*Supplementary Information*

# Activating Corticotropin-Releasing Factor Systems in Nucleus Accumbens, Amygdala, and Bed Nucleus of Stria Terminalis: Incentive Motivation or Aversive Motivation?

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## Supplementary Methods

*Stereotaxic surgery*

Rats were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance) and placed in stereotaxic apparatuses (David Kopf, CA). Pre-surgery rats received atropine (0.05mg/kg; i.p.; Henry Schein) and post-surgery received cefazolin (75mg/kg, s.c.; Henry Schein) and carprofen, which was also provided for 2 days post-surgery (5mg/kg; s.c.; Henry Schein). Bilateral infusions in NAc, CeA, or BNST contained either active AAV-DIO-ChR2-eYFP virus (n=19 female, n=14 male), or optically-inactive control virus AAV-DIO-eYFP (n=10 female, n=9 male), both driven by EF1a promoters to infect only neurons containing Cre-recombinase. A separate group received halorhodopsin AAV-DIO-NpHR-eYFP (n=8 female, n=11 male) virus for CRF-containing neuronal inhibition (Fig. S6). NAc shell coordinates were: flat skull, from bregma A/P: +1.0 to +2.0, M/L: ±2.5 to 3.3, D/V: -6.5 to -7.2 (10º-16º; n=13 female, n=11 male). Lateral CeA coordinates were: A/P: -2.2 to -2.8, M/L: ±4.2 to 4.7, D/V: -7.2 to -7.6 (n=12 female, n=12 male). Dorsolateral BNST coordinates were: A/P: +0.24 to -0.24, M/L: ±3.6, D/V: -6.9, (16º; n=12 female, n=11 male). Sites were bilaterally identical within individuals but staggered across rats (Fig.1, Table 1). Rats received bilateral 1.0µl virus infusions (0.1µl/min) with 10min for diffusion, and optic fibers (200µm) 0.3mm above virus were secured with surgical screws and acrylic. Rats were monitored 7 days post-surgery, with 3 weeks for viral incubation.

*Two-choice sucrose*

Rats underwent an instrumental two-choice task to evaluate whether associative pairing of CRF-containing neuronal stimulation with earning one sucrose reward made it more or less desirable than an identical sucrose reward received without laser (1). Rats were first habituated to

sucrose pellets in home cages and underwent 1 day where pellets were delivered to the operant box sucrose dish freely every minute for 25min. Next, rats received 5 days of Pavlovian lever training in ~45min sessions, where one of two levers appeared in alteration every minute for 8-sec paired with a distinct tone or white noise assigned to each lever, which was followed by a sucrose pellet for rats to associate these levers and rewards.

Next, one lever was permanently assigned *Laser+Sucrose* for each rat (counter-balanced) and the other was assigned the *Sucrose-alone*. Training included one day of fixed ratio 1 (FR1) reinforcement, where rats could freely choose between both available levers in 30min sessions. Each *Laser+Sucrose* lever press earned a sucrose pellet, assigned tone, and laser illumination (8-sec). Responses on *Sucrose alone* lever earned a sucrose pellet and assigned tone only. Rats next underwent 3 days of FR1 with each session now beginning with a forced-exposure to each lever: only one lever was presented (random order) until the rat pressed it, that lever was repeated a second time, and then it was withdrawn while the other lever was presented twice. This was to remind rats of each lever outcome daily before choosing freely. The remainder of the 30min session had both levers available for free choice. Levers retracted for an 8-sec time out period following each reward earned. On days 4-8, the beginning forced-exposure to both levers continued and the schedule of reinforcement escalated: FR4 (day 4), random ratio 4 (RR4, day 5), and RR6 (days 6-8). Three additional RR6 days followed at the alternate laser frequency for each rat (10Hz or 40Hz). The separate group with inhibitory halorhodopsin virus underwent identical procedures with constant yellow laser illumination.

*Progressive ratio*

Progressive ratio (PR) tests assessed whether ChR2 stimulation of CRF-expressing neurons changed the magnitude of incentive motivation to earn sucrose reward. Rats were tested one day

with laser stimulation, using parameters identical to those in the two-choice task, and with only *Laser+Sucrose* lever available in a 30min session. A second test on a separate day was run withonly *Sucrose-alone* lever available, and without laser (counter-balanced order). A third test on *Laser+Sucrose* day followed but used the alternate laser frequency (10Hz/40Hz). Within each session, the number of responses required to earn next reward increased after each reward received, following PR=$[5e^{(reward\ number \times 0.2)}] - 5$, rounded to the nearest integer (1). Breakpoint or ratio-reached were compared between days. Separate halorhodopsin rats underwent similar PR tests with laser inhibition.

