projection to the ventral hippocampus
- Inhibiting this circuit reduces anxiety-like behaviors in male Long Evans rats
- Inhibition of this pathway also decreases operant alcohol drinking-related behaviors

ABSTRACT

Alcohol use disorder (AUD) and anxiety/stressor disorders frequently co-occur and this dual diagnosis represents a major health and economic problem worldwide. The basolateral amygdala (BLA) is a key brain region that is known to contribute to the etiology of both disorders. Although many studies have implicated BLA hyperexcitability in the pathogenesis of AUD and comorbid conditions, relatively little is known about the specific efferent projections from the BLA that contribute to these disorders. Recent optogenetic studies have shown that the BLA sends a strong monosynaptic excitatory projection to the ventral hippocampus (vHC) and that this circuit modulates anxiety- and fear-related behaviors. However, it is not known if this pathway influences alcohol drinking. Here, we employed a rodent operant drinking regimen that procedurally separates appetitive (seeking) and consummatory (intake) behaviors, chemogenetics, and brain region-specific microinjections, to determine if BLA-vHC circuitry influences alcohol drinking-related behaviors. We first confirmed prior optogenetic findings that silencing this circuit reduced anxiety-like behaviors on the elevated plus-maze. We then demonstrated that inhibiting the BLA-vHC pathway significantly reduced both appetitive and consummatory alcohol drinking behaviors. Sucrose seeking and intake were also reduced following chemogenetic inhibition of this circuit, albeit to a lesser extent than alcohol drinking measures. Taken together, these findings provide the first indication that a BLA-vHC circuit may regulate both appetitive and consummatory alcohol drinking behaviors and add to a growing body of evidence suggesting that dysregulation of this pathway may contribute to the pathophysiology of AUD and anxiety/stressor-related disorders.

1.0 INTRODUCTION

Alcohol use and misuse represent major health and socioeconomic concerns in the United States and around the world. A recent analysis from the World Health Organization found that nearly 6% of all global deaths could be attributed to alcohol and the 2016 Surgeon General's Report on Alcohol, Drugs and Health noted that alcohol contributes to 88,000 deaths each year in the United States at a cost of almost $250 billion to our economy [1-3]. Despite these devastating numbers, some populations are particularly vulnerable to alcohol's deleterious health effects. One notable group is individuals diagnosed with anxiety and stressor-related disorders, like generalized anxiety disorder and post-traumatic stress disorder (PTSD). For example, 40-60% of individuals with post-traumatic stress disorder (PTSD) also suffer from alcohol use disorder (AUD) [4-6]. Moreover, these comorbidities are associated with greater symptom severity for each disorder and a much poorer prognosis in recovery [7-9]. Importantly, despite the high prevalence of these comorbid conditions, there is marked variability in the likelihood of developing these disorders [10,11] and very little is known about the specific neural circuits that might confer vulnerability or resilience to these diseases [4].

One possible reason for the frequent co-occurrence of these disorders is that their etiology may involve maladaptive changes in common neural pathways. For example, many studies have reported that both AUD and PTSD are associated with dysregulated activity and connectivity in circuits that include the prefrontal cortex, amygdala, and hippocampus [12-15]. Notably, recent studies have demonstrated that these brain regions are not homogenous structures but, rather, are comprised of distinct populations of cells with unique afferent and efferent connectivity that often encode behaviors with opposing valence [16,17]. A better understanding of the pathophysiology of AUD and comorbid affective disorders will require clear insight into the specific circuits that drive the maladaptive behaviors associated with these diseases.

One circuit that has received considerable attention, of late, involves a monosynaptic projection from glutamatergic pyramidal cells in the basolateral amygdala (BLA) to the CA1 region of the ventral hippocampus (vHC). Recent optogenetic studies have demonstrated that activation of this circuit increases unconditioned anxiety-like behaviors and reduces social interaction in mice and that silencing this pathway promotes anxiolysis [18,19]. Moreover, optogenetic inhibition of the BLA-vHC circuit disrupts footshock, but not contextual, fear learning in rats [20]. Despite the important role that this pathway plays in a number of behaviors associated with AUD and comorbid conditions, whether this pathway can modulate alcohol drinking is not known. To that end, we integrated DREADDs and brain region-specific microinjection methods to assess the effect of chemogenetic silencing of the BLA-vHC circuit on alcohol drinking behaviors using a limited-access operant regimen that procedurally separates appetitive and consummatory drinking behaviors.

2. METHODS

2.1 Subjects: Three separate cohorts of 8 male, Long Evans rats were used in these studies, all obtained from Envigo (Indianapolis, IN). Animals arrived at 150-175g, and were singly housed in clear Plexiglas cages (25.4 cm X 45.7 cm). Rats were weighed and handled daily. Rats had *ad libitum* access to food (Prolab RMH 3000, LabDiet; PMI Nutrition International, St. Louis, MO) and water throughout, and were maintained on a 12-h light/dark cycle in the same colony room. All experiments were conducted between 6-10 a.m. (EST), 5 days/week. One rat was euthanized due to loss of its head cap. Animal care procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Wake Forest University Animal Care and UseCommittee.

2.2 Viral Vectors and Drugs: All animals underwent surgery to bilaterally inject one of the following two constructs into the BLA: AAV5-CamKIIa-hM4D(Gi)-mCherry (Gi-DREADD; UNC Vector Core, Chapel Hill, NC) and AAV5-CaMKIIa-EGFP (UNC Vector Core, Chapel Hill, NC).

Clozapine-N-oxide (CNO; Tocris Bioscience), the DREADD ligand, or artificial cerebral spinal fluid (aCSF; Tocris Bioscience) were microinjected into the ventral hippocampus.

