


Pharmacogenetic Manipulation of the Nucleus Accumbens Alters Binge-Like Alcohol Drinking in Mice

Kush Purohit, Puja K. Parekh, Joseph Kern, Ryan W. Logan, Zheng Liu, Yanhua Huang, Colleen A. McClung, John C. Crabbe, and Angela R. Ozburn 

Background: Chronic alcohol intake leads to long-lasting changes in reward- and stress-related neuronal circuitry. The nucleus accumbens (NAc) is an integral component of this circuitry. Here, we investigate the effects of DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) on neuronal activity in the NAc and binge-like drinking.

Methods: C57BL/6J mice were stereotaxically injected with AAV2 hSyn-HA hM3Dq, -hM4Di, or -eGFP bilaterally into NAc [core + shell, core or shell]. We measured clozapine-*n*-oxide (CNO)-induced changes in NAc activity and assessed binge-like ethanol (EtOH) or tastant/fluid intake in a limited access Drinking in the Dark (DID) schedule.

Results: We found that CNO increased NAc firing in hM3Dq positive cells and decreased firing in hM4Di cells, confirming the efficacy of these channels to alter neuronal activity both spatially and temporally. Increasing NAc core + shell activity decreased binge-like drinking without altering intake of other tastants. Increasing activity specifically in the NAc core reduced binge-like drinking, and decreasing activity in the NAc core increased drinking. Manipulation of NAc shell activity did not alter DID. Thus, we find that increasing activity in the entire NAc, or just the NAc core is sufficient to decrease binge drinking.

Conclusions: We conclude that the reduction in EtOH drinking is not due to general malaise, altered perception of taste, or reduced calorie-seeking. Furthermore, we provide the first evidence for bidirectional control of NAc core and binge-like drinking. These findings could have promising implications for treatment.

Key Words: Alcohol, Binge Drinking, Nucleus Accumbens Shell or Core, DREADDs.

ALCOHOL USE DISORDERS (AUDs) are devastating to individuals and their families, with substantial medical and societal impact. There exists a serious public health need for (i) better understanding of disease mechanisms and (ii) improved treatments. Koob and Volkow (2010) reviewed decades of clinical and preclinical studies that address the neural circuitry associated with 3 stages of the addiction cycle: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation. Chronic alcohol abuse leads to long-lasting changes in reward- and stress-related neuronal circuitry (Koob and Volkow, 2010). A significant point of convergence for this circuitry is the nucleus accumbens (NAc). There are 2 subregions of the NAc, the shell (medial and ventral aspect) and core (lateral and dorsal

aspect). There are also 2 subtypes of GABAergic medium spiny neurons (MSNs) in the NAc, differentiated by expression of dopamine receptor D1 (Gs-coupled receptor) or D2 (Gi-coupled receptor). Human and animal studies thus far have indicated that both subregions and both MSN subtypes are important for drug-related behaviors, although they may contribute to different behaviors and during different stages of dependence (Ambroggi et al., 2011; Chaudhri et al., 2010; Koob and Volkow, 2010; Thanos et al., 2004; Voges et al., 2013). Previous studies show that binge use increases dopamine release in the NAc shell, and withdrawal/negative affect recruits activity in the extended amygdala (including the NAc shell), whereas regions involved during the preoccupation/anticipation stage project (glutamate) to the NAc core (among other brain regions) (Koob and Volkow, 2010).

Over the last several years, evidence has emerged showing that manipulation of the NAc (via lesion, deep brain stimulation [DBS], or pharmacological inactivation) reduces alcohol- and drug-related behaviors (Ozburn et al., 2015b). For instance, in humans, NAc lesions reduce relapse rates in alcohol-dependent patients (but may cause irreversible cognitive deficits) (He et al., 2008; Wu et al., 2010). There is also evidence that stimulating or increasing activity in the NAc reduces alcohol drinking, craving, and relapse in humans (Pierce and Vassoler, 2013). To further study the therapeutic potential of changing NAc activity, clinical studies have applied DBS to the NAc and found that electrical

From the Department of Psychiatry (KP, PKP, JK, RWL, ZL, YH, CAM), University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; Research and Development Service (JCC, ARO), Portland Veterans Affairs Medical Center, Portland, Oregon; and Department of Behavioral Neuroscience (JCC, ARO), Oregon Health & Science University, Portland, Oregon.

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Reprint requests: Angela R. Ozburn, PhD, VA Portland Health Care Center, 3710 SW US Veterans Hospital Road (R&D26), Portland, OR, 97239; Tel.: 503-220-8262, ext. 54265; Fax: 503-721-1029; E-mail: ozburn@ohsu.edu

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stimulation dramatically reduced alcohol craving and consumption (Heinze et al., 2009; Kuhn et al., 2011; Voges et al., 2013).

A number of studies implicate both the NAc core and shell subregions as important for alcohol drinking and alcohol craving. Previous animal studies have found that lesioning the NAc core or shell reduced limited access drinking in mice (Cassataro et al., 2014; Dhaher et al., 2009). Knapp and colleagues (2009) found that DBS of either the NAc core or shell reduces alcohol drinking in rats (using a continuous access paradigm), and Wilden and colleagues (2014) found that DBS of the NAc shell reduced ethanol (EtOH) consumption in alcohol-preferring (P) rats (Knapp et al., 2009; Wilden et al., 2014). The mechanisms by which stimulation exerts its behavioral effects are unknown. Although DBS of other brain regions can result in inactivation, this does not appear to be the case for the NAc. DBS of NAc core or shell increased c-Fos immunoreactivity in these nuclei, as well as the medial prefrontal cortex (mPFC) (Vassoler et al., 2013). Further, distal effects of acute NAc core DBS (for 2 hours) in rats include increases in dopamine and serotonin in the mPFC and dopamine and norepinephrine in the orbital frontal cortex (van Dijk et al., 2012). Notably, reductions in alcohol craving (human studies) and drinking (animal studies) occur only during stimulation. Thus, DBS effects on craving and drinking are not lasting.

