SUPPLEMENTAL MATERIAL

Nucleus accumbens cholinergic interneurons oppose cue-motivated behavior

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SUPPLEMENTAL MATERIALS AND METHODS

Surgery.

Standard surgical procedures described previously (58) were used for all surgeries. Rats were anesthetized with isoflurane (4–5% induction, 1–2% maintenance) and a nonsteroidal anti-inflammatory agent was administered pre- and post-operatively to minimize pain and discomfort. Following surgery rats were individually housed. Virus was infused into the NAc core at a rate of 0.1µl/min via an infusion needle. Following infusion, the injectors were left in place for 10 min, then slowly removed to prevent undesired viral spread.

Chemogenetic inhibition of NAc cholinergic interneurons. Rats were randomly assigned to a viral group prior to onset of behavioral training and were infused bilaterally with adeno-associated virus (AAV) cre-dependently encoding the inhibitory designer receptor *human M4 muscarinic receptor* (hM4D(Gi)); AAV2-hSyn-DIO-hM4D(Gi)-mcherry; Titer 3.7 x 10^{12}; Cohort 1: UNC-CH Vector Core, Chapel Hill, NC; Cohort 2: Addgene, Cambridge, MA) or control fluorophore only (AAV2-hSyn-DIO-mcherry; titer 5.6 x 10^{12} particles/ml; UNC-CH Vector Core). Virus (0.8µl) was infused into the NAc core (AP: +1.5 mm, ML: +/- 3.0, DV: -6.5, at a 9° angle).

Optical stimulation of NAc cholinergic interneurons. Rats were randomly assigned to a viral group prior to onset of behavioral training and were infused bilaterally with AAV cre-dependently expressing the excitatory opsin *channelrhodopsin-2* (ChR2; AAV5-EF1a-DIO-ChR2-eYFP; titer 4.8-7 x 10^{12} particles/ml; UNC-CH Vector Core) or control fluorophore only (AAV5-EF1a-DIO-eYFP; titer 6.5 x 10^{12} particles/ml, UNC-CH Vector Core). Virus was infused at 3 separate coordinates (AP: +1.3 mm, ML: $\pm$1.3, V: -6.8; AP: +1.3 mm, ML: $\pm$1.3, V: -6.0; AP: +1.3 mm, ML: $\pm$3.0, V: -6.5, at a 9° angle; 1 µl/infusion site; (58, 102)). At the same surgery, custom-, in-house-made optical fibers were implanted targeted at the NAc core (AP: +1.3 mm; ML: $\pm$1.3; V: -6.5). Prior to surgery, all optical fibers were tested for loss of power and only optical fibers with a loss of power >5% were used.

Optical stimulation of NAc cholinergic interneurons with inactivation of NAc β2-containing nAChRs. Prior to behavioral training rats were infused bilaterally with AAV cre-dependently expressing ChR2 as above. Custom-made microinfusion injector/optical fiber guide cannula (Doric Lenses, Quebec, QC, Canada) were implanted bilaterally targeted above the NAc core (AP: +1.3 mm; ML: ±1.3; V: -4.0).

Validation of chemogenetic manipulation of NAc cholinergic interneurons. Rats received 2 separate surgeries, spaced 5 days apart. In one, rats were infused unilaterally with AAV cre-dependently expressing ChR2. In the other, rats were unilaterally in the same hemisphere with AAV cre-dependently expressing hM4D(Gi). One rat received the ChR2 AAV first and hM4D(Gi) second, and the other received the hM4D(Gi) AAV first. In each case, AAV was infused at 3 separate coordinates (AP: +1.3 mm, ML: ±1.3, V: -6.8; AP: +1.3 mm, ML: ±1.3, V: -6.0; AP: +1.3 mm, ML: ±3.0, V: -6.5, at a 9° angle; 1 µl/infusion site; (58, 102)).