2.3 Surgery and Microinjection Procedures: Animals were anesthetized with sodium pentobarbital (50mg/kg intraperitoneally) and placed into a rodent stereotaxic instrument. After exposure of the skull, virus was injected intothe BLA (coordinates: anteroposterior -2.8 from bregma; mediolateral +5.0 mm, and ventral from the top of the brain -7.3 mm). Following this, stainless-steel guide cannulas (26 gauge) were implanted to terminate 1mm above the vHC (coordinates: anteroposterior -5.6mm from bregma; mediolateral from the midline +5.0 mm; ventral from the top of the brain -5.0mm). Usingstainless-steel screws and dental cement, the guide cannulas were attached to the skull. After 3-4 weeks of viral incubation, microinjections began. Animals were placed in a small plastic container and restrained by hand while the obturators were removed and injectors were inserted. The injectors extended 1mm beyond the end of the guide cannula and the drug solution (aCSF or CNO dissolved in aCSF at 10ng, 33ng or 100ng per side in a Latin Square design) was bilaterally infused for 1 min (0.5µL per side). The dose range selected for CNO injections was based on a prior report that local microinjection of 50 ng CNO into the dorsal striatum could influence goal-directed behavior (Gremel et al., 2016). Following the one minuteinjections, the injectors were left in place for an additional 30 seconds. Five minutes following the microinjections, animals were placed in the elevated plus-maze or operant chambers tobegin behavioral testing.

2.4 Behavioral Tasks

2.4.1 Operant Self-Administration

Daily sessions were performed in commercially available, sound-attenuated operant chambers (Med Associates, East Fairfield, VT) as previously described (Samson et al., 1999). Each chamber contained a house light to signal the beginning of a session, a retractable leverand a sipper tube that extended into the chamber. The system was computer controlled

and appetitive and consummatory responses were collected at 2Hz and analyzed using MedPC software (Med Associates, St. Albans, VT).

To train rats to self-administer alcohol (ethanol), we used an abbreviated sucrose-substitution protocol based on the method of Samson (Samson, 1986). Briefly, on the first day of shaping, animals were acclimated to chambers for two hours using a 10% sucrose solution on a fixed ratio (FR1) schedule that resulted in 45 second access to the sipper. Over the next consecutive days, session time, fixed ratio, and sipper access time were modified and the sucrose concentration was decreased by 1% each day and replaced with increasing concentrations of ethanol. The fixed ratio schedule was increased from a FR1 to a FR4 over an 8 day period. On day 9, the schedule was changed such that completion of a response requirement of 8 lever presses (RR8) resulted in a twenty minute presentation of the reinforcer. Over the next 10 sessions, the response requirement was increased to an RR30 and the final solution used in testing was 10% ethanol. A similar protocol was also used to train rats to an RR30 for a 3% sucrose solution. Appetitive and consummatory behaviors were monitored daily and data was collected using MedAssociates software. Animals had 20 minutes to complete the RR30, although subjected typically completed the lever press response requirement in less than five min.

2.4.2 Microinjections: After 3-4 weeks of viral incubation, and continual sessions in the operant chambers, (5 days/week, Mon-Fri), microinjections began. Animals were placed in a small plastic container and restrained by hand while the obturators were removed and injectors were inserted prior to the start of the operant session microinjections. Microinjections were given once/week and rats were placed into the operant chambers and allowed to respond to the lever for 20 minutes. Three doses of CNO (10 ng/side; 33 ng side, 100 ng/side) were tested on standard drinking sessions using a within-subjects, Latin Square design. Importantly, on microinjection days, all rats were given 20 min access to the reinforcer

solutions,regardless of whether they completed the RR30, in order to assess the effects of CNO injections on consummatory behaviors. Cohorts injected with the Gi-DREADD construct received 8 microinfusions of ACSF or CNO and the cohort injected with a construct that only expressed a reporter protein received 6 microinjections.

2.4.3 Extinction Probe Trials: To obtain an appetitive measure devoid of any consummatory behaviors, extinction probe trials were conducted. On the days when extinction probe trials were performed, rats were placed into the operant chamber and allowed to respond on the lever for the entire 20 minutes, regardless of the number of lever presses completed. Following this period, all subjects were presented with the reinforcer and allowed to drink for 20 min. Three extinction probe trials were conducted on each cohort, at least two weeks apart. The first was a baseline trial (no microinjections) and the other two trials followed intra-vHC microinfusion of CNO (33 ngper side) or vehicle with order of testing controlled using a Latin-Square design.

2.4.4 Elevated Plus-Maze: To measure unconditioned anxiety-like behavior, identical microinjection procedures were used to test behavioral responding on standard elevated plus-mazes (Med Associates, St.Albans, VT). The elevated plus-mazes were raised 72.4 cm from floor level, with runways measuring 10.2 cm wide by 50.8 cm long. Open runways had 1.3cm high lips and closed runways were detected via infrared sensors attached to the opening of each arm of the maze. Data were obtained and recorded via computer interfaced with control units and MED-PC programming (Med Associates). Animals received vehicle or CNO (33 ng per side) and, after five minutes, were placed at the junction of the four arms, facing one of the open arms and allowed to freely explore the maze for five minutes. Open arm time and open arm entries were used as measures of anxiety-like behavior and the number of closed arm entries was measured to assess general locomotor activity. Testing was conducted using a within-subject, Latin Square design, with tests separated by 3 weeks, a procedure that

eliminates the one-trial tolerance effect that can occur with some anxiolytic drugs upon repeated exposure to the elevated plus-maze (File et al., 1990; File and Zangrossi, 1993; Silberman et al., 2010).