These DBS studies provide a solid basis for moving forward with the development of similar, and possibly more selective and long-lasting treatments. Another strategy to increase activity is through cell-signaling cascades associated with G-protein-coupled receptors (GPCRs). Stimulation of GPCRs invokes cell-signaling mechanisms that underlie plasticity, including transient and long-term potentiation of neurons (Anisuzzaman et al., 2013; Carr et al., 2003). DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) are mutagenized M3 (Gq-coupled; excitatory) or M4 (Gi-coupled; inhibitory) muscarinic GPCRs selectively activated by clozapine-*n*-oxide (CNO) that can be used to specifically manipulate neuronal firing in an acute or chronic manner (Rogan and Roth, 2011). We focused here on determining whether CNO/DREADD-mediated changes in NAc neuronal activity alter binge-like drinking in mice. The National Institute on Alcohol Abuse and Alcoholism defines binge drinking as a pattern of drinking in a short period of time that results in a blood EtOH level >0.08 mg% or above. Binge-like drinking can be modeled in mice using limited access paradigms, such as Drinking in the Dark (DID; where mice are offered EtOH early in the dark phase of their circadian cycle) (Rhodes et al., 2005). In the present set of studies, we tested the hypotheses that increasing NAc (core and/or shell subregions) activity via CNO/DREADDs would reduce binge-like drinking, whereas decreasing activity would increase drinking. Changes in EtOH drinking can sometimes be generalized to other tastants or fluids, and

thus, we further investigated whether altering NAc activity changed tastant or fluid intake.

MATERIALS AND METHODS

Animals

Adult C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) female mice aged 2 to 5 months old were used for all experiments. C57BL/6J mice are genetically predisposed to high EtOH drinking, as shown by several inbred strain studies revealing that C57BL/6J female mice exhibit high alcohol-preferring/drinking behaviors in DID and 2-bottle choice paradigms (Belknap et al., 1993; Rhodes et al., 2007; Yoneyama et al., 2008). We opted to perform these studies in female mice because females often consume higher levels of alcohol and are understudied. All mice were group housed in a reverse 12:12 hour light/dark cycle with food and water ad libitum unless otherwise specified. All procedures were approved by local Institutional Animal Care and Use Committees and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Stereotaxic Surgeries

All mice received stereotaxic surgery (performed as in Ozburn et al., (2015a)). Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in saline (0.9% NaCl). Bilateral stereotaxic injections of 1 μ l of purified high titer AAV2-hSyn-HA-hM3D(Gq)-IRES-mCitrine (hM3Dq), AAV2-hSyn-HA-hM4D(Gi)-IRES-mCitrine (hM4Di), or AAV2-hSyn-eGFP was injected into the NAc core + shell (from bregma, in mm: angle 10°, AP +1.5, Lat +1.5, DV -4.4), NAc core (from bregma: AP +1.34, Lat +1.0, DV -4.0 and -4.5; where 0.5 μ l was infused at both DV -4.0 and -4.5 to infuse above and below the anterior commissure), or NAc shell (from bregma: AP +1.46, Lat +0.5, DV -4.75) using a 33 gauge Hamilton syringe (Reno, NV). AAV titers were 10E11–10E12 vg and were produced by Dr. R. Jude Samulski at University of North Carolina Viral Vector Core. Injection speed was 0.1 μ l/min, and the needle was kept in place for an additional 5 minutes before it was slowly withdrawn. Mice recovered for 2 to 3 weeks in their home cage to allow for full viral expression prior to experimental testing.

Electrophysiology

Two weeks after viral infusions of AAV vectors into the NAc, animals ($n = 4$ to 6/group) were sacrificed and 200 μ m coronal brain slices were taken. Slices were maintained at 30 to 32°C in artificial cerebrospinal fluid (in mM: 119 NaCl, 2.5 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgCl₂, 11 D-glucose 290 to 300 mOsm; 95% O₂/5% CO₂) and standard whole-cell current clamp recordings were made from infected MSNs in the NAc identified by differential interference contrast optics using the anterior commissure as a landmark. Evoked action potential firing was measured with resting membrane potential adjusted to -80 mV and current steps from 100 to 450 pA. A current step sufficient to evoke spikes was delivered every 10 seconds and spike numbers were averaged from stable baseline recordings (5 to 10 sweeps) and following 5 minutes of CNO application (10 μ M). A K⁺ based internal solution was used (in mM: 119 K-MeSO₄, 2 KCl, 1 MgCl₂, 1 EGTA, 0.1 CaCl₂, 10 HEPES, 2 Mg-ATP, 0.4 Na-GTP 275 to 285 mOsm; pH 7.2 to 7.4). Series resistance for all recordings was monitored continuously. Cells with a change in series resistance beyond 20% were excluded from data analysis. Evoked action potentials were recorded with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Signals were filtered at 2.6 to 3 kHz and amplified 10 times and then

digitized at 20 kHz with a Digidata 1322A analog-to-digital converter (Molecular Devices). Data were analyzed using pClamp 10 software (Molecular Devices).

Drinking in the Dark

EtOH consumption is determined by many factors, including palatability; therefore, we tested the effects of altering NAc activity on intake of EtOH, as well as other tastants and water using a modified DID schedule. As in Rhodes and colleagues (2005), mice were habituated to individual housing and a new sipper bottle for 1 week prior to testing (Rhodes et al., 2005). For the EtOH DID assay, mice were then offered a single bottle of 20% EtOH (v/v, in tap water; Decon Laboratories, Inc., King of Prussia, PA), starting 3 hours after lights off. For tastant and fluid DID assays, mice were offered a single bottle containing a solution of quinine hemisulfate salt monohydrate (0.03 mM or 0.06 mM in tap water), sucrose (2.5% or 5% in tap water), or tap water. Fluid intake was measured for either 2 or 4 hours; see specific experiment details below. Water was then restored for the remainder of the day (unmeasured). Mice were injected intraperitoneal (IP) daily with vehicle (1% DMSO in saline) or CNO (0.5 or 1 mg/kg; RTI International, Raleigh, NC) 15 to 30 minutes prior to measured fluid access. Details for each study follow below.

Acute Study - NAc Core + Shell (Excitatory DREADD vs. GFP). Groups of mice were injected IP with CNO (0.5 mg/kg on days 1, 2, 3, 4) 15 to 30 minutes before DID. The 0.5 mg/kg dose of CNO was chosen based on a study showing this dose increased neuronal activity in mice expressing hM3Dq (Alexander et al., 2009). The water tube was removed and a 20% EtOH solution was presented for 2 hours (3 hours after lights off) on days 1, 2, 3, and for 4 hours on day 4. This typical DID paradigm was then modified by adding 1 day of abstinence with no treatment (day 5) followed by administration of CNO (1 mg/kg) before 2 hours of EtOH availability on day 6. We administered a higher dose of CNO on day 6 because we anticipated employing the inhibitory DREADD (hM4Di) in our next experiment and CNO has a slightly lower affinity for this receptor, requiring the 1 mg/kg dose to be administered. Further, we wanted to determine whether the 1 mg/kg CNO dose produced a more robust effect ($n = 7$ to 8 /group).