Validation of optical stimulation of nucleus accumbens cholinergic interneurons. Rats were infused bilaterally with AAV cre-dependently expressing ChR2 or the control fluorophore eYFP at 3 separate coordinates (AP: +1.3 mm, ML: ±1.3, V: -6.8; AP: +1.3 mm, ML: ±1.3, V: -6.0; AP: +1.3 mm, ML: ±3.0, V: -6.5, at a 9° angle; 1 µl/infusion site; (58, 102)).

Behavioral training and testing.

Apparatus. Training and testing took place in Med Associates conditioning chambers (East Fairfield, VT) housed within sound- and light-attenuating boxes, described previously (103). Behavioral testing for chemogenetic manipulations also occurred in these chambers. For optogenetic experiments, behavioral tests were conducted in Med associates conditioning chambers outfitted with an Intensity Division Fiberoptic Rotary Joint (Doric Lenses) connecting

the output fiberoptic patchcords to a laser (Dragon Lasers, ChangChun, JiLin, China) positioned outside the conditioning chamber.

All chambers contained a retractable lever that could be inserted to the left of a recessed food-delivery port in the front wall. A photobeam entry detector was positioned at the entry to the food port. The chambers were equipped with a pellet dispenser that delivered a single 45-mg chocolate pellet (Bio-Serv, Frenchtown, NJ) into the food port. Both a tone (1.5 kHz) and white noise generator were attached to individual speakers on the wall opposite the lever and food-delivery port. A 3-watt, 24-volt house light mounted on the top of the back wall opposite the food-delivery port provided illumination.

Chemogenetic inactivation of NAc cholinergic interneurons prior to consumption test. Following PIT testing (see main text), hM4D(Gi)-expressing subjects were tested for the influence of NAc cholinergic interneuron inactivation on food consumption. All subjects were habituated to the feeding chambers (home cages devoid of bedding). After this, they were given 2, 1-hr test sessions in which they had free access to chocolate pellets (the food paired used for conditioning) following an injection of vehicle or CNO, counterbalanced for order. A subset of hM4D(Gi)-expressing subjects (*N*=8) also received a set of tests for consumption of home chow. A subset (N=9) of the mCherry-control subjects also received these consumption tests. Food was weighed prior to an after the consumption test to quantify amount consumed.

Chemogenetic inactivation of NAc cholinergic interneurons prior to progressive ratio test. Following PIT testing, a subset of subjects (hM4D(Gi) *N*=8; mCherry *N*=9) were shifted to a progressive ratio schedule of reinforcement for which lever pressing earned a chocolate pellet and after each earned reinforcer the response requirement increased by 50% (rounded up, beginning with a single press requirement). The session ended when 3 min elapsed without a lever press. Breakpoint was recorded as the highest ratio achieved. Rats received 3 days of

progressive ratio training prior to 2 progressive ratio tests, with intervening retraining, one each following vehicle or CNO, order counterbalanced.

Drugs.

Clozapine-N-oxide. For the first cohort of hM4D(Gi) and mCherry subjects, Clozapine-N-oxide (CNO) was obtained from Tocris (Minneapolis, MN) and was dissolved in Dimethyl-sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), then diluted with 0.9% saline for a final 5% concentration of DMSO and a 5mg/mL concentration of CNO. Vehicle consisted of 5% DMSO in a 0.9% saline solution. For the second cohort, water soluble CNO was obtained from Enzo Life Sciences (Farmingdale, NY) and dissolved in sterile saline vehicle. In both cases, CNO was given at a 5mg/kg. This dose was selected based on prior demonstration of its effective and sufficiently long duration hM4D(Gi) activation (104). Vehicle and CNO were injected i.p. at 1 ml/kg 30 minutes prior to test.

