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

Author Names and Affiliations:
Sarah E. Ewin, Antoine G. Almonte, Chelcie F. Heaney, Ann M. Chappell, Kimberly F. Raab-Graham, Jeffrey L. Weiner

[1]Wake Forest School of Medicine
Department of Physiology and Pharmacology
Winston-Salem, NC 27157

[2]Wake Forest Translational Alcohol Research Center
Wake Forest University Health Sciences
Winston-Salem, NC 27157

#Corresponding Author:

Jeff Weiner
Fax: 336-716-8501
Email: jweiner@wakehealth.edu

Number of pages: 27

Number of figures: 6

Number of words:

- Abstract: 244
- Introduction: 521
- Discussion: 1499

Conflict of Interest: The authors declare no competing financial interests.

Acknowledgements: This work was supported by the NIH/NIAAA awards: AA25819 (SEE); AA7565 (AGA); AA26117, AA17531, AA26455 and AA26551 (JLW).

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.

Significance Statement

Alcohol use disorder and anxiety disorders frequently co-occur and emerging evidence suggests that maladaptive changes in neural circuits that govern anxiety-related behaviors may contribute to this comorbidity. Here we examined the role of one of these circuits in alcohol

drinking behaviors in male Long-Evans rats. Using chemogenetics and an operant procedure, we found that inhibiting a projection from the basolateral amygdala to the ventral hippocampus decreased anxiety measures and suppressed drinking behaviors to a greater extent in rats responding for alcohol vs. sucrose. These are the first data demonstrating that a basolateral amygdala-ventral hippocampal circuit modulates alcohol self-administration and provide additional evidence that dysregulation of this pathway may contribute to the etiology of AUD and comorbid affective disorders.

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 (Stahre et al., 2014; Sacks et al., 2015; U.S. Department of Health and Human Services (HHS), 2016). 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 anxiety 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) (Shorter et al., 2015; Gilpin and Weiner, 2017; Petrakis and Simpson, 2017). Moreover, this comorbidity is associated with greater symptom severity for each disorder and a much poorer prognosis in recovery (Schafer and Najavits, 2007; Evren et al., 2011; Ipser et al., 2015). Importantly, despite the high prevalence of this comorbid condition, there is marked variability in the likelihood of developing these disorders (Marmar et al., 2015; Walker et al., 2017) and very little is known about the specific neural circuits that might confer vulnerability or resilience to these diseases (Gilpin and Weiner, 2017).

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 (Almonte et al., 2017; Skelly et al., 2017; Bloodgood et al., 2018). 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 (Kim et al., 2016; Beyeler et al., 2018). 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 (Felix-Ortiz et al., 2013; Felix-Ortiz and Tye, 2014). Moreover, optogenetic inhibition of the BLA-vHC circuit disrupts footshock, but not contextual, fear learning in rats (Huff et al., 2016). Despite the critical role of this circuit in many 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.

Methods

Subjects:

A total of 24 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. 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 Use Committee.

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.

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 into the 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). Using stainless-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 minute injections, 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 to begin behavioral testing.

Behavioral Tasks

Operant Self-Administration

Training

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 lever and 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

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. 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 injections on consummatory behaviors.

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 (33ng per side) or vehicle.

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 (33ng 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).

CNO Control Cohorts

To control for non-specific effects of CNO, a separate cohort of rats received intra-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).

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 was then collected from a tail snip of each rat. Blood ethanol concentrations were determined using a commercially available ethanol dehydrogenase enzymatic assay kit (Diagnostic Chemicals, Oxford, CT).

Immunohistochemistry

After the final operant session, animals were anesthetized (0.05 mg/kg euthanasia drug) and transcardially perfused with phosphate-buffered saline (PBS), followed by 10% formalin in PBS. The brains were removed and stored in 30% sucrose solution for at least 1 week before being sectioned. The brains were sectioned on a freezing microtome (40 µm sections) and immediately placed in cyroprotectant consisting of 30% ethylene glycol and 20% glycerol in 0.05 M PBS. Slices were then blocked in 10% natural goat serum (NGS) (ImmunoReagents Inc, Raleigh, NC) with 0.3% Tween 20 for 2 hours and then incubated with primary antibodies (vHC: rabbit anti-mCherry 1:250 and mouse anti-neurofilament medium (NFM)1:500; BLA: rabbit anti-mCherry; anti-mCherry, Alomone Labs, Jerusalem, Israel; NFM, Cell Signaling Technology, Danvers, MA) in 10% NGS overnight. Slices were washed with PBS 4 times for 10 minutes each wash before incubating with the secondary antibodies (Alexa Fluor 555 goat anti-rabbit, and AlexaFluor 647 goat anti-mouse 1:400) in 10% NGS for 2 hours. Following this, the slices were washed 2 times for 10 minutes each with PBS. The third wash, only in the BLA, included DAPI (300 nM) which was washed on for 25 minutes. Finally, one last wash for 10 minutes with PBS concluded the slice preparation. Slices were mounted and allowed to sit for 2-4 hours at room temperature before being placed in 4 degree refrigerator. Slices were imaged using a Nikon A1 Confocal Laser Microscope System.

Experimental Design and 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.