Chronic Study - NAc Core + Shell (Excitatory or Inhibitory DREADD, or GFP). DID was carried out as in Rhodes and colleagues (2005), with a modification to limit access to 2 h/d. Groups of mice were habituated to individual housing and a new sipper bottle 1 week prior to testing. There were 4 periods of EtOH testing with 2 weeks of recovery between each period. This schedule was designed to assess the effect of changing NAc activity on (i) binge-like EtOH drinking (14 days: vehicle pretreatment daily for 7 days, 1 mg/kg CNO pretreatment daily for 7 days), intake of (ii) a bitter solution (4 days: quinine, 2 days each concentration), (iii) a sweet, caloric solution (4 days: sucrose, 2 days each concentration), and (iv) water (4 days). Prior to each 4-day period of tastant/fluid testing, all mice were injected with CNO (1 mg/kg) each day. Each measured solution was presented (alone, no choice) for 2 hours ($n = 8$ to 9 /group).

Chronic Study - NAc Core or Medial Shell (Excitatory or Inhibitory DREADD, or GFP). To determine whether increasing or decreasing NAc activity chronically and specifically in core or medial shell could alter binge-like alcohol drinking, we assayed the effects of CNO/DREADDs on binge-like drinking using a 2-week DID paradigm (2 h/d EtOH access). During the first week of DID access to 20% EtOH, groups of mice were pretreated with vehicle to establish baseline levels of intake, and during the second week, mice were administered CNO (1 mg/kg) prior to the drinking session ($n = 11$ to 13 /group [core]; $n = 9$ to 14 /group [shell]).

Immunohistochemistry

After the completion of behavioral studies, mice were deeply anesthetized with 250 mg/kg ketamine and 25 mg/kg xylazine and transcardially perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde in PBS. The brains were allowed to postfix in 4% paraformaldehyde in PBS for 24 hours and then placed in 30% glycerol in PBS for an additional 24 hours before being stored in PBS-0.01% sodium azide. Using a freezing microtome (Leica, Wetzlar, Germany), 30- μ m brain sections were obtained and immunohistochemical staining was carried out using standard procedures. Antibodies used include rabbit anti-GFP (1:20,000; Abcam, cat#ab290; Cambridge, MA) or rabbit anti-HA (1:1,000; Cell Signaling Technology, cat#3724; Danvers, MA) and anti-rabbit conjugated with Alexa 488 (1:500; Molecular Probes, Carlsbad, CA). Brain sections were mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA) and observed using a fluorescence microscope. Animals were excluded from our study if expression was not localized to the NAc.

Statistics

Data are presented as the mean \pm SEM. Data with normal distributions were analyzed using Student's *t*-test, or 1- or 2-way analyses of variance (ANOVAs), followed by Bonferroni's post hoc tests; data with nonnormal distributions were analyzed using Mann-Whitney *U*-tests (data presented in Fig. 2). Data analysis was performed and graphs were generated using GraphPad Prism (GraphPad Software, La Jolla, CA).

RESULTS

Pharmacogenetic Manipulation of Neuronal Activity in the NAc

To assess the effect of CNO on hM3Dq-, hM4Di-, or GFP-expressing neurons, we performed standard whole-cell current clamp recordings from virally transduced MSNs in the NAc. Evoked action potential firing was measured with resting membrane potential adjusted to -80 mV and current steps from 100 to 450 pA.

Figure 1A shows an example of viral placement in core + shell and Fig. 1B shows representative traces from recordings, where CNO application decreased the number of evoked action potentials in hM4Di (top)-expressing MSNs, did not alter firing in GFP (middle)-expressing MSNs, and increased evoked action potentials in hM3Dq (bottom)-expressing MSNs. Data showing individual and average (\pm SEM) responses to successive current injections at baseline and following CNO application are shown in Fig. 1C, where a treatment \times group interaction was identified, $F(2, 12) = 22.8$, $p < 0.0001$. Bonferroni post hoc testing revealed CNO reduced firing for cells expressing hM4Di ($p < 0.01$) and significantly increased firing in cells expressing hM3Dq ($p < 0.001$).

Percent change in firing rate after CNO application is shown in Fig. 1D (1-way ANOVA $F(2, 12) = 42.8$, $p < 0.0001$); Bonferroni post hoc $p < 0.05$ (hM4Di vs. GFP), $p < 0.001$ (hM3Dq vs. GFP). CNO (i) significantly decreased evoked firing rates by approximately 25% in hM4Di-expressing MSNs, (ii) increased evoked firing rates by approximately 35% in hM3Dq-expressing MSNs, and (iii) produced no significant changes in firing rates in GFP-expressing MSNs.