Dihydro-β-erythroidine. The selective α4β2-containing nicotinic receptor antagonist Dihydro-β-erythroidine (DhβE) was obtained from R&D systems (Minneapolis, MN), dissolved in sterile ACSF and infused as described previously (58, 105) bilaterally into the NAc at 15 µg/0.5 µl/side over 1 min. Given our previous work demonstrating that bilateral general blockade of nicotinic acetylcholine receptors with mecamylamine can augment cue-motivated behavior (58), we selected a subthreshold dose of DhβE that would not on its own influence motivated behavior when infused into the NAc (106). This avoided the confound of an independent effect of the drug when it was paired with optical stimulation of NAc cholinergic interneurons.

Optical stimulation.

Light was delivered to the NAc using a laser (Dragon Lasers, ChangChun, JiLin, China) connected through a ceramic mating sleeve (Thorlabs, Newton, NJ) to the ferrule implanted on

the rat. We used a 473 nm laser to activate ChR2-transfected projection neurons. For optical stimulation of NAc cholinergic interneurons in behaving subjects, blue (473 nm) light pulses (5 msec pulse width, 10 mW) were delivered at 10 Hz for 120s duration. Stimulation parameters were selected to match the upper range of the endogenous firing rate of striatal cholinergic interneurons (64, 65) and were found to reliably elicit acetylcholine release in the NAc *in vivo* (Figure 2D). Light effects were estimated to be restricted to the NAc core based on predicted irradiance values (https://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php).

We also assess the influence of chemogenetic inhibition of NAc cholinergic interneurons over optically-evoked acetylcholine release. In this case, blue light pulses blue (473 nm) light pulses of varying magnitudes (10 msec pulse width, 5-30 mW) were delivered at 20 Hz for 5-s duration to evoke transient acetylcholine release.

Choline biosensors.

Biosensor fabrication. Silicon wafer-based platinum microelectrode array (MEA) probes were fabricated in the Nanoelectronics Research Facility at UCLA as described previously (103, 107, 108) and modified for choline detection using a method similar that we have described for glutamate detection (62, 103, 107, 108). These biosensors use choline oxidase (ChOx) as the biological recognition element for choline and rely on electro-oxidation, via constant-potential amperometry (0.7 V versus a Ag/AgCl reference electrode), of enzymatically-generated hydrogen peroxide reporter molecule to provide a current signal. This current output is recorded and converted to choline concentration using a calibration factor determined *in vitro* prior to sensor implantation. Choline sensing allows for an accurate proxy measure of extracellular acetylcholine, which is rapidly hydrolyzed by endogenous acetylcholinesterase (109-112). Indeed, adding acetylcholinesterase onto the sensing electrode does not enhance detection of cholinergic activity (110). Enzyme immobilization was accomplished by manually loading enzyme mixture consisting of ChOx (4 µl of 0.5 u/µl ChOx) and bovine serum albumin (2 µl of 60mg/ml BSA) on the

microelectrode sites, following by topping a layer of bis(sulfosuccinimidyl)suberate (100mg/ml BS3) for crosslinking. Interference from electroactive anions and cations is effectively excluded from the amperometric recordings, while still maintaining a subsecond response time, by electropolymerization of m-phenylenediamine (m-PD), as well as dip-coat application of Nafion to the electrode sites prior to enzyme immobilization. Electrodes were coated with 5 mM m-PD at 850 mV, followed by dip-coating in 2% Nafion, and cured at 115° C for 20 min. Each MEA had two non-enzyme-coated sentinel electrodes for the removal of correlated noise from the choline sensing electrodes by signal subtraction, as described previously (103, 108). These electrodes were prepared identically with the exception that the BSA solution did not contain ChOx.

Reagents. Choline oxidase (ChOx, from Alcaligenes sp), Nafion (5 wt.% solution in lower aliphatic alcohols/H_2O mix), bovine serum albumin (BSA, min 96%), m-phenylenediammine (m-PD), choline chloride (>99%), L-ascorbic acid, 3-hydroxytyramine (dopamine) were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Bis(sulfosuccinimidyl)suberate (BS3) was purchased from Thermo Fisher Scientific (Pittsburgh, PA).