*Spout-touch self-stimulation*

Incentive properties of CRF-expressing neuronal stimulation alone without sucrose were tested in an instrumental spout-touch self-stimulation test. With two empty waterspouts available, each touch on a designated *Laser-spout* provided stimulation (3-sec; 10Hz/40Hz; 30min). Touches on the other *Inactive-spout* earned nothing, as a baseline exploration measure. Rats were classified on Day 1 as robust self-stimulators if they made 2x more touches on *Laser-spout* as on *Inactive-spout,* and made >50 *Laser-spout* touches (2). Others were classified as low-level self-stimulators if they made at least 10 *Laser-spout* touches and 2x more *Laser-spout* than *Inactive-spout* touches. Days 2-3 evaluated the consistency of self-stimulation. MedPC programs recorded responses. Pilot NpHR groups underwent similar testing with inhibitory yellow laser (constant Hz, 8-sec stimulation; Fig. S6)

*Place-based self-stimulation*

In another, relatively passive, place-based self-stimulation test, rats could earn laser self-stimulations by entering and remaining in a designated chamber within a 3-chamber apparatus (2

major, 1 smaller center). Rats started sessions in the center chamber. An initial session without laser evaluated baseline preference. Then for 3 test days, one side was designated the *Laser-delivering* chamber, with distinct contextual cues (dots/stripes, floor textures), and the opposite side was another no-laser chamber with distinct cues. Entry into the *Laser-delivering* chamber (>half-body) triggered onset of laser stimulation, which continued to cycle for as long as rats remained in that chamber (3-sec-on/4-sec-off; 10Hz/40Hz; triggered via MATLAB program), and terminated upon exit. Time spent in each chamber was scored by video (Noldus Observer XT 12). Difference-scores (*Laser-delivering* - *No-laser* seconds) were compared between groups. Pilot NpHR groups underwent similar testing with inhibitory yellow laser (constant Hz, cycling 8-sec-on, 4-sec-off; Fig. S6)

*Histology*

Brains were sectioned into 40 micrometer slices using a cryostat (Leica, Wetzlar, Germany). Tissue was rinsed for 10min in 0.1M sodium phosphate buffer (NaPB) three times and blocked with 5% normal donkey serum (60 min). Tissue was incubated overnight at room temperature in rabbit anti-cFos (1:2500; Catalog#: 226 003; Lot #: 4-63; RRID:AB_2231974; Synaptic Systems, Göttingen, Germany) and chicken anti-GFP (1:2000; Catalog#: AB13970; Lot #: GR3190550-30; RRID:AB_300798; Abcam, Cambridge, MA). Slices were rinsed 3x for 10min in 0.1M NaPB before incubation with biotinylated donkey anti-rabbit secondary (1:300; Catalog #: AB2340593; Lot #: 128703; RRID:AB_2340593; Jackson Immunoresearch, West Grove, PA) and donkey anti-chicken Alexa Fluor 488 (1:300; Code #: AB2340375; Lot #: 144438; RRID:AB_2340375; Jackson Immunoresearch, West Grove, PA) for 120min. Tissue was rinsed 3x for 10min in 0.1M NaPB before incubation with Streptavidin Cy3 (1:300; Catalog #: AB2337244, Lot #: 141873, RRID: AB_2337244; Jackson Immunoresearch, West Grove, PA) for

90min. Tissue was rinsed 3x for 10min in 0.1M NaPB, mounted onto slides, and coverslipped with Pro-long gold (Invitrogen). Images were taken using a digital camera (Qimaging, Surrey, BC, Canada) attached to a fluorescence microscope (Leica, Wetzlar, Germany) at sites surrounding optic fibers. Immunoreactivity was visualized with filters with excitation bands 515-545 for Fos protein and 490-510 for virus. Adobe Photoshop was used to adjust contrast and brightness.

*Local Fos plumes*

Local Fos plumes were evaluated by counting Fos+ neurons in 15 successive blocks (50x50um) along eight radial arms surrounding the fiber tip (2, 3). Neuron counting stopped once 2 consecutive blocks without Fos+ cells occurred, marking that arm's radius. Fos elevation was assessed as percent change from levels of respective illuminated inactive-eYFP virus controls who underwent identical conditions.