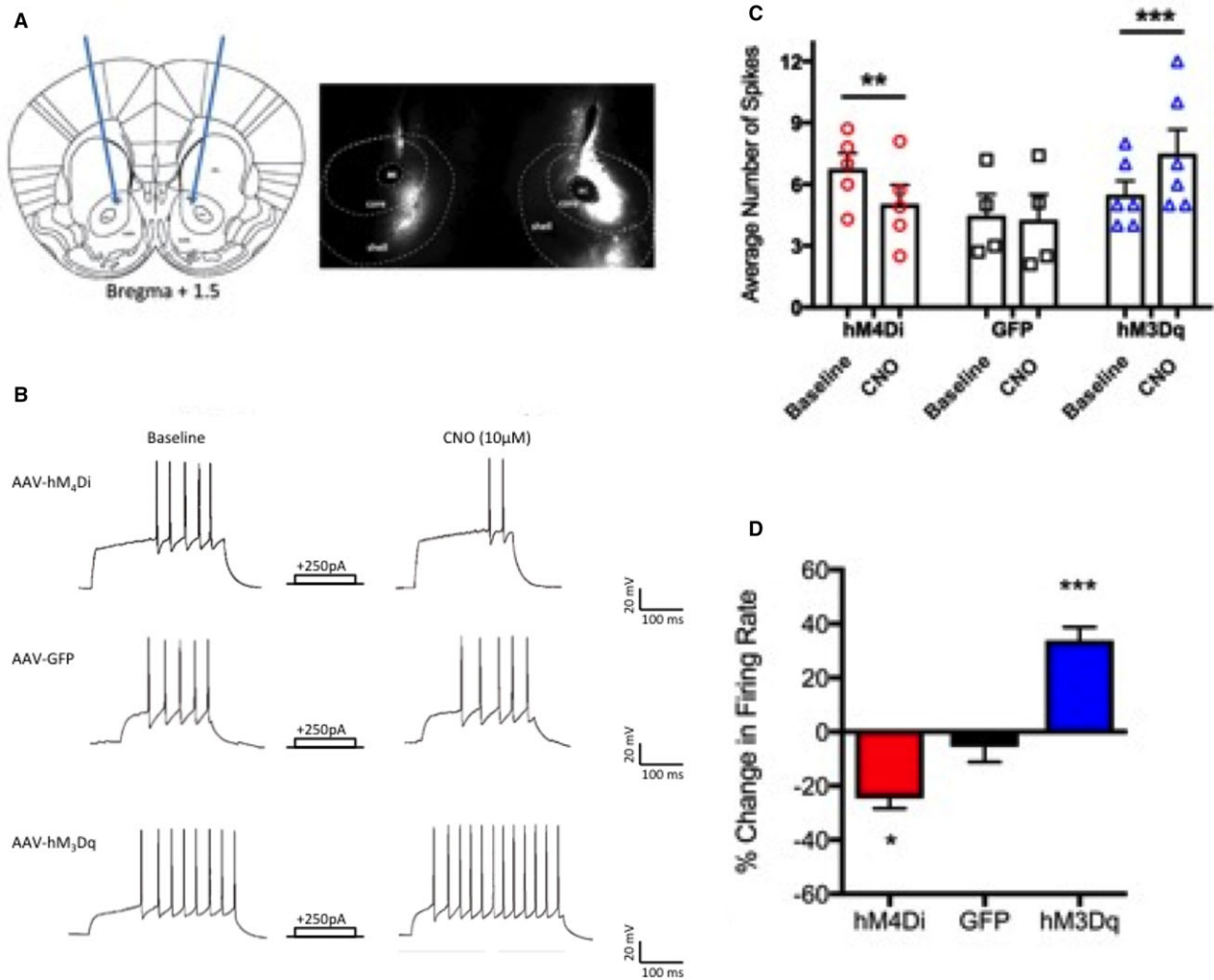


Fig. 1. Pharmacogenetic manipulation of neuronal activity in the NAc. **(A)** Targeted region for stereotaxic delivery of AAV2 hM3Dq, hM4Di, or GFP to the NAc core + shell (**left**) and representative $2\times$ image showing viral expression in the NAc core and shell. **(B)** Representative traces show CNO application reduced the number of evoked action potentials in hM4Di-expressing neurons (**top**), did not change firing rates in GFP-expressing neurons (**middle**), and increased firing in hM3Dq neurons. **(C)** Individual and mean firing rates for each group at baseline and after CNO, $n = 4$ to 6 /group; 2-way ANOVA treatment \times group interaction, $F(2, 12) = 22.8$, $p < 0.0001$. Bonferroni post hoc $**p < 0.01$, $***p < 0.001$ (CNO vs. baseline). **(D)** Percent change in firing rate after CNO application, 1-way ANOVA $F(2, 12) = 42.8$, $p < 0.0001$; Bonferroni post hoc $*p < 0.05$ (hM4Di vs. GFP), $***p < 0.001$ (hM3Dq vs. GFP). [Color figure can be viewed at wileyonlinelibrary.com]

Increasing NAc (Core + Shell) Activity Decreased Binge-Like EtOH Drinking in an Acute DID Schedule

Results from this study indicate that acute and repeated stimulation of NAc activity (via CNO/DREADDs) significantly reduced EtOH intake in the DID paradigm for 3 days (Fig. 2A; average 2-hour intake for individuals on DID days 1, 2, 3: Mann–Whitney U -test, median EtOH intake was 2.33 g/kg for GFP mice ($n = 8$) and 1.79 g/kg for hM3Dq mice ($n = 7$); the distributions in the 2 groups differed significantly (Mann–Whitney U -test = 7, $p = 0.01$, 2-tailed). However, when the DID access was increased to 4 hours on day 4, the effect was noticeably absent (Fig. 2B). Due to the short half-life of CNO (< 2 hours; see Discussion section), and because mice were injected with CNO 15 to 30 minutes prior to DID, we presume

that there was no bioavailable CNO during the last 2 hours of the session on day 4. After a 1-day break, we assayed the effects of 1 mg/kg CNO on 2-hour DID and again found that it reduced intake in mice expressing the excitatory DREADD, hM3Dq (Fig. 2B). We did not expect the 1 mg/kg dose to have a more robust effect of DID intake than the 0.5 mg/kg CNO dose, specifically because there is limited expression of DREADDs in the membrane for the CNO to bind to and exert an effect (notably, these doses are in the low range of published studies with DREADDs). Results from this study show that increasing activity in the NAc reduced binge-like drinking by $\sim 34\%$. However, one caveat to this experimental design is that it lacked a vehicle control (all mice were given CNO), which we addressed in the next experiment.