Instrumentation. Electrochemical preparation of the sensors was performed using a Versatile Multichannel Potentiostat (model VMP3) equipped with the 'p' low current option and low current N' stat box (Bio-Logic USA, LLC, Knoxville, TN). *In vitro* calibration and *in vivo* measurements were conducted using a low-noise multichannel Fast-16 mkIII potentiostat (Quanteon LLC, Nicholasville, KY), with reference electrodes consisting of a glass-enclosed Ag/AgCl wire in 3 M NaCl solution (for *in vitro*, Bioanalytical Systems, Inc., West Lafayette, IN) or a 200 µm diameter Ag/AgCl wire (*in vivo*). All potentials are reported versus the Ag/AgCl reference electrode. Oxidative current was recorded at 80 kHz and averaged over 0.25-s intervals.

In Vitro Biosensor Characterization. All biosensors were calibrated *in vitro* to test for sensitivity and selectivity to choline prior to implantation. A constant potential of 0.7 V was applied to this

working electrodes against a Ag/AgCl reference electrode in 40 ml of stirred PBS at pH 7.4 and 37° C within a faraday cage. After the current detected at the electrodes equilibrated (~15-30 min), aliquots of choline were added to the beaker to reach final concentrations in the range of 20-100 μM choline. A calibration factor based on analysis of these data was calculated for each electrode. The average calibration factor the sensors used in these studies was 37.42 (s.e.m.=6.77) μM/nA. Control electrodes, coated with P(m-PD), Nafion, and BSA/BS3, but not ChOx, showed no detectable response to choline. Aliquots of ascorbic acid (250 μM final concentration) and dopamine (5-10 μM final concentration) were added to the beaker as representative examples of readily oxidizable potential anionic and cationic interferent neurochemicals, respectively, to confirm selectivity for choline. For the sensors used in these studies, no current changes above the level of the noise were detected to the addition of cationic (dopamine) or anionic (ascorbic acid) interferents, as reported previously (103, 113). To assess uniformity of H_2O_2 sensitivity across control and ChOx-coated electrodes, aliquots of H_2O_2 (10 μM) were also added to the beaker. There was less than a 10% difference in the H_2O_2 sensitivity on control electrode sites relative to enzyme-coated sites, indicating that any changes detected *in vivo* on the enzyme-coated biosensor sites following control channel signal subtraction could not be attributed to endogenous H_2O_2.

Approach validation.

Validation of chemogenetic inhibition of cholinergic interneurons. Pre-calibrated, silicon wafer-based platinum microelectrode array choline biosensors were packaged with an optical fiber affixed to the back surface of the sensor (to reduce the photovoltaic artifact) such that the tip of the optical fiber was ~100 μm above the sensor tip. Rats expressing ChR2 and hM4D(Gi) in cholinergic interneurons of the NAc were anesthetized with isoflurane (induced at 5% and maintained at 1-2%) and placed in a stereotaxic frame. Breaths per minute (bpm) were maintained at 1 bpm by adjusting isoflurane level. The biosensor/optical fiber probe was lowered into the NAc

core (AP: +1.3 mm; ML: ±1.3; V: -6.5 - 6.8) and connected to the potentiostat for application of a 0.7 V potential. Oxidative current was recorded at 80 kHz and averaged over 0.25 s intervals. Following equilibration of the amperometric signal (~30 min) laser stimulations were delivered via the optical fiber to evoke choline fluctuations and sensor placement was optimized along the DV axis to a recording location with reliable evoked choline fluctuations. Rats were then injected with sterile saline vehicle. Blue light (473 nm, 20 Hz, 5-30 mW, 10-ms pulse width, 5-s duration) was delivered to evoke acetylcholine release. Stimulations were spaced at least 30 s apart and the average current response across a 1-s period, 5 s prior to stimulation onset served as the baseline. Following completion of the stimulation protocol, rats were given CNO i.p., as described for the behavioral experiments above. 45 min later the stimulation protocol was repeated to determine the extent to which CNO activation of hM4D(Gi) attenuated optically-evoked acetylcholine release. Final ChR2+hM4D(Gi) *N*=4 recording locations in 2 subjects.