*RNAScope® Fluorescent In Situ Hybridization (ISH)*

Brains were rapidly dissected, and flash frozen in dry ice. Brains were equilibrized for at least 1 hour in a Leica cryostat and sectioned into 17µm slices. A total of ~12-20 slices per rat (n=3 female, n=3 male) were collected from *Crh*-Cre+ rats. Sections across the three slides per rat included sections of 1) nucleus accumbens shell and dorsal striatum, 2) dorsolateral BNST and globus pallidum, and 3) central amygdala and nearby amygdala nuclei. Slices were thaw mounted on Superfrost plus slides (Fischer) and stored at -80 C with desiccators. Procedures for ISH followed Advanced Cellular Diagnostics (ACD) manual for RNAscope® 2.0 assay and followed previous reports (4, 5).

Briefly, slides were fixed for 20 min (4º C) in 10% neutral buffered formalin and washed twice for 1 min each with PBS. Slides were dehydrated for 5 min with 50% ethanol, for 5 min with

70% ethanol, and twice for 5 min with 100% ethanol before overnight incubation at -20º C in 100% ethanol. The next day slides were first dried for 10 min (room temperature) and a hydrophobic barrier was drawn around the sections and dried for 15 min. Section were incubated with Protease Pretreat-4 for 20 min, washed twice for 1 min each with ddH20, and incubated with ACD probes Rn-*Crem*-03 (Catalogue# 530001) and Rn-*Crh*-C3 (Catalogue # 318931-C3) for 2 hours in the ACD HybEZ oven (40º C). Slides then underwent amplification steps in the HybEZ oven (40º C) with two 2 min washes between steps (at room temperature). These amplification (at 40º C) steps included 1) Amp 1 for 30 min, 2) Amp 2 for 15 min, 3) Amp 3 for 30 min, and 4) Amp 4-Alt A for 15 min. Sections were stained with a DAPI-containing solution at room temperature, coverslipped with ProLong Gold Antifade, and stored at 4º C until imaging.

Sections were imaged with a digital camera (Qimaging, Surrey, BC, Canada) attached to a fluorescence Leica DM microscope (Leica, Wetzlar, Germany). Images at 40x were taken of the NAc shell, CeA, and BNST with the same hardware and software settings for quantification, titrated for each probe. The number of cells expressing either *Cre* mRNA or *Crh* mRNA (containing >5 particles) were manually counted in core sample volumes (0.1mm x 0.1mm x 17µm boxes; placed to contain at least 1 CRF+ cell) of tissue in CeA, NAc shell, and BNST (CeA: n=3 female, n=3 male; NAc: n=3 female, n=4 male; BNST: n=3 female, n=3 male) (5).

## Supplementary Results

*Local Fos plumes*

Laser excitation of CRF-containing neurons in NAc ChR2 rats elevated local Fos expression surrounding optic fiber tips by 150-200% in Fos plumes of 0.22-0.36mm radius, over NAc eYFP control level at corresponding sites (Fig. 2). In CeA, ChR2 stimulation of CRF-containing neurons produced 150-200% elevated Fos plumes of 0.25–0.38mm radius, and in BNST produced 150-200% Fos plumes of 0.26-0.43mm radius (Fig. 2). These Fos plume sizes suggest that laser illumination of ChR2-infected CRF-containing neurons induced local zones of neural activation ~0.6-0.8mm in all three structures. Therefore 0.7mm diameter size was used for placement symbols in localization-of-function maps (Fig. 1).

*Fluorescent in situ hybridization*

The number of cells expressing either *Cre* mRNA or *Crh* mRNA (containing >5 particles) were counted in core sample volumes (0.1mm x 0.1mm x 17μm; placed to contain at least 1 CRF+ cell) of tissue in CeA, NAc shell, and BNST (CeA: n=3 female, n=3 male; NAc: n=3 female, n=3 male; BNST: n=3 female, n=3 male) (5). In CeA, CRF+ neurons and Cre+ neurons were densely concentrated within the lateral division of CeA (CeL), with an average density of 10.1±0.9 co-labeled Cre+/CRF+ neurons in a 0.1mm x 0.1mm area. CRF+ neurons made up 31.3% of neurons sampled within the CeL, with an average of 10.5±1.0 Cre+ and 10.6±1.0 CRF+ neurons per box. Similar densities were seen in females (34.0%) and in males (29.8%). Non-specific Cre was not typically observed as 96.4% of Cre+ neurons in CeA were co-labeled with *Crh* mRNA, and *Cre* mRNA was present in 95.3% of CRF+ neurons (Fig. 3).