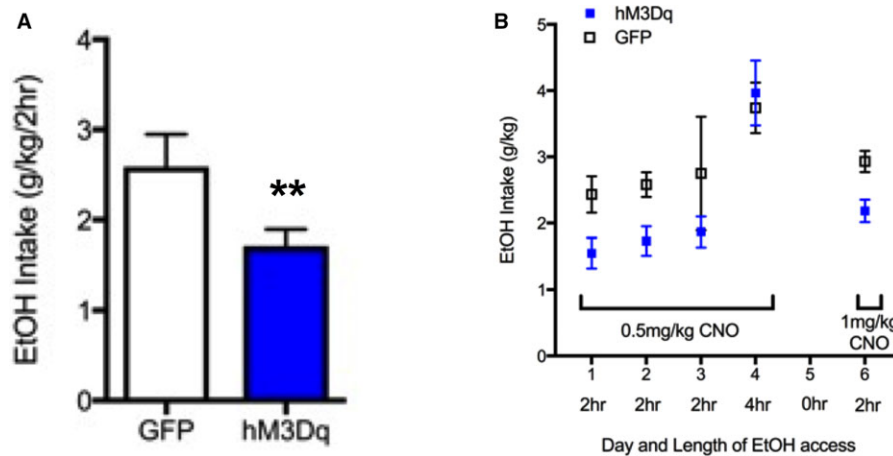


Fig. 2. Increasing NAc core and shell activity decreased acute binge-like EtOH drinking. **(A)** Average 2-hour intake for individuals on DID days 1, 2, 3: Mann–Whitney *U*-test, median EtOH intake for GFP and hM3Dq mice were 2.33 g/kg ($n = 8$) and 1.79 g/kg ($n = 7$); the distributions in the 2 groups differed significantly (Mann–Whitney *U*-test = 7, $**p = 0.01$, 2-tailed). **(B)** Increasing NAc core + shell activity decreased acute binge-like EtOH drinking on days 1, 2, 3, and 6. Two-way ANOVA (only includes data from 2-hour drinking days 1, 2, 3, and 6) revealed main effect of AAV group $F(1, 52) = 9.70$, $p < 0.01$. The effect of CNO on mice expressing hM3Dq was noticeably absent on day 4, when EtOH access was 4 hours. There was not a significant difference in intake between 0.5 and 1 mg/kg CNO. Because the half-life of CNO is ~2 hours, the length of access to EtOH drinking was adjusted to 2 hours in subsequent studies. $n = 7$ to 8/group. [Color figure can be viewed at wileyonlinelibrary.com]

Increasing NAc (Core + Shell) Activity Reduced Chronic Binge-Like EtOH Drinking

To determine whether increasing or decreasing NAc (core and shell) activity could more chronically alter binge-like alcohol drinking, we used a 2-week DID paradigm. Mice were pretreated with vehicle to establish baseline levels of intake during the first week, and then in the second week, mice were treated daily with CNO prior to DID. Average daily EtOH intakes for each treatment week (vehicle and CNO) data are presented in Fig. 3B. Repeated-measures 2-way ANOVA revealed a significant main effect of CNO treatment to reduce drinking, $F(1, 22) = 4.29$, $p = 0.05$, and a trend toward a group \times treatment interaction, $F(2, 22) = 3.11$, $p = 0.06$. To pursue the interaction, we expressed the response of each animal during CNO treatment as a change from its own individual average baseline intake score. These difference scores are shown in Fig. 3A. One-way ANOVA revealed a trend toward significant group differences, $F(2, 22) = 3.112$, $p = 0.06$. We hypothesized that increasing activity would reduce binge-like drinking, and a direct test of this hypothesis revealed that mice in the hM3Dq group showed a significant reduction as compared to mice in the GFP group ($t = 2.114$, $df = 15$, $p < 0.05$). Chronically increasing activity in the NAc thus reduced binge-like drinking by ~29%, similar to the acute effects (Fig. 2).

After the alcohol DID and a period of abstinence, we assessed whether changing NAc MSN activity altered intake of specific tastants or fluid more generally by measuring intake of a bitter quinine solution (0.03 and then 0.06 mM quinine), a sweet sucrose solution (2.5% and then 5%, w/v), and tap water. Separate ANOVAs were carried out for each

solution. As expected, mice consumed low levels of the quinine solutions. There was a significant decrease in intake at the higher concentration for all groups, $F(1, 23) = 13.54$, $p < 0.01$ (Fig. 3C). Mice consumed high levels of sucrose solutions and moderate levels of the neutral solution (water). While CNO appeared to reduce consumption of 5% sucrose in hM3Dq mice, no significant effects of treatment were observed for either sucrose or water (Fig. 3D,E). These results suggest altering NAc activity did not alter tastant or fluid intake.

Altering NAc Core Activity Bidirectionally Altered Chronic Binge-Like EtOH Drinking

To determine whether chronically increasing or decreasing activity specifically in the core subregion of the NAc could alter binge-like alcohol drinking, we used drinking in a 2-week DID paradigm. During the first week of DID, mice were pretreated with vehicle to establish baseline levels of intake prior to the CNO pretreatment during the second week. Mean individual difference scores (intake after CNO treatment as a change from the baseline intake score) are shown in Fig. 4A. One-way ANOVA revealed a significant effect of CNO treatment, $F(2, 33) = 3.582$, $p < 0.05$. This appeared to result from differentially altering NAc core activity: Although post hoc testing revealed that neither DREADD group differed significantly from the GFP group, they differed significantly from each other ($p < 0.05$, hM3Dq vs. hM4Di). Average EtOH intakes for each treatment week (vehicle and CNO) are presented in Fig. 4B. Representative image from immunofluorescent verification of AAV injection placement is shown in Fig. 4C.