Validation of optical stimulation of cholinergic interneurons. Rats expressing ChR2 or eYFP in cholinergic interneurons of the NAc were anesthetized and implanted with a biosensor/optical fiber probe into the NAc (as above). Amperometric signal was recorded and sensor placement optimized as above. Blue light (473 nm, 10 Hz, 10 mW, 5-ms pulse width, 120-s duration) was delivered at the exact parameters used for the behavioral experiment to evaluate its ability to evoke acetylcholine release in ChR2-expressing subjects. Stimulations were spaced at least 60 s apart and the average current response across a 1-s period, 10 s prior to stimulation onset served as the baseline. Final ChR2 *N*=5 recording locations in 4 subjects, eYFP *N*=5 recording locations in 3 subjects.

Histology.

Rats were deeply anesthetized with Pentasol (390mg/mL; 100mg/kg) and transcardially perfused with PFA. Brains were removed and post-fixed in 4% formalin for 20 hours, after which they were

placed in 30% sucrose solution for 2-3 days. Brains were then frozen and sliced on a cryostat into 40-µm sections and stored in PBS.

We used ChAT immunoreactivity to visualize cholinergic interneurons. Sections were washed in PBS and blocked in 3% normal donkey serum with 0.3% triton X-100 for 2 hours at room temperature. Following washes in PBS, the tissue was incubated in goat-anti-ChAT (1:200; Millipore Sigma, Burlington, MA) at 4° Celsius for 20 hrs. It was then washed in PBS and incubated for 2 hrs at room temperature with either donkey-anti-goat RHD red (1:400; Millipore Sigma) or donkey anti-goat Cy2 (1:400; Abcam, Cambridge, UK).

To verify ChR2-eYFP expression, tissue sections were washed in PBS and blocked in 3% normal goat serum and 0.5% triton X-100 for 1.5 hrs. Following 3 PBS washes, tissue was incubated with mouse anti-GFP (1:1000, Abcam) 3% NGS and 0.5% triton X-100 at 4° Celsius for 20 hrs. Following 3 PBS washes, sections were incubated in goat anti-mouse Alexa 488 (1:1000, Abcam).

To verify hM4D(Gi)-mCherry expression, tissue sections were washed in PBS and blocked in 10% normal goat serum and 0.5% triton X-100 for 1.5 hrs. Following 3 washes at 5 min each, tissue was incubated with rabbit anti-mCherry (1:1000, ThermoFisher Scientific, Waltham, MA) at 4° C for 20 hrs. Following 3 blocking washes at 10 min each, tissue was incubated for 2 hrs with goat anti-rabbit alexa fluor 594 (1:500, ThermoFisher Scientific).

All sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and imaged using a Keyence (BZ-X710) microscope (Osaka, Osaka Prefecture, Japan) with a 4X, 10X, or 20X objective (CFI Plan Apo), CCD camera, and BZ-X Analyze software. Tiled images were taken of whole sections, including 6-8 sections per rat containing the NAc across the A/P axis based on the Paxinos and Watson brain atlas (101).

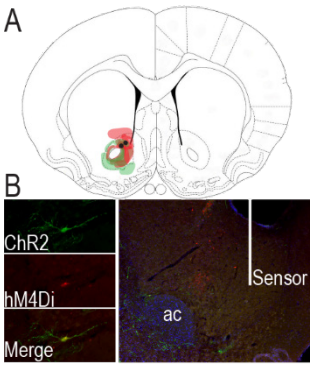
Data analysis.

Progressive ratio behavioral analysis. For the progressive ratio tests, lever press rate (presses/min) was calculated for the entire session and breakpoint was calculated as the highest ratio achieved before a >3-min break in lever pressing.