In NAc, CRF+ neurons were sparsely distributed throughout the rostro-caudal axis of medial shell (+2.52 to +1.08mm AP). The density of Cre+/CRF+ neurons in NAc shell was 6.0±0.7 cells per 0.1mm x 0.1mm box, or approximately half that of CeA density. NAc CRF+ neurons made up 18.7% of neurons present in sample boxes, with an average of 6.3±0.7 Cre+ and 6.9±0.7 CRF+ neurons per box. Similar *Crh* mRNA expression was seen in the rostral (19.0% of neurons) and caudal (18.2%) accumbens shell. Similar densities were seen in females (18.4%) and in males (19.0%). Significant non-specific Cre expression was not observed, with 95.3% of Cre+ neurons also containing *Crh* mRNA, and 87.1% of CRF+ neurons also were Cre+.

In BNST, neurons expressing *Crh* mRNA were distributed throughout the dorsolateral BNST with an average density of 10.0±0.7 co-labeled Cre+/CRF+ neurons per 0.1x0.1mm box, similar as in CeA. BNST CRF+ neurons made up 23.2% of neurons sampled within the dorsolateral BNST, with an average of 11.0±0.9 Cre+ and 10.4±0.7 CRF+ neurons per box. Substantial non-specific Cre expression was not observed as 90.1% of Cre+ neurons were co-labeled with *Crh* mRNA, and *Cre* mRNA was present in 95.7% of CRF+ neurons. Similar densities were seen in females (19.7%) and in males (27.6%).

*Further analysis of potential sex differences for NAc & CeA groups*

Females and males both showed similar stimulation-induced incentive effects in NAc & CeA ChR2 groups on sucrose two-choice, sucrose breakpoint, and laser self-stimulation tests, but the N's of sex groups within each structure were too small for statistical comparison. Therefore, it seemed of interest to further combine data from the two structures, and to statistically compare females vs males for combined CeA and NAc groups (n=9 *Crh*-Cre+ ChR2 females, n=11 *Crh*-Cre+ ChR2 males). A power analysis based on our observed laser CRF-containing neuron ChR2 incentive effect sizes of 0.379 – 0.865 (partial $\eta^2$) in two-choice, breakpoint, and self-stimulation

results, indicated that groups of 2-4 of each sex would be required to achieve actual power of 0.97 – 0.99. Similarly, a related power analysis based CRF-related sex differences in a recent study (6), indicated that groups of n=6 of each sex would be required for actual power of 0.98. Our combined CeA/NAc sex groups at least exceeded these minimum N sizes.

In the two-choice sucrose test (laser effect size, partial $\eta^2 = 0.865$), female (n=7) and male (n=10) ChR2 rats did not differ in their strength of laser-induced preference for *Laser+Sucrose* over *Sucrose-alone* ($F_{1,15} = 1.06$, $p = 0.319$; CeA & NAc females = 9:1±1 ratio preference for *Laser+Sucrose* (452 ± 55 presses) over *Sucrose-alone* (57 ± 18); males = 8:1±1 ratio preference for *Laser+Sucrose* (386 ± 46 responses) over *Sucrose-alone*: 46 ± 15). Female and male ChR2 rats also did not differ in overall lever-pressing in the two-choice task ($F_{1,15} = 0.545$, $p = 0.472$).

In the progressive ratio test of sucrose motivation for CeA/NAc ChR2 rats (laser effect size, partial $\eta^2 = 0.832$), females and males did not differ in magnitude of enhancement of incentive motivation, both showing roughly 200% laser-induced increases in effort breakpoint (*Laser+Sucrose* females: 91 ± 10 breakpoint, n=6; males: 113 ± 9, n=7; *Sucrose alone* females: 44 ± 10; males: 56 ± 9; $F_{1,11} = 0.837$, $p = 0.380$). There was also no apparent sex difference in effort breakpoints achieved during progressive ratio regardless of laser effects ($F_{1,11} = 1.961$, $p = 0.189$).