RESULTS

Immunohistochemical confirmation of Gi-DREADD expression in the basolateral amygdala and ventral hippocampus

To confirm reporter protein expression in both the BLA and terminal fields in the vHC, we used immunohistochemistry and confocal microscopy. After sectioning the rat brain in coronal slices, the BLA was stained for DAPI, a nuclei marker and mCherry, the fluorescent tag for the Gi DREADD. We confirmed that the reporter protein was expressed in the BLA, primarily in cell bodies, as evidenced by the overlay of mCherry and DAPI (Fig 1A). We examined the terminal fields in the vHC by using NFM and mCherry and saw robust co-staining in the CA1 stratum radiatum (Fig 1B).

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 2A) and open arm entries ($t_{1,14}$=-3.197, $p<0.01$; Fig 2A), 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 2A). 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 2B).

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 (Butler et al., 2014; McCool et al., 2014). 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 3A). 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 and ACSF injection days. This analysis also demonstrated that CNO, at the two highest doses tested, significantly reduced ethanol intake ($q_{1,14} = 3.291$, $p<0.05$; $q_{1,14} = 10.588$, $p<0.001$) relative to ACSF. 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 expressed 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. 3B). In addition, all subjects completed the 30 lever press response requirement following each of the CNO infusions.

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 4A). Post-hoc comparisons indicated no difference in sucrose intake between baseline and ACSF sessions but a significant decrease in sucrose intake at the highest CNO dose tested ($q_{1,14} = 3.948$, $p<0.05$)(Fig 4A). 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, $p < 0.01$).

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 (Czachowski et al., 2001; Samson and Chappell, 2004; Butler et al., 2014). 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 5A). 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 5B). 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%).

Additional 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 was significantly 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 examined the effects of CNO on two additional appetitive (latency to first lever press, time to complete the 30 lever press response requirement; Fig. 6 A) and consummatory (latency to first lick, licks/min; Fig. 6B) measures obtained during the standard drinking sessions. A two-way analysis of variance revealed no

difference in latency to lever press between the ethanol and sucrose cohorts but a significant effect of CNO ($F_{1,28}$ = 4.979, $p < 0.05$)(Fig 6A). Post-hoc analysis indicated that CNO significantly increased the latency to lever press for ethanol ($q_{1,14}$ = 3.996, $p < 0.01$) but not sucrose. A similar analysis of lever press completion time revealed a significant effect of both reinforcer (F1,28 = 4.643, $p < 0.05$) and CNO (F1,28 = 20.301, $p<0.001$)(Fig. 6A), but no interaction between these factors. For this measure, CNO significantly increased the time needed to complete the lever press response requirement for both ethanol- ($q_{1, 14}$ = 5.546, $p < 0.001$) and sucrose-drinking ($q_{1,14}$ = 3.466, $p < 0.05$) subjects (Fig. 6A). A two-way analysis of variance for latency to the first lick revealed no difference between ethanol and sucrose drinking subjects but a significant effect of CNO ($5_{1, 28}$ = 5.444, $p< 0.05$)(Fig. 6B). Post-hoc comparisons confirmed that CNO significantly increased latency to the first lick for ethanol ($q_{1,14}$ = 3.909, $p < 0.05$) but not sucrose, drinking subjects. The same analysis of licks/min indicated a significant effect of reinforcer ($F_{1,28}$ = 33.491, $p < 0.001$) and CNO ($F_{1,28}$ = 6.507, $p < 0.05$) and no interaction between these factors. Post-hoc analyses revealed that CNO significantly reduced licks/min for ethanol- ($q_{1,14}$ = 4.279, $p < 0.01$) but not sucrose drinking subjects (Fig. 6B).

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 (Stewart et al., 1993; Spanagel et al., 1995; Morales et al., 2015; Ewin et al., 2018; Morales et al., 2018). Acute alcohol is a highly effective anxiolytic, even after chronic exposure (McCool et al., 2003; Liang et al., 2006). 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)

(Silberman et al., 2010; de Beaurepaire, 2012; Fox et al., 2012; Blodgett et al., 2014; Butler et al., 2014). 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 (Felix-Ortiz et al., 2013; Janak and Tye, 2015; Yang et al., 2016), 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 (Felix-Ortiz et al., 2013). Similarly, optogenetic inhibition of this pathway increased center time on the open field and decreased latency to feed on the

novelty suppressed feeding task (Felix-Ortiz et al., 2013). 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 (Samson et al., 2003; Samson and Chappell, 2004). 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 (Sinclair et al., 2012; Butler et al., 2014; McCool et al., 2014)). 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 (Butler et al., 2014). 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 (McCool et al., 2014).

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 (Janak and Tye, 2015; Tye, 2018). For example, while optogenetic stimulation of some BLA-NAc circuits reinforce operant responding, photoactivation of BLA projections to the central amygdala can be aversive (Kim et al., 2016; Beyeler et al.,

2018). 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 (Stuber et al., 2011; Millan et al., 2017). 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, during operant sessions, chemogenetic silencing of this BLA-vHC pathway selectively altered several additional appetitive and consummatory measures in alcohol drinking, but not sucrose drinking, subjects. For example, latency to the first lever press (an appetitive measure) as well as latency to first lick and lick rate (consummatory measures) were significantly reduced by intra-vHC CNO in alcohol-drinking rats but not affected in the sucrose cohort. 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 could account for the greater effects of BLA-vHC silencing on alcohol vs. sucrose drinking? 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 (Bachmanov et al., 2003; Wolfe et al., 2016). 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 (Higgs and Cooper, 1996, 1998; Silveira et al., 2000). 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 (Edwards et al., 2013; Rau et al., 2015; Gilpin and Weiner, 2017). 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 (Almonte et al., 2017; Ewin et al., 2018). 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.