2.4.5 CNO Control Cohorts: To control for non-specific effects of CNO, a separate cohort of rats receivedintra-vHC microinjections and behavioral treatments in a manner identical to subjects that received the received the viral construct expressing hM4D(Gi). The only difference was that these subjects received BLA microinjections of a virus that only expressed a reporter protein (AAV5-CaMKIIa-EGFP).

2.4.6 Blood Ethanol Determination: Blood ethanol concentrations were measured during the final operant session. Animals were allowed to complete the operant self-administration session and a 10µL blood sample wasthen collected from a tail snip of each rat. Blood ethanol concentrations were determined usinga commercially available ethanol dehydrogenase enzymatic assay kit (Diagnostic Chemicals, Oxford, CT).

2.4.7 Statistical Analysis: Statistical analyses were performed in SigmaPlot version 14.0. Data were analyzed using two-tailed t tests, one-way repeated measures ANOVAs, and two-way ANOVAs. When a significant interaction and/or main effect was detected, Neuman-Keuls post-hoc analysis was used. A p value of 0.05 or less was considered significant with the exception of an exploratory analyses of differences between the magnitude of CNO effects on ethanol vs. sucrose drinking-related measures, where the threshold for interactions was set at 0.1.

3.0 RESULTS

3.1 Chemogenetic silencing of the BLA-vHC circuit reduces anxiety-like behaviors in the elevated plus-maze

To confirm prior optogenetic studies, we first tested the effect of silencing the BLA-vHC pathway on anxiety-like behaviors using the elevated plus-maze. In rats expressing hM4D(Gi)-DREADD,

intra-vHC infusion of CNO (33ng per side) significantly increased open arm time ($t_{1,14}$=-3.445, p < 0.02; Fig 1A) and open arm entries ($t_{1,14}$=-3.197, p<0.01; Fig 1A), two well-validated measures of anxiety-like behavior. Closed arm entries, a measure of non-specific locomotor activity in this assay, was not affected ($t_{1,14}$=1.336, p>0.05; Fig 1A). To ensure that these anxiolytic effects were dependent on Gi-DREADD expression, we generated a separate cohort of rats that received intra-BLA infusion of a virus that only expressed a reporter protein. Intra-vHC infusion of CNO in these animals had no effect on open arm time ($t_{1,14}$=38.50, p>0.05), open arm entries ($t_{1,14}$ = 0.234, p>0.05) or closed arm entries ($t_{1,14}$ = 1.657, p > 0.05)(Fig 1B).

3.2 Chemogenetic silencing of the BLA-vHC circuit reduces alcohol intake

We next sought to test the hypothesis that inhibition of the BLA-vHC projection would reduce operant ethanol drinking behaviors. To examine this question, we employed a drinking regimen optimized to separate appetitive and consummatory behaviors, as prior studies by us and others have shown that these measures do not correlate with each other and that pharmacological manipulations that inhibit BLA excitability primarily reduce appetitive drinking-related behaviors [22,23]. In this procedure, rats were trained to complete a 30 lever press response requirement (RR30) to gain access to a sipper tube containing a 10% ethanol solution for daily 20 min drinking sessions. Ethanol intake/session was used as the primary consummatory measure and averaged 0.83 +/- 0.07 g/kg (N=8) in the nine sessions prior to CNO infusions. This level of intake generated near binge-like blood ethanol levels (73.29 +/- 6.81 mg%; N = 7). The number of lever presses during extinction probe trials was used as the main measure of appetitive behavior. In these modified sessions, subjects were allowed to lever press for 20 min and the ethanol solution was withheld until after this time period had elapsed.

We first examined the effect of bilateral intra-vHC infusions of CNO in rats expressing hM4D(Gi)-DREADD using the standard daily drinking sessions. On microinjection days, sessions were reinforced regardless of whether subjects completed the RR30, as we predicted

that intra-vHC CNO infusions might reduce lever pressing. A one-way repeated measures ANOVA, with treatment as the factor, revealed that intra-vHC CNO dose-dependently decreased ethanol intake ($F_{4,35}$=17.751, p <0.001)(Fig 2A). Post-hoc comparisons confirmed that there was no difference in ethanol intake between the average session intake during the nine sessions prior to the start of microinjections (baseline) and ACSF injection days. This analysis also demonstrated that CNO, at the two highest doses tested, significantly reduced ethanol intake relative to baseline ($q_{1,14}$ = 5.864, p <0.002; $_{q1,14}$ = 10.588, p<0.001) and ACSF (q1,14 = 3.291, p<0.05; q1,14 = 7.902, p<0.001). Ethanol consumption returned to baseline levels within 1-2 days following CNO microinjection. As predicted, CNO infusions did result in some of the subjects failing to complete the lever press response requirement (low: 4/8, middle: 5/7, high: 4/8). To determine if this reduction in ethanol intake was dependent on Gi-DREADD expression, we tested the highest dose of CNO in a separate cohort of subjects that were treated in an identical manner except that they received injections of a viral construct that expressing a reporter protein but not the DREADD. In this group, a one-way repeated measures ANOVA revealed no effect of treatment on ethanol intake (Fig. 2B). In addition, all subjects completed the 30 lever press response requirement following each of the CNO infusions.