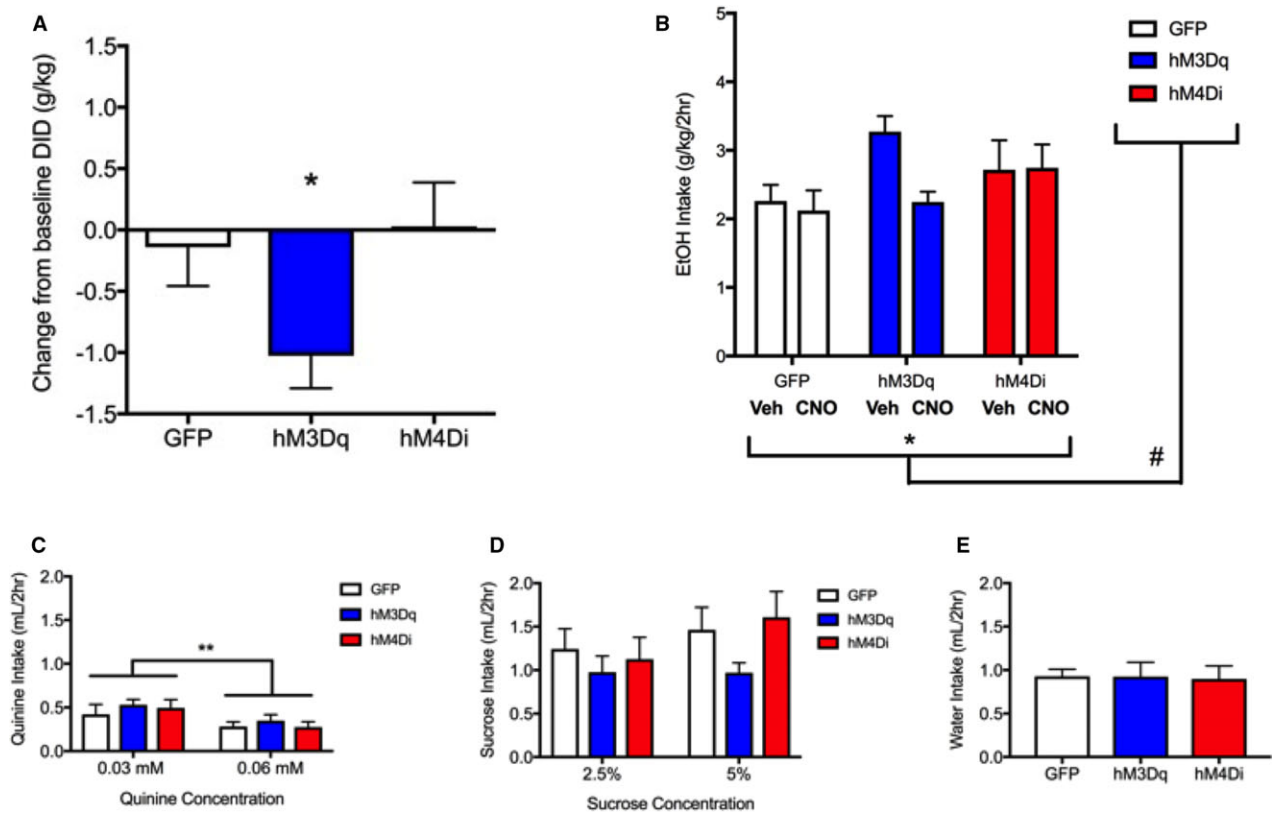


Fig. 3. Increasing NAc core + shell activity decreased chronic binge-like EtOH drinking without altering intake of other fluids. **(A)** EtOH intake presented as average individual difference scores (intake during treatment with CNO – intake during treatment with vehicle). As in the previous experiment (see Fig. 2), mice in the hM3Dq group showed a significant reduction as compared to mice in the GFP group (Student's *t*-test, $t = 2.114$, $df = 15$, $p < 0.05$). **(B)** Average weekly EtOH intake after treatment with vehicle or CNO. Two-way ANOVA revealed a significant main effect of CNO treatment, $F(1, 22) = 4.29$, $p = 0.05$ (*), and a nonsignificant trend toward a group \times treatment interaction, $F(2, 22) = 3.11$, $p = 0.06$ (#). **(C)** Altering NAc activity does not change intake of a bitter solution. There was a significant decrease in quinine intake at the higher concentration for all groups, $F(1, 23) = 13.54$, $**p < 0.01$. Mice consumed high levels of sucrose solutions **(D)** and moderate levels of the neutral solution, water **(E)**. Separate ANOVAs were carried out for **(D)** and **(E)**, where no trends or significant effects of treatment were observed for either fluid. $n = 8$ to 9 /group. [Color figure can be viewed at wileyonlinelibrary.com]

Altering NAc Shell Activity Did Not Affect Chronic Binge-Like EtOH Drinking

The difference scores for DREADD groups are presented in Fig. 5A, where we observed no significant effects, suggesting that changing NAc medial shell activity did not affect binge-like drinking. Average EtOH intakes for each treatment week (vehicle and CNO) are presented in Fig. 5B. Representative image from immunofluorescent verification of AAV injection placement is shown in Fig. 5C.

DISCUSSION

These experiments demonstrate that neuronal activity can be controlled in a spatial and temporal manner using CNO/DREADDs. Increasing activity in the entire NAc, or just the NAc core was sufficient to decrease binge-like drinking. The ability to change brain activity with spatial and temporal precision has opened up new avenues for therapeutics, as well as for basic studies in neuroscience, where the NAc is currently a promising target for addiction (Luigjes et al., 2012). Acute and chronic alcohol alters electrical membrane properties of

MSNs, and produces a dysregulation of neurotransmission in the NAc (Marty and Spigelman, 2012a,b; Volkow et al., 2004). Individuals with substance use disorders exhibit hypoactive mesolimbic/cortical circuitry (Volkow et al., 2004). Stimulation of the NAc may mitigate hypoactive circuitry in the brain of individuals with AUD, acting as a substitute for the effects of drug action.

Clinical studies in treatment-resistant alcoholics suggest DBS of the NAc shell relieves symptoms of craving and reduced relapse (Heinze et al., 2009). Additionally, several preclinical studies found that stimulating the NAc reduced alcohol intake and relapse-like drinking (Henderson et al., 2010; Knapp et al., 2009; Wilden et al., 2014). van Dijk and colleagues (2012) evaluated the effects of DBS in the NAc core in rats on monoamine neurotransmitter concentrations in cortical regions. Acute stimulation (2 hours) significantly increased dopamine and serotonin in the mPFC, and dopamine and noradrenaline in the orbital frontal cortex (van Dijk et al., 2012). This important study provides further evidence for the distal effects of increasing brain activity via DBS. Stimulating the NAc has also been shown to effectively