References

Almonte AG, Ewin SE, Mauterer MI, Morgan JW, Carter ES, Weiner JL (2017) Enhanced ventral hippocampal synaptic transmission and impaired synaptic plasticity in a rodent model of alcohol addiction vulnerability. Sci Rep 7:12300.

Bachmanov AA, Kiefer SW, Molina JC, Tordoff MG, Duffy VB, Bartoshuk LM, Mennella JA (2003) Chemosensory factors influencing alcohol perception, preferences, and consumption. Alcohol Clin Exp Res 27:220-231.

Beyeler A, Chang CJ, Silvestre M, Leveque C, Namburi P, Wildes CP, Tye KM (2018) Organization of Valence-Encoding and Projection-Defined Neurons in the Basolateral Amygdala. Cell Rep 22:905-918.

Blodgett JC, Del Re AC, Maisel NC, Finney JW (2014) A meta-analysis of topiramate's effects for individuals with alcohol use disorders. Alcohol Clin Exp Res 38:1481-1488.

Bloodgood DW, Sugam JA, Holmes A, Kash TL (2018) Fear extinction requires infralimbic cortex projections to the basolateral amygdala. Transl Psychiatry 8:60.

Butler TR, Chappell AM, Weiner JL (2014) Effect of beta3 adrenoceptor activation in the basolateral amygdala on ethanol seeking behaviors. Psychopharmacology (Berl) 231:293-303.

Czachowski CL, Chappell AM, Samson HH (2001) Effects of raclopride in the nucleus accumbens on ethanol seeking and consumption. Alcohol Clin Exp Res 25:1431-1440.

de Beaurepaire R (2012) Suppression of alcohol dependence using baclofen: a 2-year observational study of 100 patients. Front Psychiatry 3:103.

Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW (2013) Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. Transl Psychiatry 3:e296.

Evren C, Sar V, Dalbudak E, Cetin R, Durkaya M, Evren B, Celik S (2011) Lifetime PTSD and quality of life among alcohol-dependent men: impact of childhood emotional abuse and dissociation. Psychiatry Res 186:85-90.

Ewin SE, Morgan JW, Niere F, McMullen NP, Barth SH, Almonte AG, Raab-Graham KF, Weiner JL (2018) Chronic Intermittent Ethanol Exposure Selectively Increases Synaptic Excitability in the Ventral Domain of the Rat Hippocampus. Neuroscience.

Felix-Ortiz AC, Tye KM (2014) Amygdala inputs to the ventral hippocampus bidirectionally modulate social behavior. J Neurosci 34:586-595.

Felix-Ortiz AC, Beyeler A, Seo C, Leppla CA, Wildes CP, Tye KM (2013) BLA to vHPC inputs modulate anxiety-related behaviors. Neuron 79:658-664.

File SE, Zangrossi H, Jr. (1993) "One-trial tolerance" to the anxiolytic actions of benzodiazepines in the elevated plus-maze, or the development of a phobic state? Psychopharmacology (Berl) 110:240-244.

File SE, Mabbutt PS, Hitchcott PK (1990) Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. Psychopharmacology (Berl) 102:98-101.

Fox HC, Anderson GM, Tuit K, Hansen J, Kimmerling A, Siedlarz KM, Morgan PT, Sinha R (2012) Prazosin effects on stress- and cue-induced craving and stress response in alcohol-dependent individuals: preliminary findings. Alcohol Clin Exp Res 36:351-360.

Gilpin NW, Weiner JL (2017) Neurobiology of comorbid post-traumatic stress disorder and alcohol-use disorder. Genes Brain Behav 16:15-43.

Gremel CM, Chancey JH, Atwood BK, Luo G, Neve R, Ramakrishnan C, Deisseroth K, Lovinger DM, Costa RM (2016) Endocannabinoid Modulation of Orbitostriatal Circuits Gates Habit Formation. Neuron 90:1312-1324.

Higgs S, Cooper SJ (1996) Effects of the benzodiazepine receptor inverse agonist Ro 15-4513 on the ingestion of sucrose and sodium saccharin solutions: a microstructural analysis of licking behavior. Behav Neurosci 110:559-566.

Higgs S, Cooper SJ (1998) Effects of benzodiazepine receptor ligands on the ingestion of sucrose, intralipid, and maltodextrin: an investigation using a microstructural analysis of licking behavior in a brief contact test. Behav Neurosci 112:447-457.

Huff ML, Emmons EB, Narayanan NS, LaLumiere RT (2016) Basolateral amygdala projections to ventral hippocampus modulate the consolidation of footshock, but not contextual, learning in rats. Learn Mem 23:51-60.

Ipser JC, Wilson D, Akindipe TO, Sager C, Stein DJ (2015) Pharmacotherapy for anxiety and comorbid alcohol use disorders. Cochrane Database Syst Rev 1:CD007505.

Janak PH, Tye KM (2015) From circuits to behaviour in the amygdala. Nature 517:284-292.

Kim J, Pignatelli M, Xu S, Itohara S, Tonegawa S (2016) Antagonistic negative and positive neurons of the basolateral amygdala. Nat Neurosci 19:1636-1646.

Liang J, Zhang N, Cagetti E, Houser CR, Olsen RW, Spigelman I (2006) Chronic intermittent ethanol-induced switch of ethanol actions from extrasynaptic to synaptic hippocampal GABAA receptors. J Neurosci 26:1749-1758.