3.3 Chemogenetic silencing of the BLA-vHC circuit reduces intake of a natural reinforcer

We also asked whether silencing the BLA-vHC circuit altered intake of a natural reinforcer. To address this question, another cohort of rats was trained to self-administer 3% sucrose using the same limited-access operant procedure used to examine ethanol drinking behaviors. This sucrose concentration engenders similar extinction probe trial responding as 10% ethanol in this drinking regimen. A one-way repeated measures ANOVA indicated a significant overall effect of treatment ($F_{4,35}$=4;296, p <0.008)(Fig 2C). Post-hoc comparisons indicated no difference in sucrose intake between baseline and ACSF sessions. However, the highest dose of CNO tested (100 ng/side) did significantly decrease sucrose intake relative to baseline ($q_{1,14}$ = 5.606,

$p<0.004$) and ACSF ($q_{1,14} = 3.948$, $p<0.05$)(Fig 2C). Notably, the magnitude of the inhibitory effect of CNO on sucrose intake was significantly less than that observed with ethanol drinking (ethanol = 51.8 +/- 7.8%, sucrose = 23.7 +/- 4.7%; $t_{1,14} = 3.081$, $p < 0.01$).

3.4 Chemogenetic silencing of the BLA-vHC circuit reduces extinction probe trial responding for ethanol and sucrose

To test the hypothesis that inhibition of the BLA-vHC pathway would decrease appetitive behaviors, we tested the effect of CNO on the number of lever presses completed during extinction probe trials, where subjects had 20 minutes to lever press but sessions were not reinforced until the end of this period. The number of lever presses completed in these sessions is a well-validated appetitive measure reflecting a subject's motivation or craving to obtain the reinforcer [21,22,24]. In the ethanol-drinking cohort of rats expressing Gi-DREADD, there was a significant effect of treatment (one-way ANOVA, $F_{2,16} = 5.448$, $p <0.05$). Post-hoc comparisons revealed no difference between the number of lever presses completed during the baseline period and following intra-vHC infusion of ACSF but a significant reduction in lever press responding following CNO microinjections, relative to either the baseline ($q_{2,16} = 4.320$, $p < 0.05$) or ACSF ($q_{2, 16} = 3.481$, $p < 0.05$) sessions (Fig 3A). In contrast, there was no significant treatment effect in the cohort of rats expressing only a reporter protein, confirming that the reduction in this appetitive ethanol drinking behavior was dependent on vHC expression of Gi-DREADD (Fig 3B). Finally, a similar analysis in the sucrose drinking cohort also revealed a significant treatment effect on extinction probe trial responding ($F_{2,18} = 6.059$, $p < 0.05$) and post-hoc comparisons showed that CNO reduced lever press responding relative to the ACSF ($q_{2,18} = 4.923$, $p < 0.05$), but not the baseline, sessions. In contrast to the observed difference in the magnitude of the effect of intra-vHC CNO on the intake of ethanol and sucrose, no difference was observed in its effects on extinction probe trial responding for these reinforcers (ethanol = 47.1 +/- 20.1%, sucrose = 41.5 +/- 8.1%).

3.5 Additional exploratory comparisons of chemogenetic silencing of the BLA-vHC circuit on appetitive and consummatory responding for ethanol and sucrose

The preceding analyses revealed that chemogenetic inhibition of the BLA-vHC pathway significantly reduced the primary appetitive and consummatory measures of ethanol and sucrose drinking-related behavior assessed in these studies. However, CNO reduced ethanol intake at a lower dose than that needed to inhibit sucrose intake and the magnitude of the inhibitory effect of the highest dose of CNO on intake trended to be greater in ethanol drinking subjects. To gain further insight into possible differences in the role of this circuit in ethanol and sucrose drinking-related behaviors, we conducted an secondary, exploratory analysis of the effects of CNO on two additional appetitive (latency to first lever press, time to complete the 30 lever press response requirement; Fig. 4A) and consummatory (latency to first lick, licks/min; Fig. 4B) measures obtained during the standard drinking sessions. Due the relatively small number of subjects in each cohort, we set the threshold to pursue post-hoc testing for two-way interactions for these exploratory analyses at 0.1. As evidenced by the graphical illustration of these datasets, it is readily apparent that CNO effects on three of the measures were largely restricted to the alcohol-drinking cohort. Indeed, using the exploratory threshold, a significant two-way interaction was detected for latency to first lever press ($F_{1,28} = 3.113$, $p = 0.09$) and lick rate ($F_{1,28} = 2.985$, $p = 0.09$) and a trend of an interaction was also observed for latency to first lick ($F_{1,28} = 2.483$, $p = 0.13$). Notably, for the two measures that met the exploratory interaction threshold, post-hoc analyses revealed that CNO significantly modulated ethanol, (latency to first lever press: $q_{1,14} = 3.996$, $p < 0.01$; ($q_{1,14} = 4.279$, $p < 0.01$), but not sucrose, drinking-related measures.

4.0 DISCUSSION

There is compelling evidence, from preclinical and clinical studies, that the etiology of anxiety/stressor disorders and AUD may involve maladaptive changes in common neural circuits. In addition to the frequent co-occurrence of these disorders, withdrawal from chronic alcohol promotes a negative affective state, of which anxiogenesis is often an integral component [25-29]. Acute alcohol is a highly effective anxiolytic, even after chronic exposure [30,31]. Moreover, many medications with anxiolytic properties reduce dependence-induced increases in alcohol drinking in animal models and have shown promise in human AUD clinical trials (e.g. topiramate, baclofen, adrenoceptor modulators) [22,32-35]. Given emerging evidence that the monosynaptic projection from the basolateral amygdala to the ventral hippocampus (BLA-vHC) regulates a broad spectrum of anxiety-related behaviors [19,36,37], here we sought to examine whether this circuit influences operant alcohol drinking measures. We first confirmed recent optogenetic findings, demonstrating that intra-vHC infusion of CNO in animals expressing Gi-DREADD within the BLA-vHC circuit significantly decreased anxiety-like behaviors on the elevated plus-maze. Then, using a limited access, operant drinking regimen, we found that intra-vHC CNO microinfusion robustly suppressed appetitive and consummatory alcohol drinking behaviors. This treatment also significantly reduced measures of sucrose self-administration, although most of these effects were more modest and required a higher CNO dose than the reductions in operant behaviors observed in alcohol drinking rats. Importantly, intra-vHC CNO treatments did not alter locomotor activity and CNO microinfusions had no effect on anxiety-like behaviors or alcohol drinking in animals only expressing a reporter protein in the BLA-vHC circuit. Taken together, these findings provide the first evidence that silencing BLA-vHC synaptic communication strongly attenuates appetitive and consummatory alcohol drinking-related behaviors, possibly via a decrease in its pharmacological properties.