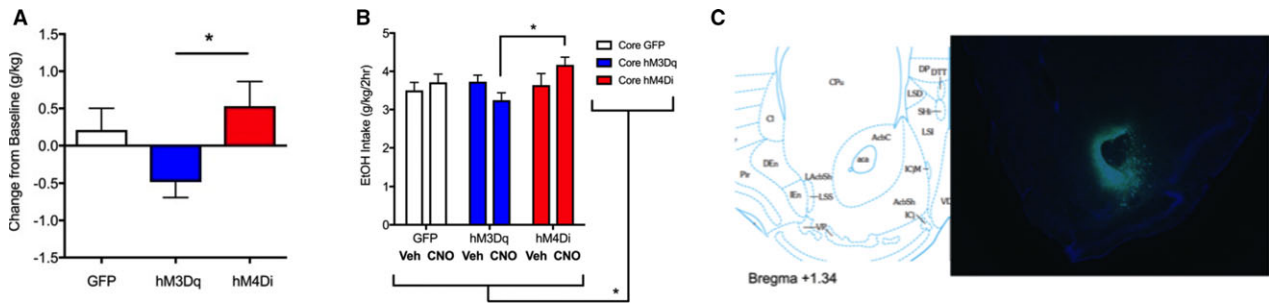


Fig. 4. Bidirectional modulation of NAc core activity and chronic binge-like EtOH drinking. **(A)** EtOH intake presented as average individual difference scores (intake during treatment with CNO – intake during treatment with vehicle) suggests bidirectional modulation of binge-like drinking in the mice treated with hM3Dq as compared with hM4Di, 1-way ANOVA, $F(2, 33) = 3.6$, $p < 0.05$. Bonferroni post hoc $*p < 0.05$. **(B)** Two-way ANOVA identified a significant group \times treatment interaction, $F(2, 33) = 3.57$, $*p < 0.05$, with a Bonferroni post hoc test revealing significant differences between hM3Dq and hM4Di groups in response to CNO treatment ($*p < 0.05$). **(C)** Diagram (left; from Franklin and Paxinos, 2007) and 4 \times image (right) of coronal section showing targeting of NAc core (where green indicates viral transduction and blue indicates DAPI stain). $n = 11$ to 13/group. [Color figure can be viewed at wileyonlinelibrary.com]

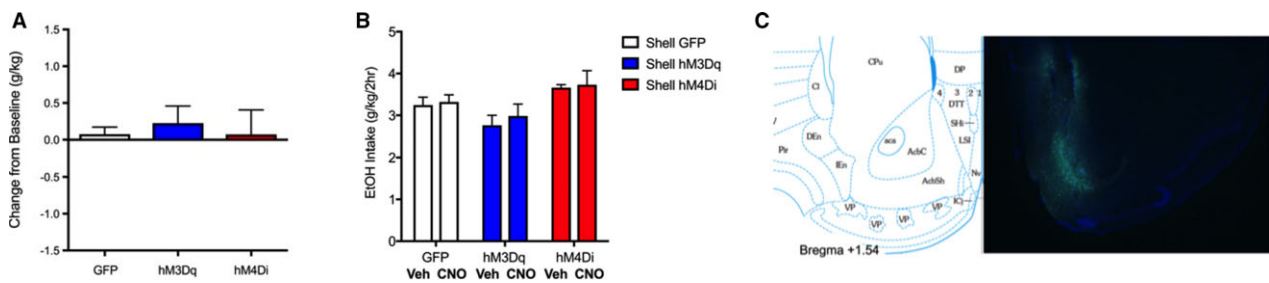


Fig. 5. Altering NAc shell activity did not alter chronic binge-like EtOH drinking. **(A)** EtOH intake presented as difference score (individual intake during treatment with CNO). One-way ANOVA N/S. **(B)** Two-way ANOVA identified a significant main effect of group, $F(2, 30) = 3.97$, $p < 0.05$, but not treatment or treatment \times group interaction (suggesting changing NAc shell activity did not affect binge-like drinking). **(C)** Diagram (left; from Franklin and Paxinos, 2007) and 4 \times image (right) of coronal section showing targeting of NAc shell (where green indicates viral transduction and blue indicates DAPI stain). $n = 9$ to 14/group. [Color figure can be viewed at wileyonlinelibrary.com]

reduce craving for morphine, heroin, and cocaine (Guo et al., 2013; Ma et al., 2013; Vassoler et al., 2013). One potential therapeutic caveat to DBS is that its effects can be short lasting. The studies we report here with DREADDs offer the potential for longer lasting modulation of neuroplasticity via GPCR manipulations. It will require further studies for us to determine how long the increased NAc activity can be maintained, and whether its effect to reduce binge-like drinking will persist.

Studies report that stimulation of either the NAc core or shell (or both) is effective in reducing drug-related behaviors. The core-shell-dichotomy has been elaborated mainly in rodents, whereas in humans, these subregions are less well characterized. Here, we report subregion specific effects of manipulating activity via CNO/DREADDs on binge-like drinking in C57BL/6J mice. We found that increasing activity in the NAc core reduced binge-like drinking, whereas decreasing activity appeared to increase drinking. These “bidirectional” effects were significant, but modest. It is possible that the modest effect size could be attributed to the short duration of action of CNO, which limits the time of the scheduled alcohol access period, and makes it difficult to observe large increases or decreases in alcohol intake. The inhibitory DREADD effects were not observed when

manipulating activity either in both the core and shell of the NAc or in the NAc shell alone. This phenomenon could be due to the role of the NAc core subregion in specific neurocircuitry engaged in binge-like drinking. For example, the core has reciprocal projections with other regions known to be important for motivated behaviors, as well as alcohol behaviors (e.g., prefrontal and infralimbic cortex, ventral pallidum, and basolateral amygdala) (Ozburn et al., 2015a,b). The effects appeared more robust for mice from the NAc (core + shell) hM3Dq group. It is not clear if this is because viral transduction was also present in the core and shell border region. The core-shell border region is not well studied in binge-like drinking. Howard and colleagues (2009) reported increases in DA in the border area between the core and shell when male rats are exposed to alcohol self-administration cues and during self-administration, whereas increases in DA were observed in the core and shell only during cue exposures. As discussed in Namburi and colleagues (2016), there exist specific populations of neurons which have been found to differentially respond to or impact appetitive and aversive behaviors. It is important to note there exist functional, anatomical, and genetic characteristics which all contribute to the representation of valence in the NAc shell. Perhaps the reason we did not observe effects of medial shell

manipulation on binge-like drinking is because our manipulations spanned rostral to caudal regions of the medial shell (generally, rostral medial shell has been associated with positive valence and caudal medial shell is associated with negative valence). Together, these findings suggest further study of the organization of the NAc as it relates to binge-like drinking is warranted.

An additional consideration in the interpretation of these findings is that we used CNO/DREADDs, which are GPCRs, to produce physiologically relevant changes in activity. Recent work has shown that lesions, pharmacological inactivation, and DBS of the NAc decrease alcohol intake in animal models (Cassataro et al., 2014; Dhaher et al., 2009; Wilden et al., 2014). These manipulations likely have much more robust consequences on activity and circuits than CNO/DREADD-induced alterations in neuronal activity (which result in approximately a 30% increase or decrease in activity in the accumbens, which may only last for a few hours). Thus, we propose the effects of CNO/DREADDs on DID we observed here are mediated via more subtle effects on cell signaling and circuit level. Cassataro and colleagues (2014) reported that chronic NAc inhibition (using CNO stimulation of inhibitory DREADDs) reduced limited access drinking in male mice. It is possible that there exist sex differences in response to CNO/DREADDs effects on binge-like drinking as the results of our experiments in female mice are the opposite of those reported by Cassataro and colleagues (2014). Future work will focus on carrying out these experiments in both male and female mice. Further differences between these studies include CNO dose (not reported by Cassataro et al., 2014) and routes of administration. Cassataro and colleagues (2014) administered CNO via unmeasured tap water (not during EtOH drinking), thus it is possible NAc activity was not inhibited during the EtOH access (CNO plasma levels are not detectable 2 hours after injection; Guettier et al., 2009).