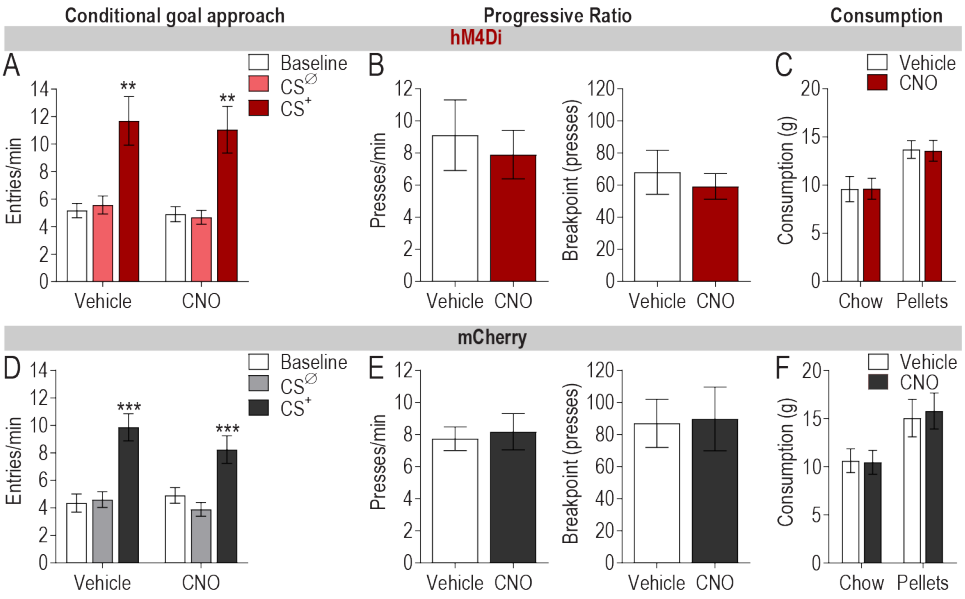
Amperometric analysis. Analysis details and characterization of release events have been described previously (62, 103, 108, 114). Electrochemical data were baseline-subtracted. Detected current was averaged across a 1-s period 5 or 10 s (as above) prior to stimulation and was subtracted from current output at each time point. Current changes from baseline on the PPD/Nafion-coated sentinel electrode were then subtracted from current changes on the PPD/Nafion/ChOx choline biosensor electrode to remove correlated noise. This signal was then converted to choline concentration using an electrode-specific calibration factor obtained *in vitro*.

	Pavlovian		Instrumental	
	preCS	**CS**	**Entries**	**Presses**
hM4D(Gi)	9.55	18.55**	8.66	19.11
	1.55	*3.67*	*1.43*	*1.55*
mCherry	10.73	19.436***	12.63	16.09
	1.08	*2.13*	*1.33*	*1.57*
ChR2	10.29	17.62*	9.07	16.47
	1.32	*3.05*	*1.18*	*2.72*
eYFP	9.08	13.64*	7.77	14.50
	1.19	*2.98*	*0.93*	*1.20*
ChR2 + pharmacology	8.52	16.62**	11.32	19.69
	0.91	*2.48*	*1.80*	*1.80*

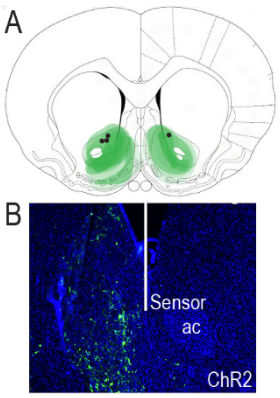
Supplemental Table 1. Training data. Training data from the last day of Pavlovian and instrumental training averaged across subjects for each experimental group. Pavlovian data are shown as food-port entries/min (s.e.m. below) averaged across the 2-min baseline periods immediately prior to CS^+ onset and the food-port entries/min during CS^+ probe period (after CS onset, but prior to reward delivery). In all cases, rats entered the food port more during the CS probe period than during the baseline periods. Instrumental data are shown as food-port entries/min (Entries) and lever presses/min (Presses) averaged across the entire session. *P<0.05, **P<0.01, ***P<0.001.