We found that chemogenetically silencing an excitatory projection from the BLA to the vHC significantly decreased open arm time and open-arm entries on the elevated plus-maze

with no change in closed arm entries, a measure of nonspecific locomotor activity. Importantly, intra-vHC microinfusion of CNO had no effects on any measures in subjects only expressing a reporter protein in this circuit. These results align with the work of other recent studies that have examined the behavioral role of this circuitry. For example, optogenetic activation of BLA-vHC synapses increased anxiety-like behaviors on the elevated plus-maze while inhibiting this pathway reduced anxiety measures [19]. Similarly, optogenetic inhibition of this pathway increased center time on the open field and decreased latency to feed on the novelty suppressed feeding task [19]. These studies, along with our current findings, provide further evidence that monosynaptic projections from the BLA to the vHC can bidirectionally modulate a wide range of anxiety-like behaviors.

Using the same chemogenetic strategy employed to assess anxiety-like behaviors, we then demonstrated, for the first time, that silencing this BLA-vHC circuit significantly decreased operant alcohol consumption and extinction probe trial responding, an appetitive measure of seeking behavior [21,38]. Again, these effects were dependent on Gi-DREADD expression, as intra-vHC CNO infusion had no effect on either measure in subjects only expressing a reporter protein in this circuit. Notably, prior studies, using an operant procedure identical to that employed in this study, have shown that pharmacological treatments that decrease BLA excitability primarily reduce appetitive, but not consummatory, alcohol drinking-related behaviors [22,23,39]). For example, intra-BLA infusion of the β3-adrenoreceptor agonist, BRL 37344, which enhances a discrete GABAergic circuit in this brain region (Silberman et al, 2010), selectively decreased extinction responding but not alcohol intake [22]. Another study, using the same operant procedure, found that BLA microinjection of a nonselective, serotonin type-2 receptor agonist, which also reduces BLA excitability, decreased appetitive responding for a sweetened alcohol solution with minimal alterations in consummatory behaviors [23].

Why would silencing a BLA-vHC circuit inhibit both appetitive and consummatory drinking measures whereas nonselective inhibition of the BLA only affect appetitive behaviors?

These findings are likely reconciled by the growing appreciation that BLA pyramidal cells send largely non-overlapping projections to numerous downstream targets and these distinct circuits often have opposing behavioral effects [36,40]. For example, while optogenetic stimulation of some BLA-NAc circuits reinforce operant responding, photoactivation of BLA projections to the central amygdala can be aversive [16,17]. Although there is some disagreement regarding the anatomical distribution of these positively and negatively valenced BLA projection neurons, it seems plausible that experimental manipulations that non-selectively silence the BLA may alter multiple circuits that either promote or antagonize consummatory behaviors, thus resulting in no net effect on these measures. In support of this notion, optogenetic activation of BLA-NAc projections can either promote or disrupt appetitive and/or consummatory behaviors [41,42]. In light of these findings, our observations that chemogenetic silencing of a BLA-vHC circuit consistently reduced both appetitive and consummatory alcohol drinking-related measures suggests that this pathway may normally act as a positive modulator of both types of behaviors.

Interestingly, we also found that inhibition of this BLA-vHC circuit reduced appetitive and consummatory sucrose drinking behaviors, albeit to a lesser extent than alcohol drinking measures. Whereas intra-vHC CNO significantly reduced alcohol intake at a dose of 33 ng/side, only the highest dose of CNO (100 ng/side) inhibited sucrose intake and the magnitude of this effect was about half that observed in alcohol drinking animals. Moreover, a secondary exploratory analysis of additional measures recorded during operant drinking sessions revealed that some effects of CNO were only observed in alcohol drinking rats. For example, latency to the first lever press (an appetitive measure) as well as lick rate (consummatory measure) were significantly modulated by intra-vHC CNO in alcohol-drinking rats but not affected in the sucrose cohort. While additional studies will be needed to confirm these exploratory observations, these divergent effects could not be attributed to a greater reinforcing salience of alcohol as rats consumed a larger volume of sucrose and exhibited higher extinction responding for this

reinforcer. These differences also provide further evidence that locomotor impairment did not contribute to the inhibitory effects of intra-vHC on alcohol drinking measures.

What might account for these initial observations that BLA-vHC silencing may have more robust effects on alcohol vs. sucrose drinking-related measures? One important difference between these two solutions is that sucrose is a natural reinforcer whose salience is mediated primarily by its taste properties whereas alcohol reinforcement is likely mediated by both its sensory and pharmacological properties [43,44]. Although beyond the scope of these studies, it is tempting to speculate that the greater efficacy of intra-vHC CNO on alcohol vs. sucrose drinking behaviors may be because inhibition of this BLA-vHC circuit diminishes the salience of the pharmacological effects of alcohol that reinforce appetitive and consummatory behaviors directed at this reinforcer (e.g. anxiolysis). In fact, silencing the BLA-vHC pathway decreased anxiety-like behaviors in our studies and, while many anxiolytic drugs decrease alcohol drinking behaviors, these drugs actually increase consumption of sweet solutions [45-47]. Together, these findings offer initial support for the idea that the attenuation of anxiety-like behavior associated with BLA-vHC silencing may have contributed to the greater efficacy of intra-vHC CNO on alcohol vs sucrose drinking-related behaviors in these studies.