Marmar CR, Schlenger W, Henn-Haase C, Qian M, Purchia E, Li M, Corry N, Williams CS, Ho CL, Horesh D, Karstoft KI, Shalev A, Kulka RA (2015) Course of Posttraumatic Stress Disorder 40 Years After the Vietnam War: Findings From the National Vietnam Veterans Longitudinal Study. JAMA Psychiatry 72:875-881.

McCool BA, Frye GD, Pulido MD, Botting SK (2003) Effects of chronic ethanol consumption on rat GABA(A) and strychnine-sensitive glycine receptors expressed by lateral/basolateral amygdala neurons. Brain Res 963:165-177.

McCool BA, Christian DT, Fetzer JA, Chappell AM (2014) Lateral/basolateral amygdala serotonin type-2 receptors modulate operant self-administration of a sweetened ethanol solution via inhibition of principal neuron activity. Front Integr Neurosci 8:5.

Millan EZ, Kim HA, Janak PH (2017) Optogenetic activation of amygdala projections to nucleus accumbens can arrest conditioned and unconditioned alcohol consummatory behavior. Neuroscience 360:106-117.

Morales M, McGinnis MM, McCool BA (2015) Chronic ethanol exposure increases voluntary home cage intake in adult male, but not female, Long-Evans rats. Pharmacol Biochem Behav 139:67-76.

Morales M, McGinnis MM, Robinson SL, Chappell AM, McCool BA (2018) Chronic Intermittent Ethanol Exposure Modulation of Glutamatergic Neurotransmission in Rat Lateral/Basolateral Amygdala is Duration-, Input-, and Sex-Dependent. Neuroscience 371:277-287.

Petrakis IL, Simpson TL (2017) Posttraumatic Stress Disorder and Alcohol Use Disorder: A Critical Review of Pharmacologic Treatments. Alcohol Clin Exp Res 41:226-237.

Rau AR, Chappell AM, Butler TR, Ariwodola OJ, Weiner JL (2015) Increased Basolateral Amygdala Pyramidal Cell Excitability May Contribute to the Anxiogenic Phenotype Induced by Chronic Early-Life Stress. J Neurosci 35:9730-9740.

Sacks JJ, Gonzales KR, Bouchery EE, Tomedi LE, Brewer RD (2015) 2010 National and State Costs of Excessive Alcohol Consumption. Am J Prev Med 49:e73-79.

Samson HH, Chappell AM (2004) Effects of raclopride in the core of the nucleus accumbens on ethanol seeking and consumption: the use of extinction trials to measure seeking. Alcohol Clin Exp Res 28:544-549.

Samson HH, Czachowski CL, Chappell A, Legg B (2003) Measuring the appetitive strength of ethanol: use of an extinction trial procedure. Alcohol 31:77-86.

Schafer I, Najavits LM (2007) Clinical challenges in the treatment of patients with posttraumatic stress disorder and substance abuse. Curr Opin Psychiatry 20:614-618.

Shorter D, Hsieh J, Kosten TR (2015) Pharmacologic management of comorbid post-traumatic stress disorder and addictions. Am J Addict 24:705-712.

Silberman Y, Ariwodola OJ, Chappell AM, Yorgason JT, Weiner JL (2010) Lateral paracapsular GABAergic synapses in the basolateral amygdala contribute to the anxiolytic effects of beta 3 adrenoceptor activation. Neuropsychopharmacology 35:1886-1896.

Silveira PP, Xavier MH, Souza FH, Manoli LP, Rosat RM, Ferreira MB, Dalmaz C (2000) Interaction between repeated restraint stress and concomitant midazolam administration on sweet food ingestion in rats. Braz J Med Biol Res 33:1343-1350.

Sinclair CM, Cleva RM, Hood LE, Olive MF, Gass JT (2012) mGluR5 receptors in the basolateral amygdala and nucleus accumbens regulate cue-induced reinstatement of ethanol-seeking behavior. Pharmacol Biochem Behav 101:329-335.

Skelly MJ, Ariwodola OJ, Weiner JL (2017) Fear conditioning selectively disrupts noradrenergic facilitation of GABAergic inhibition in the basolateral amygdala. Neuropharmacology 113:231-240.

Spanagel R, Montkowski A, Allingham K, Stohr T, Shoaib M, Holsboer F, Landgraf R (1995) Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. Psychopharmacology (Berl) 122:369-373.

Stahre M, Roeber J, Kanny D, Brewer RD, Zhang X (2014) Contribution of excessive alcohol consumption to deaths and years of potential life lost in the United States. Prev Chronic Dis 11:E109.

Stewart RB, Gatto GJ, Lumeng L, Li TK, Murphy JM (1993) Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. Alcohol 10:1-10.

Stuber GD, Sparta DR, Stamatakis AM, van Leeuwen WA, Hardjoprajitno JE, Cho S, Tye KM, Kempadoo KA, Zhang F, Deisseroth K, Bonci A (2011) Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature 475:377-380.

Tye KM (2018) Neural Circuit Motifs in Valence Processing. Neuron 100:436-452.

U.S. Department of Health and Human Services (HHS) OotSG (2016) Facing Addiction in America: The Surgeon General's Report on Alcohol, Drugs, and Health. In. Washington, DC: HHS.