In the spout-touch self-stimulation task (laser effect size, partial $\eta^2 = 0.830$), female (n=7) and male (n=7) *Crh*-Cre+ ChR2 rats did not differ in their number of *Laser-spout* self-stimulations (female = 17 ± 4 self-stimulations; male = 18 ± 4; $F_{1,12} = 0.12$, $p = 0.915$). Females and males also did not differ in their pattern of touches across the two spouts (*Inactive spout* touches: female = 7±3; male = 9±3; $F_{1,12} = 0.167$, $p = 0.690$). In the place-based self-stimulation task laser effect size, partial $\eta^2 = 0.379$), female and male *Crh*-Cre+ ChR2 rats did not differ in their preference for

the *Laser-delivering* chamber (females: 210% more time in *Laser-delivering* chamber than in *No-laser* chamber, n=7; males: 170%, n=7; $F_{1,12} = 0.269$, $p = 0.613$). There were no sex differences in time spent in each chamber (females = 547±73 seconds in *Laser-delivering* chamber: 255±43 sec in *No-laser* chamber; males = 519±74 sec in *Laser-delivering* chamber, 301±43 in *No-laser* chamber; $F_{1,12} = 0.34$, $p = 0.857$).

We recognize that anatomical and behavioral sex differences have been reported in CRF systems, for example in extended amygdala and NAc (6–17). However, our current data suggest incentive enhancement effects of ChR2 laser stimulation for CeA *Crh*-Cre+ and NAc *Crh*-Cre+ groups were similar for both females and males here, with roughly comparable magnitudes in both sexes. We acknowledge that future studies with larger groups could potentially find subtle sex differences for these CRF ChR2 effects in future, but we conclude that the categorical effects for positively-valenced vs negatively-valenced motivation induced by CRF-containing neuronal stimulation in CeA, NAc and BNST described here appear robust and shared across sexes.