In summary, these studies provide the first evidence that an excitatory projection from the BLA to the vHC significantly modulates appetitive and consummatory alcohol drinking-related behaviors. Both clinical and preclinical studies have shown that increased BLA excitability is a common feature of AUD and comorbid diseases, like generalized anxiety disorder and post-traumatic stress disorder [4,48,49]. Interestingly, BLA projections to the hippocampus are largely restricted to the ventral domain of this brain region and we have recently reported that a model of vulnerability to AUD and a model of alcohol dependence both lead to increases in hippocampal synaptic excitability that are exclusively restricted to the ventral region of the hippocampus [14,50]. Although additional studies will be needed to establish a causal role of BLA-vHC dysregulation in the behavioral phenotypes promoted by

these models, these emerging findings are consistent with the idea that maladaptive increases in BLA-vHC synaptic communication may contribute to both the anxiogenic behaviors and excessive alcohol drinking associated with AUD and comorbid affective conditions. Our data also suggest that pharmacological interventions that reduce BLA-vHC excitation may be particularly effective treatments for individuals suffering from these disorders. Future studies will also be needed to explore the role of BLA projections to other downstream targets, like the NAc and CeA, in the pathogenesis of AUD and comorbid disorders.

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Figure legends

Figure 1

Chemogenetic silencing of the BLA-vHC results in a decrease in anxiety-like behavior on the elevated plus maze. (A) In rats expressing Gi-DREADD, intra-vHC infusion of CNO (33 ng/side) increased open arm time and open arm entries with no effect on closed arm entries, a measure of locomotor activity (N_{aCSF} =7, N_{CNO} = 7; *p<0.05, unpaired t test). (B) In rats only expressing the reporter protein, intra-vHC infusion of CNO had no effect on any measures on the elevated plus- maze (N_{aCSF} =5-7, N_{CNO} = 5-7; *p<0.05, unpaired t-test).

Figure 2

Chemogenetic silencing of the BLA-vHC reduces ethanol intake and consummaotry and appetitive measures for sucrose. (A) Ethanol intake was reduced in rats expressing Gi DREADD following intra-vHC injections of 33 and 100 ng/side CNO (N_{aCSF} =7, N_{CNO} = 7, ***p<0.001 relative to baseline, $^{\Psi}$p <0.05, $^{\Psi\Psi\Psi}$p < 0.001 relative to ACSF; One-way repeated measures ANOVA followed by the Newman Keuls post-hoc test). (B) There was no effect on ethanol intake in the reporter protein only control group following intra-vHC injections of 100ng/side CNO (N_{aCSF} =8, N_{CNO} = 8; One-way repeated measures ANOVA). (C) Sucrose intake was reduced in rats expressing Gi DREADD following intra-vHC injections of 100ng/side CNO (N_{aCSF} =8, N_{CNO} = 8,$^{\Psi}$p<0.05 relative to ACSF; One-way repeated measures ANOVA followed by the Newman Keuls post hoc test).

Figure 3

Chemogenetic silencing of the BLA-vHC reduces appetitive measures during an extinction probe trial. (A) Lever pressing for ethanol was significantly reduced following intra-vHC injections of 100ng/side CNO in rats expressing Gi DREADD (N_{aCSF} =7-8, N_{CNO} = 7-8,*p<0.05; relative to baseline $^{\Psi}$p<0.05 relative to ACSF; One-way repeated measures ANOVA followed by

the Newman Keuls post hoc test). (B) There was no effect on lever pressing for ethanol in the reporter protein only control group following intra-vHC injections of 100ng/side CNO (N_{aCSF} =8, N_{CNO} = 8; One-way repeated measures ANOVA). (C) Lever pressing for sucrose was significantly reduced following intra-vHC injections of 100ng/side CNO in rats expressing Gi DREADD (N_{aCSF} =7-8, N_{CNO} = 7-8,*$p<0.05$; One-way repeated measures ANOVA)

Figure 4

Comparisons of additional appetitive and consummatory measures in ethanol and sucrose groups. (A) The latency to the first lever press was greater in the ethanol group following intra-vHC CNO injection, while there was no difference in the latency to the first lever press in the sucrose group upon intra-vHC CNO injection. There was a significant effect of intra-vHC CNO injection in both the ethanol and sucrose group on lever press completion time. (B) The latency to the first lick was greater, while the licks per minute were reduced, in the ethanol group upon intra-vHC CNO injection. There was no effect of intra-vHC CNO injection on either measure in the sucrose group (Ethanol group: N_{aCSF} =7-8, N_{CNO} = 7-8; Sucrose group: N_{aCSF} =8, N_{CNO} =8; *$p<0.05$;**$p<0.01$; ***$p<0.001$; Two-way ANOVA followed by the Newman Keuls post hoc test).