Walker FR, Pfingst K, Carnevali L, Sgoifo A, Nalivaiko E (2017) In the search for integrative biomarker of resilience to psychological stress. Neurosci Biobehav Rev 74:310-320.

Wolfe SA, Workman ER, Heaney CF, Niere F, Namjoshi S, Cacheaux LP, Farris SP, Drew MR, Zemelman BV, Harris RA, Raab-Graham KF (2016) FMRP regulates an ethanol-dependent shift in GABABR function and expression with rapid antidepressant properties. Nat Commun 7:12867.

Yang Y et al. (2016) Opposite monosynaptic scaling of BLP-vCA1 inputs governs hopefulness- and helplessness-modulated spatial learning and memory. Nat Commun 7:11935.

Figures and Figure legends

Figure 1

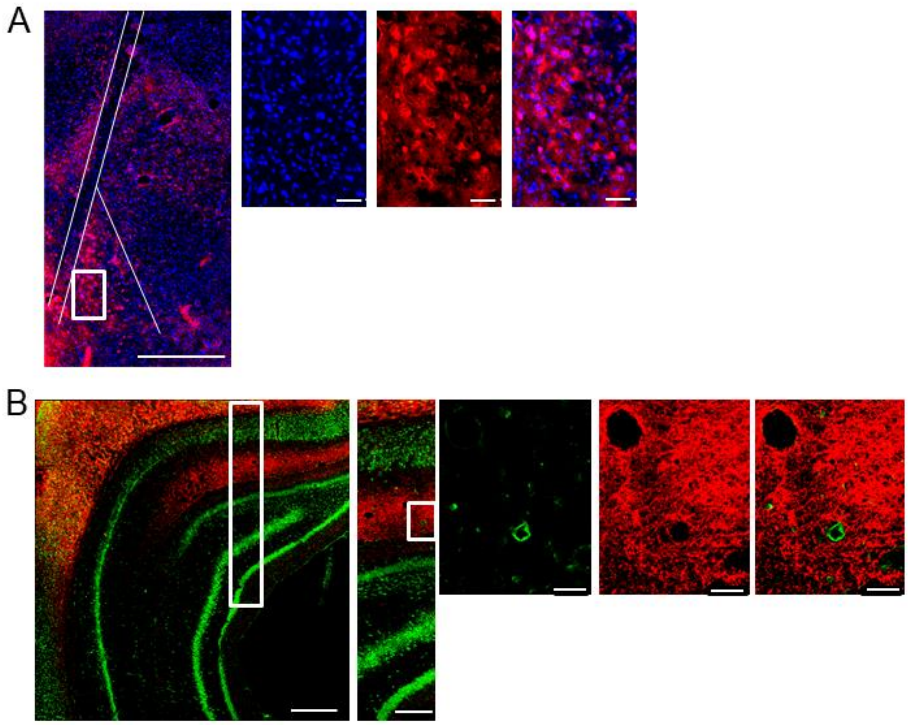

Fig. 1 Representative figures of Gi DREADD expression. Staining for mCherry and DAPI in the BLA (scale bars from left to right: 500 µm and 50 µm) (A) and mCherry and NFM in the vHC (scale bars from left to right: 500µm, 200 µm and 50 µm) (B).