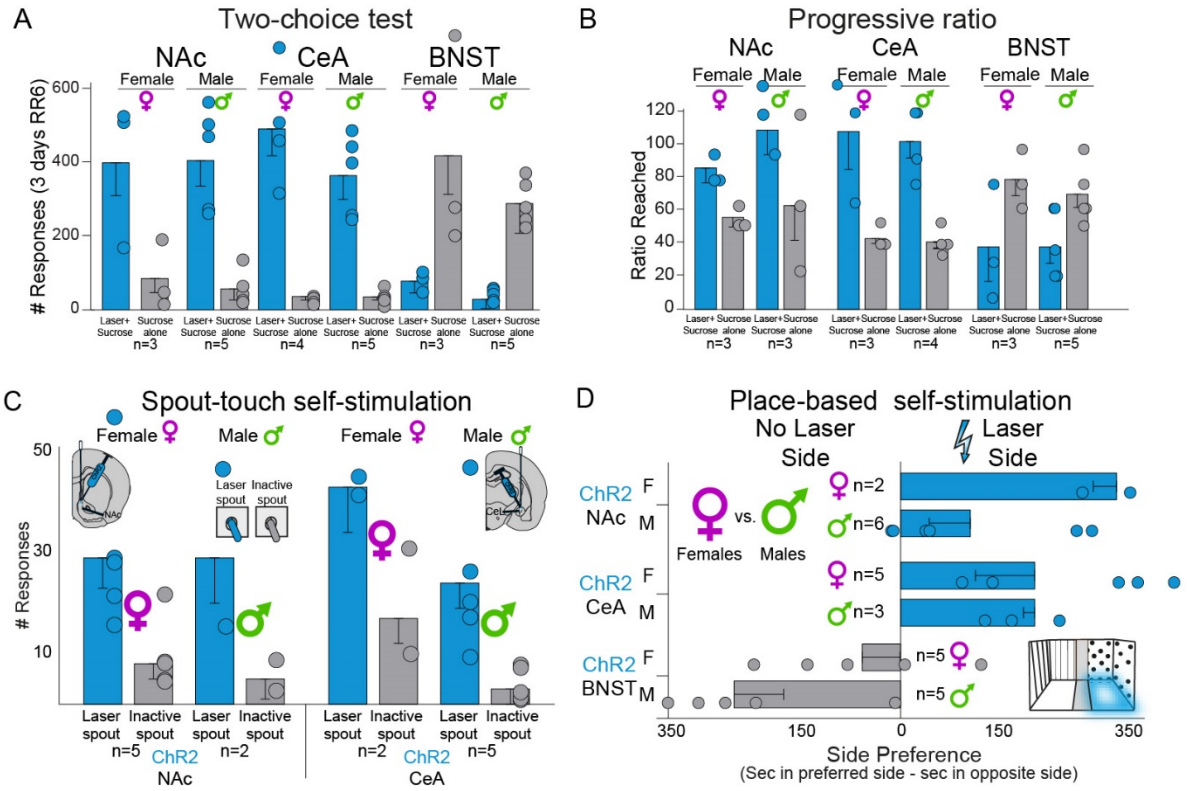


**Figure S1. Female and male groups across behavioral tests. A)** On average, male and female rats demonstrated similar levels of *Laser+Sucrose* preference in the two-choice test (NAc: n=3 female, 5:1 ratio preference; n=5 male, 7:1 ratio; CeA: n=4 female, 13:1 ratio; n=5 male, 10:1 ratio), while on average male BNST rats displayed a stronger opposite ratio preference for the *Sucrose-alone* option (n=5, 10:1 ratio) than female BNST rats (n=3 female, 5:1 ratio). **B)** On average in NAc (n=3 female, n=3 male) and CeA (n=3 female, n=4 male) groups, both female and male rats displayed ~200% increases in *Laser+Sucrose* breakpoint effort. In BNST groups, female (n=3) and male (n=5) rats showed similar ~50% suppression in *Laser+Sucrose* breakpoint effort. **C)** In the spout-touch task, laser self-stimulations were similar for NAc rats (n=5 female, n=2 male; days 2-3, 10Hz and 40Hz combined), though the small group of female CeA ChR2 rats (n=2) on average self-stimulated more than male CeA rats (n=5). **D)** On average, the small group of female NAc rats (n=2) displayed slightly higher levels of place-based self-stimulation than male

NAc rats (n=6), and male BNST ChR2 rats showed a stronger laser-avoidance on average (n=5 female; n=5 male). However, these small samples are not properly powered to detect meaningful sex differences across regions. Means and SEM reported.