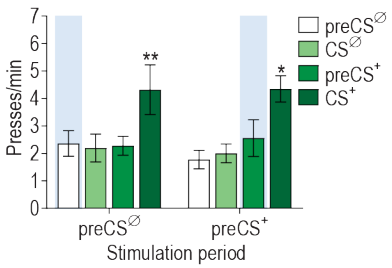
Supplemental Figure 1. Histological verification of hM4D(Gi) + ChR2 co-expression in NAc cholinergic interneurons. (**A**) Schematic representation of ChR2-eYFP and hM4D(Gi)-mCherry expression and sensor placement the NAc for both subjects. Slide represents 1.00 mm anterior to bregma. (**B**) Representative immunofluorescent image of ChR2-eYFP and hM4D(Gi)-mCherry-expressing cholinergic interneurons in the NAc with biosensor track. AC, anterior commissure.



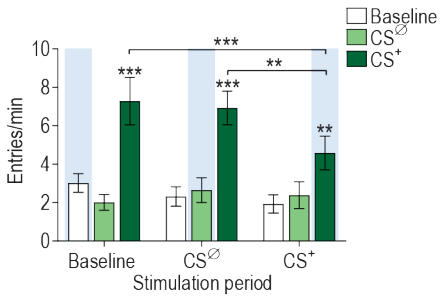
Supplemental Figure 2. Chemogenetic activation of NAc cholinergic interneurons does not alter conditional approach behavior, lever pressing during a progressive ratio test, or food consumption. (**A**) Effect of chemogenetic inactivation of NAc cholinergic interneurons on Pavlovian conditional food-port approach responding during PIT. *N*=19. Following either vehicle or CNO, the CS^{+} significantly elevated entries into the food-delivery port relative to baseline and CS$^{\varnothing}$ periods (CS period: $F_{2,36}$=19.63, *P*=0.0002; Drug: $F_{1,18}$=1.08, *P*=0.31; CS x Drug: $F_{2,36}$=0.18, *P*=0.76). (**B**) Following PIT testing, a subset of subjects (*N*=8) were given instrumental retraining then transitioned to a progressive ratio schedule of reinforcement for which after each earned reinforcer the response requirement increased by 50% (rounded up, beginning with a single press requirement). Following 3 days of progressive ratio training, rats were given 2 progressive ratio tests, with intervening retraining, one following vehicle and one following injection of CNO. Inactivation of NAc cholinergic interneurons did not influence either press rate (left; t_7=0.82, *P*=0.44) or break point (right, t_7=1.14, *P*=0.29) on the progressive ratio. (**C**) Following PIT, rats were tested for the influence of NAc cholinergic interneuron inactivation on consumption of the chocolate pellets (*N*=19) that had been paired with the CS^{+} and lever pressing and of home chow (*N*=8). NAc cholinergic interneuron inactivation with CNO relative to vehicle injection did not influence consumption of either food (Chow: t_7=0.09, *P*=0.93; Pellets: t_{18}=0.23, *P*=0.82). (**D**) There was no effect of CNO on Pavlovian conditional food-port approach responding during PIT in subjects lacking hM4D(Gi) (CS period: $F_{2,30}$=42.97, *P*<0.0001; Drug: $F_{1,15}$ =0.79, *P*=0.39; CS x Drug: $F_{2,30}$=2.51, *P*=0.12) (*N*=16). (**E**) There was no effect of CNO on lever pressing during a progressive ratio test in subjects lacking hM4D(Gi) (Press rate: t_8=0.32, *P*=0.74; Break point: t_8=0.18, *P*=0.86; *N*=9). (**F**) There was no effect of CNO on consumption of either home chow (t_8=0.32, *P*=0.76) or chocolate pellets (t_8=0.68, *P*=0.52) in subjects lacking hM4D(Gi) (N=9). Mean ±1 s.e.m. ***P<0.001.