Figure 2

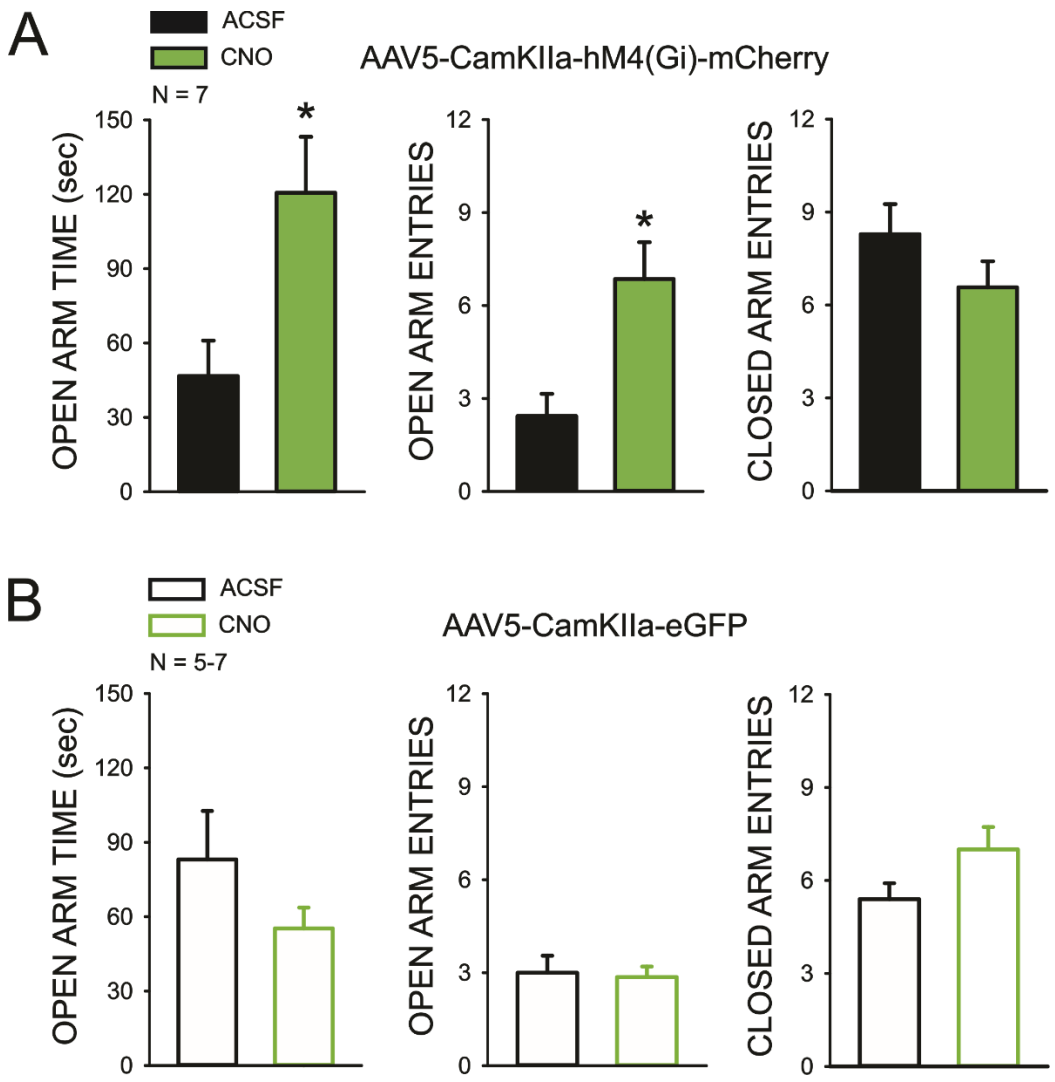

Fig. 2 Chemogenetic silencing of the BLA-vHC results in a decrease in anxiety-like behavior on the elevated plus maze. (A) In rats expressing Gi-DREADD, intra-vHC infusion of CNO (33 ng/side) increased open arm time and open arm entries with no effect on closed arm entries, a measure of locomotor activity (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 3

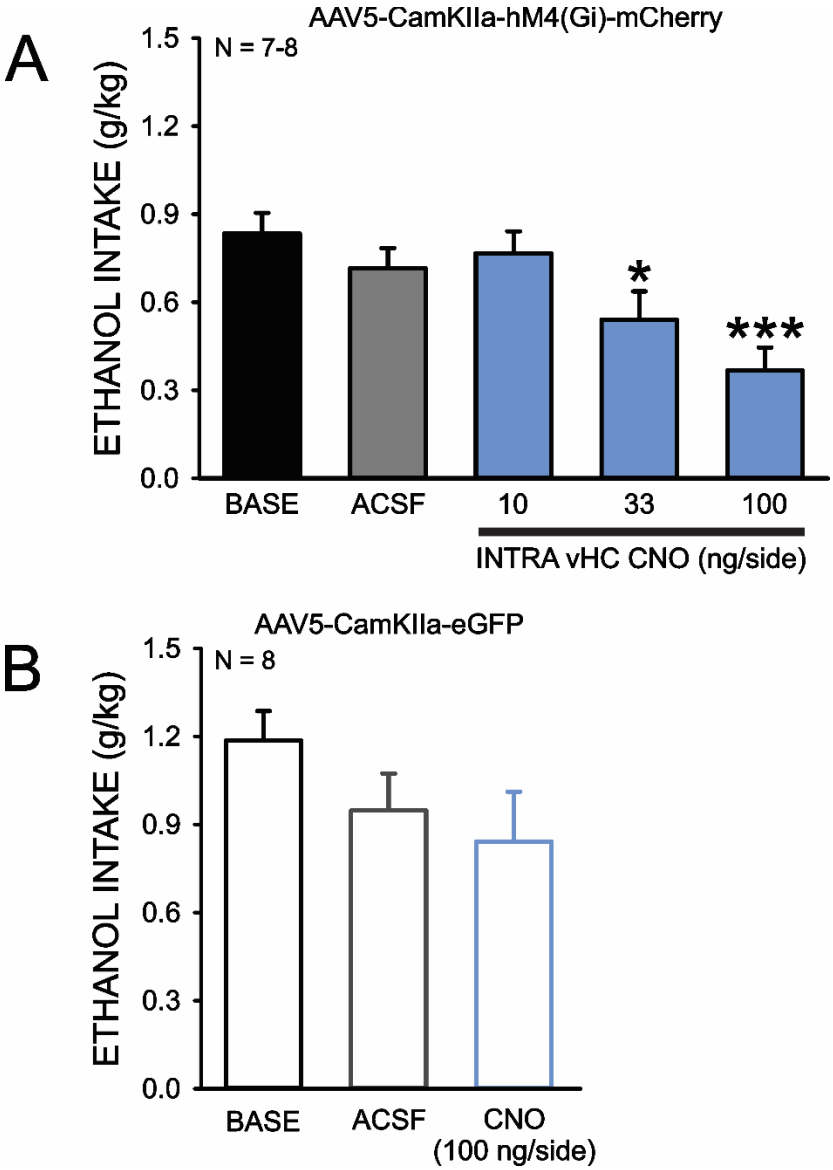

Fig. 3 Chemogenetic silencing of the BLA-vHC reduces ethanol intake. (A) Ethanol intake is reduced in rats expressing Gi DREADD following intra-vHC injections of 33 and 100 ng/side CNO (N_{aCSF} =7, N_{CNO} = 7,*$p<0.05$, ***$p<0.001$; One way repeated measures ANOVA). (B) There was no effect on ethanol 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).