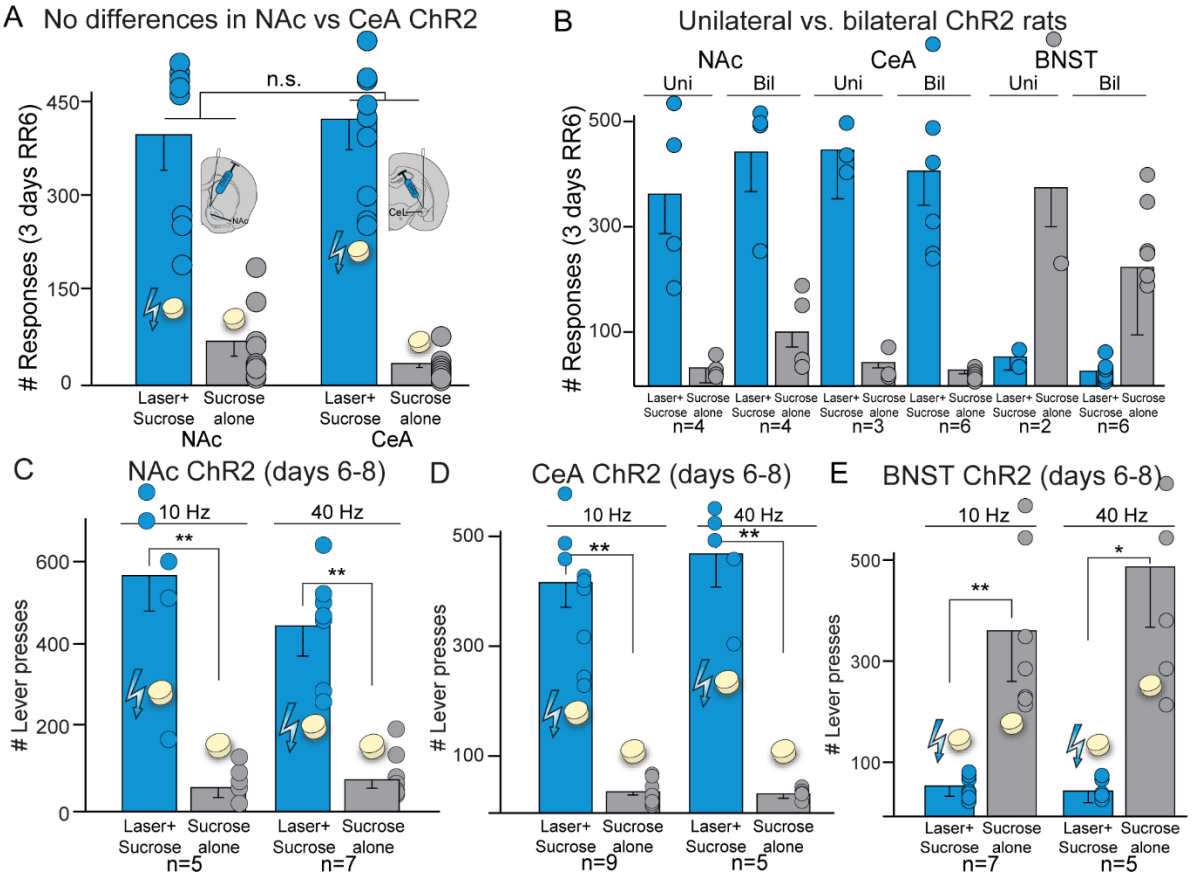


**Figure S2. Two-choice extended data. A)** When comparing incentive motivational effects on sucrose preference between brain regions, there was no difference in laser bias for NAc and CeA ChR2 rats in the two-choice task (mixed-model ANOVA, laser x group interaction, $F_{1,15} = 0.757$, $p = 0.398$) or total number of *Laser+Sucrose* lever presses induced by ChR2 pairings (two-way unpaired *t*-test, $t_{15} = 0.649$, $p = 0.526$). **B)** On average rats receiving unilateral ChR2 CRF-containing neuron excitation (NAc: n=4; CeA: n=3; BNST: n=2) demonstrated similar laser-

preference as those receiving bilateral excitation (NAc: n=4; CeA: n=6; BNST: n=6), though groups were too small to detect possible differences. **C)** Both 10Hz and 40Hz ChR2 excitation caused similar preference for *Laser+Sucrose* with no differences between frequencies for NAc laser-effects (10Hz: $F_{1,4} = 24.540$, $p = 0.008$, n=5; 40Hz: $F_{1,6} = 39.209$, $p = 0.001$, n=7; frequency x laser interaction: $F_{1,10} = 1.186$, $p = 0.302$), or **D)** CeA ChR2 laser-preferences (10Hz: $F_{1,8} = 59.101$, $p<0.001$, n=9; 40Hz: $F_{1,4} = 90.572$, $p = 0.001$, n=5; frequency x laser interaction: $F_{1,12} = 0.534$, $p = 0.479$). **E)** BNST ChR2 excitation during 3 days of RR6 showed similar *Laser+Sucrose* avoidance at both 10Hz ($F_{1,6} = 30.241$, $p = 0.002$, n=7) and 40Hz ($F_{1,4} = 9.474$, $p = 0.037$, n=5), with no differences between frequencies ($F_{1,10} = 0.996$, $p = 0.342$). Means and SEM reported. n.s., nonsignificant.