Figure 1

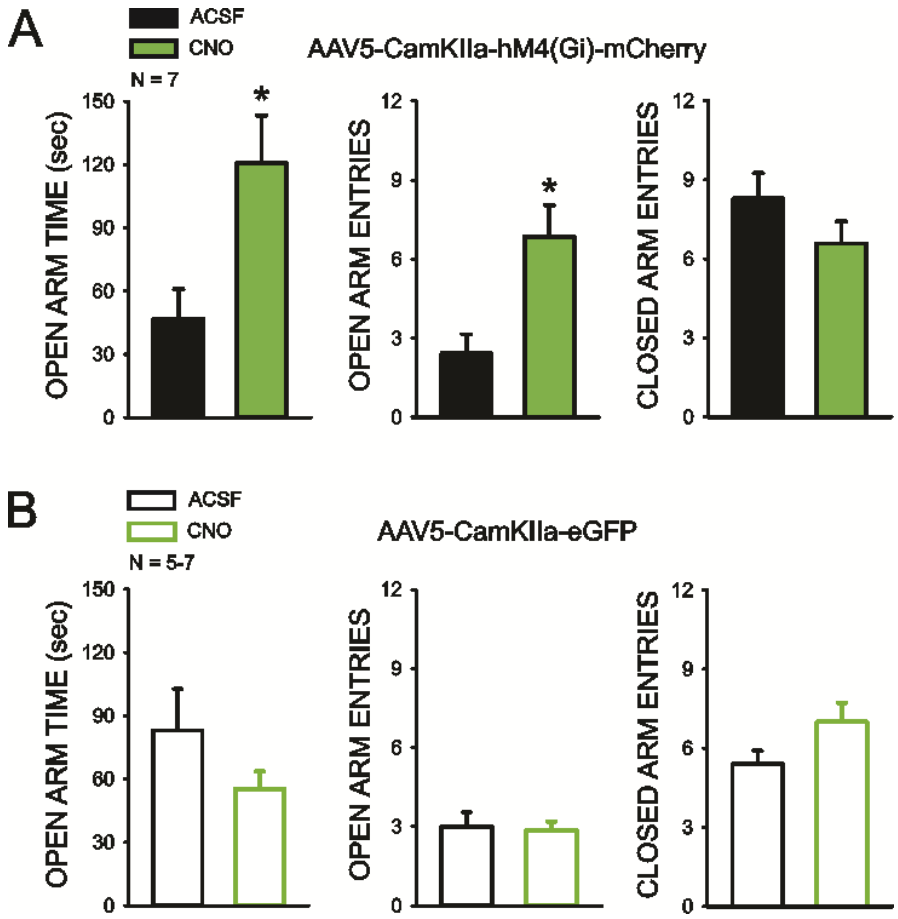

A
ACSF
CNO
AAV5-CamKIIa-hM4(Gi)-mCherry
N = 7
OPEN ARM TIME (sec)
OPEN ARM ENTRIES
CLOSED ARM ENTRIES
B
ACSF
CNO
AAV5-CamKIIa-eGFP
N = 5-7
OPEN ARM TIME (sec)
OPEN ARM ENTRIES
CLOSED ARM ENTRIES

Figure 2

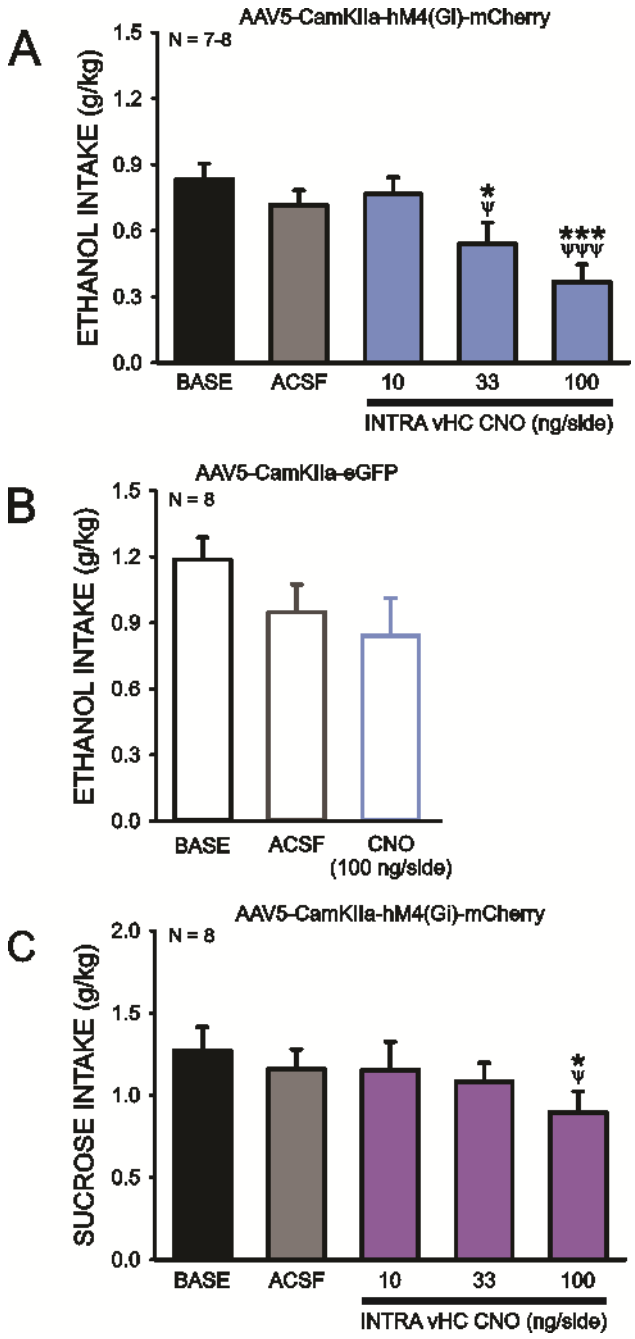

A
AAV5-CamKIIa-hM4(Gi)-mCherry
N = 7-8
ETHANOL INTAKE (g/kg)
1.5
1.2
0.9
0.6
0.3
0.0
*
ψ
***
ψψψ
BASE
ACSF
10
33
100
INTRA vHC CNO (ng/side)
B
AAV5-CamKIIa-eGFP
N = 8
ETHANOL INTAKE (g/kg)
1.5
1.2
0.9
0.6
0.3
0.0
BASE
ACSF
CNO
(100 ng/side)
C
AAV5-CamKIIa-hM4(Gi)-mCherry
N = 8
SUCROSE INTAKE (g/kg)
2.0
1.5
1.0
0.5
0.0
*
ψ
BASE
ACSF
10
33
100
INTRA vHC CNO (ng/side)