Figure 4

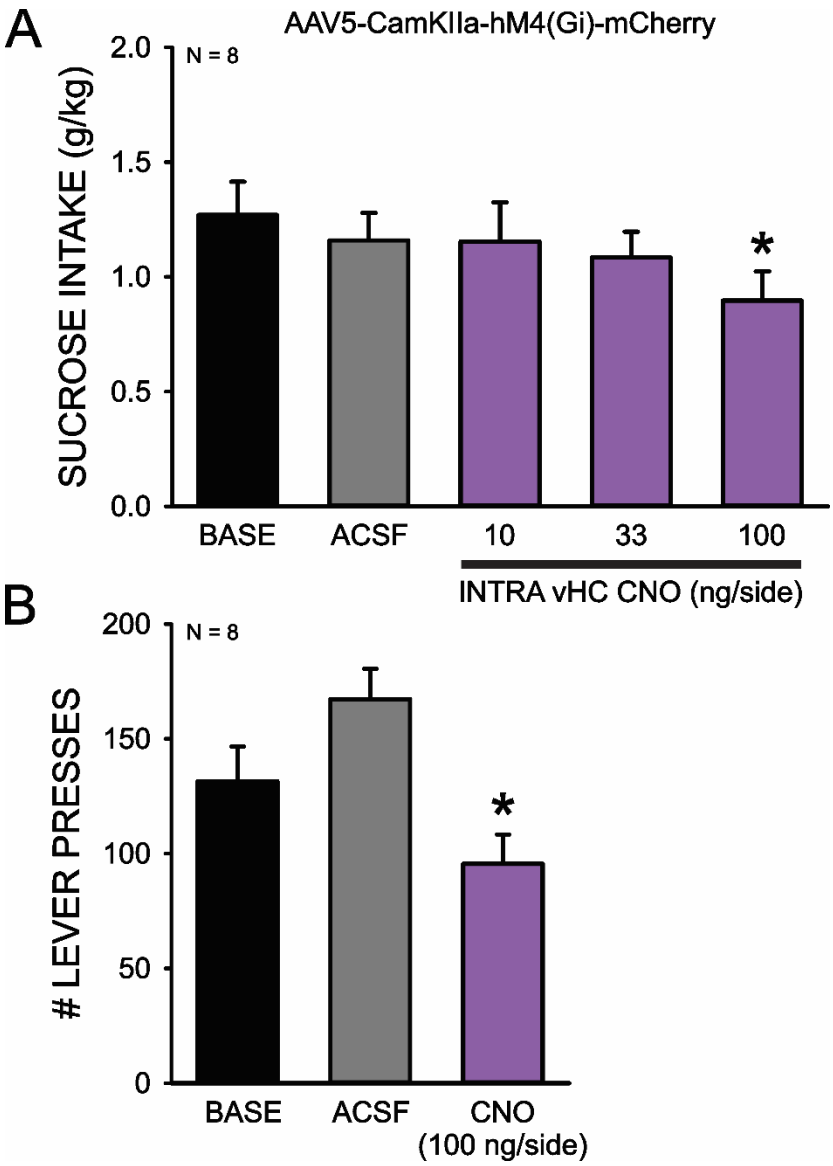

Fig 4. Chemogenetic silencing of the BLA-vHC reduces consummatory and appetitive measures for sucrose. (A) Sucrose intake was reduced in rats with the Gi DREADD following intra-vHC injections of 100ng/side CNO (N_{aCSF} =8, N_{CNO} = 8,*$p<0.05$, One way repeated measures ANOVA). (B) During an extinction probe trial, rats expressing Gi DREADD following intra-vHC injections of 100ng/side CNO decreased their lever pressing for sucrose (N_{aCSF} =8, N_{CNO} = 8; *$p<0.05$; One way repeated measures ANOVA).