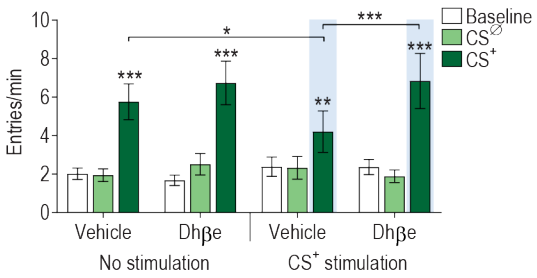
Supplemental Figure 3. Histological verification of ChR2 expression in NAc cholinergic interneurons for ChR2 validation subjects. (**A**) Schematic representation of ChR2-eYFP expression and sensor placement the NAc for all subjects. Slide represents 0.7 mm anterior to bregma. (**B**) Representative immunofluorescent image of ChR2-eYFP expressing cholinergic interneurons in the NAc with biosensor track. AC, anterior commissure.



Supplemental Figure 4. Optical stimulation of NAc cholinergic interneurons has no effect during either preCS^{+} or preCS^{Ø} baseline periods. There were two 'baseline' stimulation control periods: one in which NAc cholinergic interneurons were optically stimulated during each 2-min period prior to the CS^{+} and another in which NAc cholinergic interneurons were stimulated during the 2-min preCS^{Ø} periods. In both cases, there was no effect of stimulation on pressing during the baseline period or on the expression of PIT (CS period: $F_{3,24}$=9.60, P=0.0002; Stimulation period: $F_{1,8}$=0.05, P=0.82; CS x Stimulation period: $F_{3,24}$=0.34, P=0.79). Mean $\pm$1 s.e.m. *P<0.05, **P<0.01.



Supplemental Figure 5. Effect of optical stimulation of NAc cholinergic interneurons on food-port approach responses. Effect of optical stimulation of NAc cholinergic interneurons on food-port approach responding during PIT (CS period: $F_{2,16}$=27.9, P<0.0001; Stimulation period: $F_{2,16}$=2.04, P=0.16; CS x Stimulation period: $F_{4,32}$=2.979, P=0.03; N=9). During each test food-port entries were significantly elevated during CS^+ presentation. Stimulation of NAc cholinergic interneurons concurrent with CS^+ presentation did cause these to be fewer than during the control baseline or $CS^{\varnothing}$ stimulation sessions (P<0.01). This slight attenuation is expected given that 1) Pavlovian food-port entries are in part sensitive to the same incentive motivational processes supporting PIT and 2) because food-port entries become linked to instrumental actions during training (115, 116). Mean $\pm$1 s.e.m. **P<0.01, ***P<0.001. These data indicate that NAc CIN stimulation does not prevent the CS^+ from eliciting a significant Pavlovian conditional food-port approach response and suggest the subjects are still capable of recognizing the CS^+.



Supplemental Figure 6. Effect of optical stimulation of NAc cholinergic interneurons and intra-NAc DhβE on food-port approach responses. Effect of optical stimulation of NAc cholinergic interneurons during CS^+ following either intra-NAc vehicle or DhβE infusion on food-port approach responding during PIT (CS period: $F_{2,20}$=22.14, P<0.0001; CS^+ Stimulation: $F_{1,10}$=0.10, P=0.75; Drug: $F_{1,10}$=5.06, P=0.048; CS x Drug: $F_{2,20}$=8.21, P=0.002; Drug x Stimulation: $F_{1,10}$=0.08, P=0.78; CS x Drug x Stimulation: $F_{2,20}$=2.23, P=0.13; N=11). Food port entries were significantly elevated during the CS^+ for each test, though stimulation of NAc CINs concurrent with the CS^+ did slightly attenuate this. Mean ±1 s.e.m. *P<0.05, **P<0.01, ***P<0.001.