Figure 3

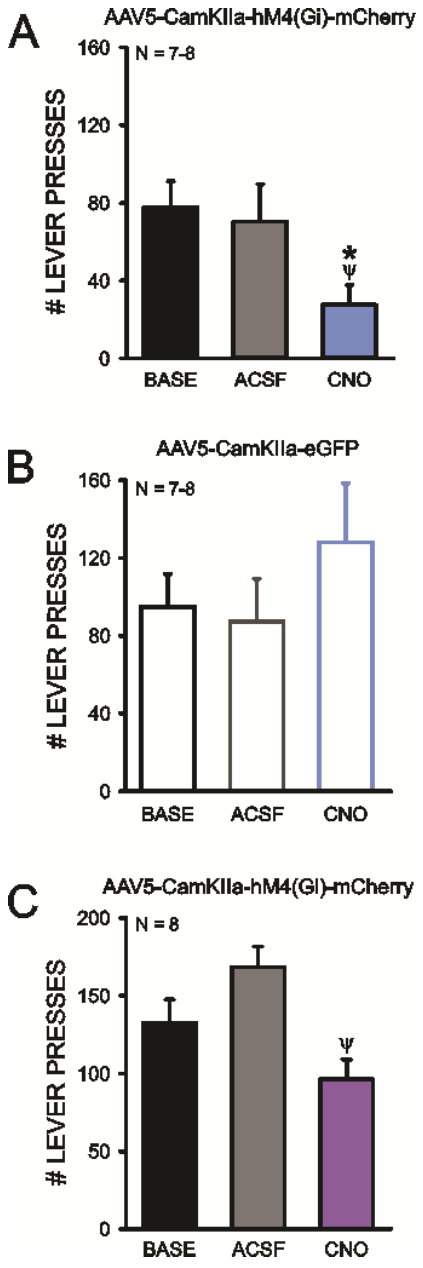
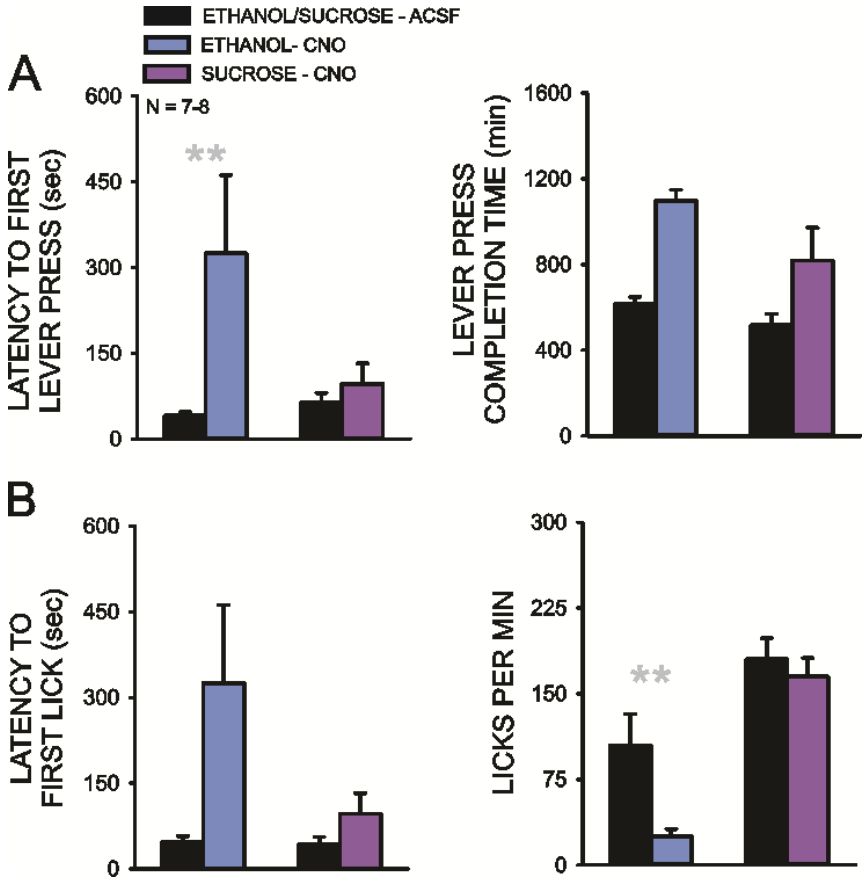

A
AAV5-CamKIIa-hM4(Gi)-mCherry
N = 7-8
# LEVER PRESSES
160
120
80
40
0
*
ψ
BASE
ACSF
CNO
B
AAV5-CamKIIa-eGFP
N = 7-8
# LEVER PRESSES
160
120
80
40
0
BASE
ACSF
CNO
C
AAV5-CamKIIa-hM4(Gi)-mCherry
N = 8
# LEVER PRESSES
200
150
100
50
0
ψ
BASE
ACSF
CNO

Figure 4


ETHANOL/SUCROSE - ACSF
ETHANOL- CNO
SUCROSE - CNO
A
N = 7-8
**
LATENCY TO FIRST LEVER PRESS (sec)
600
450
300
150
0
LEVER PRESS COMPLETION TIME (min)
1600
1200
800
400
0
B
LATENCY TO FIRST LICK (sec)
600
450
300
150
0
LICKS PER MIN
300
225
150
75
0
**