Another possible interpretation of these findings is that removal of the CNO/DREADD-induced inhibition of the NAc resulted in a rebound firing effect (consistent with findings that increased firing decreases alcohol intake). In studies where DREADDs are expressed in specific brain regions via viral-mediated gene transfer (e.g., Navarro et al., 2016; Pleil et al., 2015; Rinker et al., 2017; Vardy et al., 2015; Zhu et al., 2014), behavioral effects of CNO (administered via IP or intracranial injection) are observed for periods of time ranging from 30 minutes to 4 hours. Notably, Navarro and colleagues (2016) observed CNO-induced reductions in alcohol drinking (and other consummatory behaviors) for a period of 2, but not 4 hours (where hM4Di was expressed in the lateral hypothalamus). Rinker and colleagues (2017) observed a reduction in 2-hour alcohol intake when hM4Di was expressed in the bed nucleus of the stria terminalis (BNST) and CNO was administered directly into the ventral tegmental area. Additionally, Pleil and colleagues (2015) observed that CNO reduced alcohol intake for 4 hours in mice-expressing hM4Di in the BNST. However, in studies where

transgenic mice express DREADDs, the behavioral effects of CNO can last for several hours (Alexander et al., 2009; Guettier et al., 2009). Thus, studies to date indicate that the effects of changing activity using CNO and DREADDs may depend on a number of factors, including route of CNO administration, expression levels of DREADDs, and connectivity with other regions. Additionally, sex as a biological variable is often overlooked. Many of these types of studies have only been performed in males. We know very little about the effects of sex on these types of manipulations. The inclusion of sex as a biological variable in preclinical alcohol research will increase scientific rigor, and lead to a better understanding of alcohol-related brain circuitry, pharmacology and drug discovery, and improve translation to the clinic. And, a final caveat is the recent report (MacLaren et al., 2016) that CNO may have active metabolites. Addressing this possibility was beyond the scope of the current studies, but should be evaluated in the future.

We have long known that the NAc plays an important role in reward-related processes (Everitt et al., 1991). Thus, manipulating neuronal activity of this region may be rewarding, or alter the perceived rewarding effects of other stimuli. Reduced drinking of EtOH might result from a general reduction in interest in any rewarding stimuli. Alternatively, CNO treatment could have altered taste thresholds for sweet or bitter, or could have reduced thirst in general or induced a state of general malaise. Bull and colleagues (2014) addressed the effects of CNO/DREADDs on both reward and EtOH drinking in a recent study. They expressed excitatory DREADDs selectively in NAc core astrocytes and found that CNO increased cytosolic calcium in primary astrocytes, modestly facilitated responding for rewarding brain stimulation, and reduced the motivation for EtOH (Bull et al., 2014). These results suggest that NAc core astrocytes can modulate sensitivity to rewarding stimulation. We assessed whether the DREADD-induced changes in neuronal activity were accompanied by altered tastant or fluid intake and found no significant effects of treatment. As CNO treatment failed to reduce sucrose intake significantly, we tentatively propose that CNO/DREADDs do not alter the reinforcing effects of this natural reward. That intake of the highest concentration of sucrose appeared to be lower, however, means that we cannot entirely rule out an effect based on calories or sweet taste. Due to the challenging nature of these experiments, and the need to analyze data from only those animals with verified placements, our sample sizes were also fairly small, so increased numbers of subjects might have detected a reduction in 5% sucrose drinking. Our data show definitively that CNO did not alter aversion for the bitter taste of quinine, or produce malaise or general thirst reduction, as neither quinine nor water intake was affected.

These studies are the first to systematically study alterations in neuronal activity in the NAc and its subregions, the core and shell, using CNO/DREADDs and measuring alcohol-related behaviors. There are strengths and caveats to the studies presented here. Our approach included replication:

increasing NAc activity reduced binge-like drinking across 3 separate experiments. Importantly, we did not observe sensitization or tolerance to repeated CNO administration (data not shown). In addition to the interpretational caveats discussed above, we did not measure blood EtOH levels, as we would have needed to perform repeated blood sampling and this would have been stressful to the animals and possibly have interfered with behavior. Although the reductions in binge drinking were significant, but modest, it is likely that they are pharmacologically relevant. Notably, even modest reductions in alcohol drinking can robustly decrease achieved blood alcohol levels (see fig. 3A from Rhodes et al., 2005, reporting correlation between achieved blood alcohol concentrations and EtOH intake for C57BL/6J mice). Last, another caveat is that we were unable to pseudorandomize animals to groups as all mice underwent surgery and were injected with different AAVs (hM3Dq, hM4Di, or GFP). This can sometimes lead to variable EtOH intake between groups (e.g., Fig. 5) and experiments (e.g., Figs 2 and 4). However, only mice with confirmed placements were included in the analyses. Therefore, we could not a priori pseudorandomize individuals to specific groups prior to the beginning of treatment to minimize group differences, which can lead to group differences in EtOH intake (at baseline). Conducting these studies with specific injections of an AAV into a subregion, especially in mice, is very challenging. Including baseline (vehicle) and CNO (treatment) periods, as well as always including an AAV-GFP group allows us to focus on changes within groups in response to treatment. We replicated these findings in 3 different experiments carried out at 2 different universities, as well as in 2 experiments carried out using another strain of mice (Ozburn et al., in press).

SUMMARY

Increasing NAc core + shell medium spiny neuron firing decreased binge-like alcohol drinking (acutely and chronically), but not tastant or fluid intake. Further, we found that manipulating activity in the NAc core can bidirectionally regulate binge-like drinking. Moving forward, we are using DREADD viral approaches in combination with the Cre/loxP system to further pursue cell-type and circuit characterization of binge-like drinking. Last, it will be important to determine whether chronically altering NAc activity induces plasticity on a behavioral, neuronal, transcriptome, and/or morphological level.

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CONFLICT OF INTEREST

The authors have nothing to disclose.

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