Figure 5

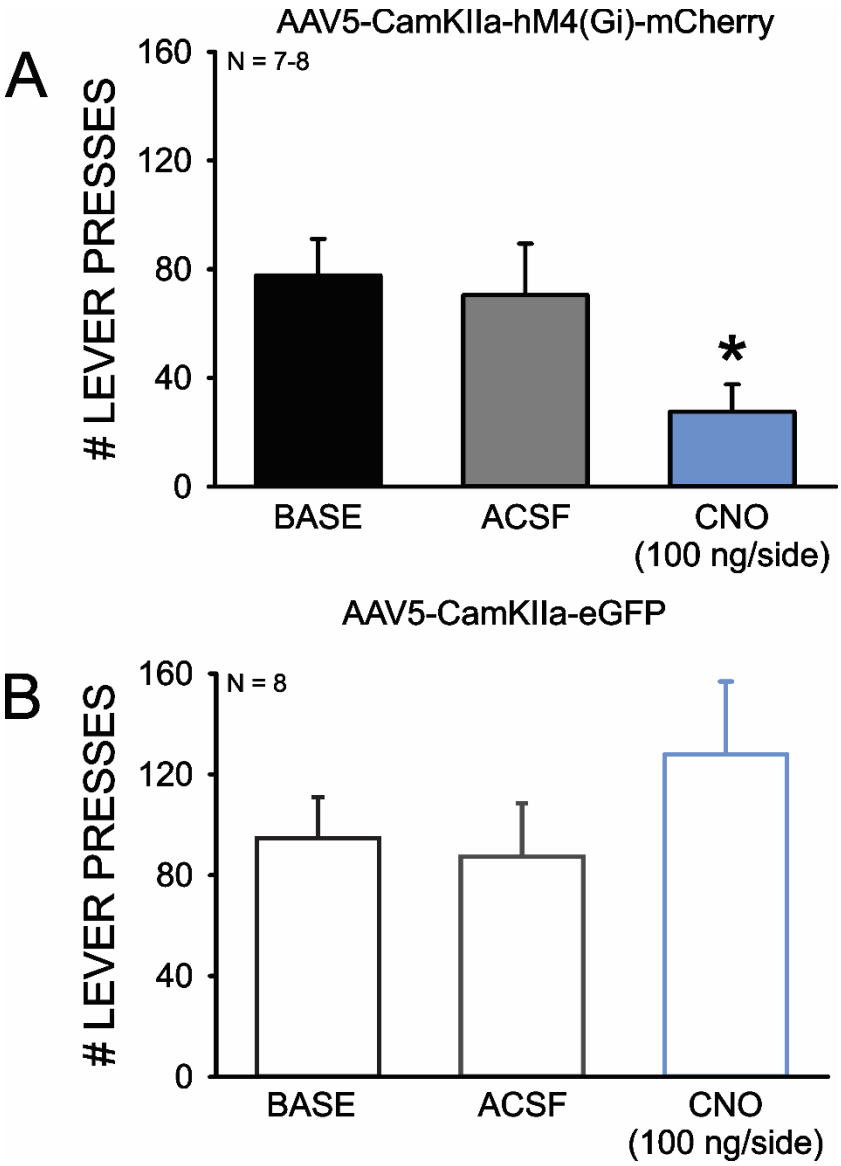

Fig 5. Chemogenetic silencing of the BLA-vHC reduces appetitive measures during an extinction probe trial. (A) Lever pressing for ethanol was significantly reduced following intra-vHC injections of 100ng/side CNO in rats expressing Gi DREADD (N_{aCSF} =7-8, N_{CNO} = 7-8,*$p<0.05$; One way repeated measures ANOVA). (B) There is 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).

Figure 6

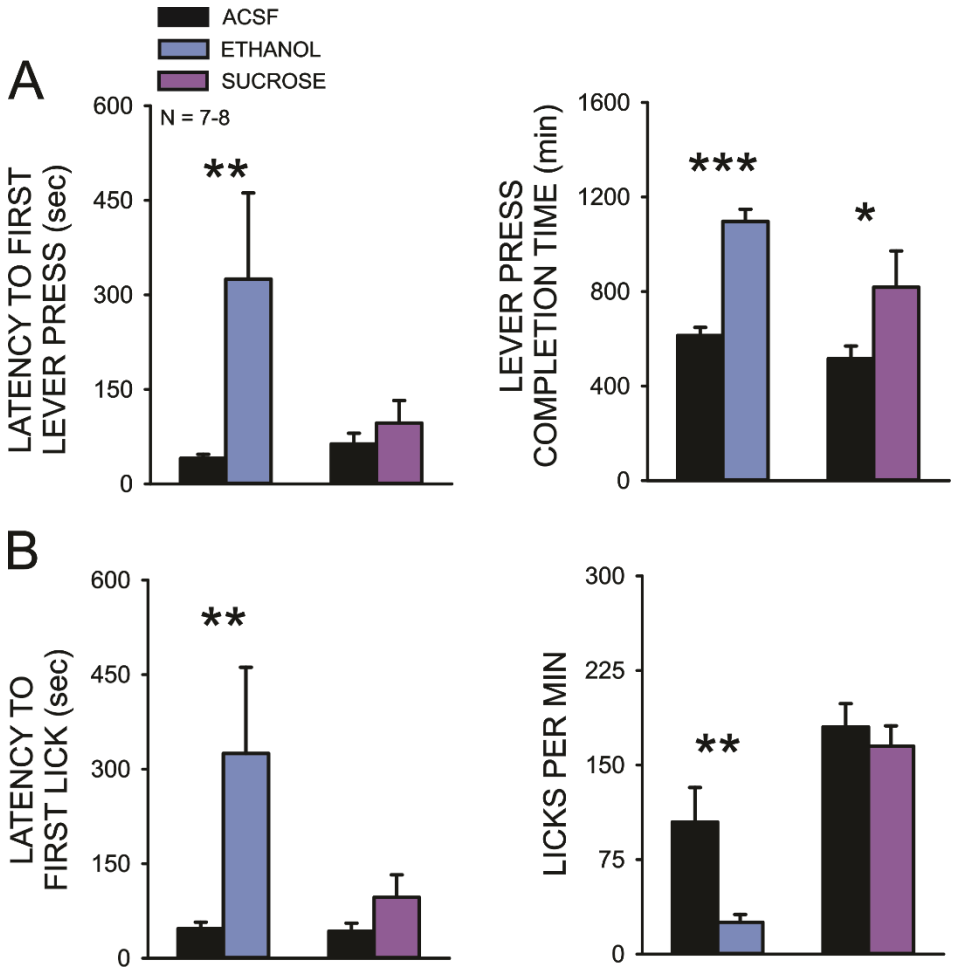

Fig. 6 Comparisons of additional appetitive and consummatory measures in ethanol and sucrose groups. (A) The latency to the first lever press was greater in the ethanol group following intra-vHC CNO injection, while there was no difference in the latency to the first lever press in the sucrose group 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).