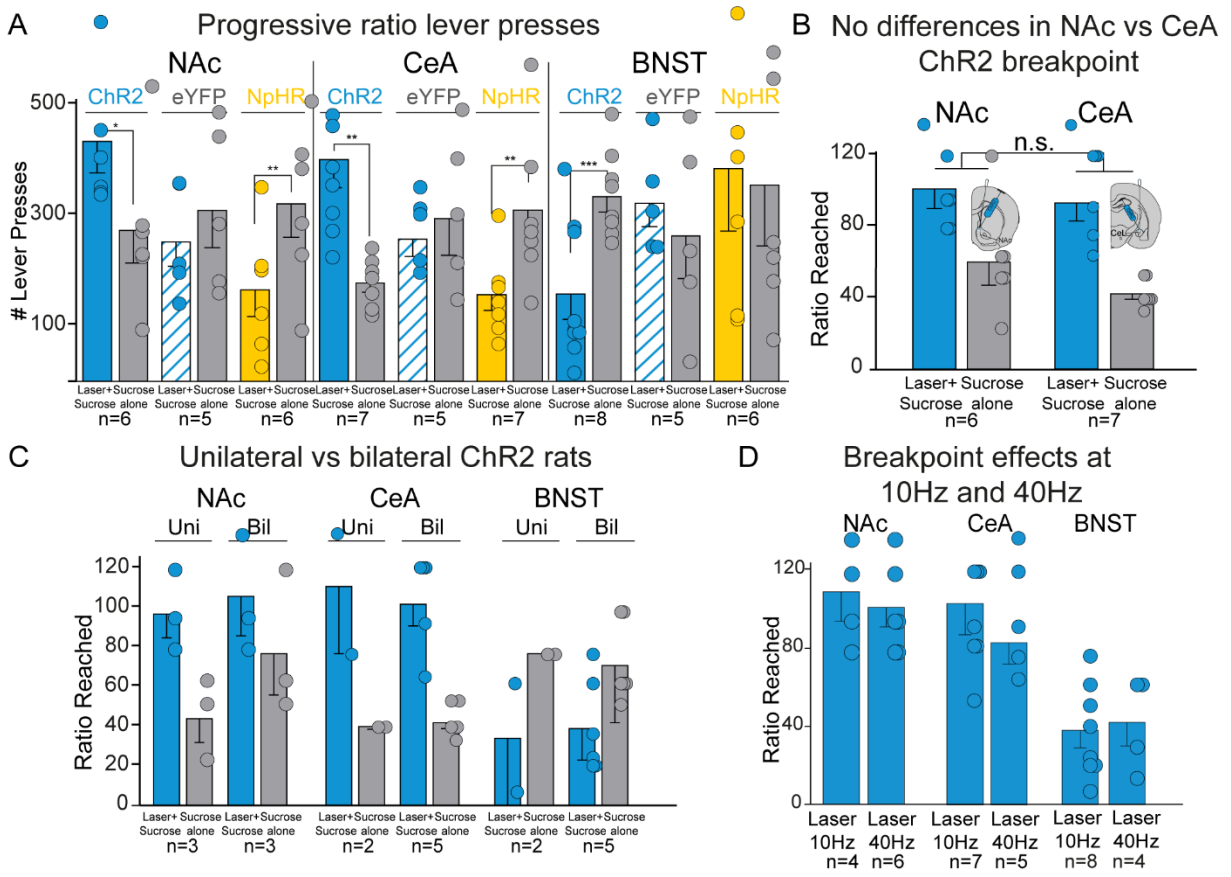


**Figure S3. Progressive ratio extended data. A)** NAc and CeA ChR2 animals pressed more for *Laser+Sucrose* than *Sucrose-alone* (two-way paired *t*-test, NAc: $t_5 = 4.015$, $p = 0.010$, 95% CI: [58,234], $d = 1.6$, n=6; CeA: $t_6 = 4.959$, $p = 0.003$, 95% CI: [113,333], $d = 2.48$, n=7), while BNST ChR2 rats responded at higher rates on the *Sucrose alone* day (two-way paired *t*-test, $t_7 = 6.178$, $p < 0.001$, 95% CI: [109,243], $d = 2.75$, n=8). eYFP rats responded equally between days across groups (two-way paired *t*-test, NAc: $t_4 = 0.788$, $p = 0.475$, n=5; CeA: $t_4 = 0.453$, p = 0.673, n=5; BNST: $t_4 = 0.506$, $p = 0.640$, n=5). CeA NpHR rats pressed less on the *Laser+Sucrose* day (two-way paired *t*-test, $t_6 = 4.631$, $p = 0.004$, 95% CI: [72,231], $d = 2.44$, n=7), as did NAc NpHR rats (two-way paired *t*-test, $t_5 = 4.659$, $p = 0.006$, 95% CI: [69,239], $d = 2.04$, n=6). BNST NpHR rats responded equally across PR days (two-way paired *t*-test, $t_5 = 0.365$, $p = 0.730$, n=6). **B)** When comparing incentive effects in sucrose motivation between brain regions, NAc and CeA ChR2 rats

demonstrated comparable levels of breakpoint enhancement from CRF-containing neuron excitation (mixed-model ANOVA, laser x group interaction, $F_{1,11}$ = 0.010, $p$ = 0.921). **C)** On average rats that received unilateral ChR2 stimulation (NAc: n=3; CeA: n=2; BNST: n=2) demonstrated comparable laser-based effects on sucrose motivation as rats that bilateral ChR2 (NAc: n=3; CeA: n=5; BNST: n=5), though groups are too small to meaningfully compare effects. **D)** Both 10Hz ($t_3$ = 4.841, $p$ = 0.017, n=4) and 40Hz ChR2 ($t_5$ = 6.010, $p$ = 0.002, n=6) excitation in NAc caused ~200% enhancements of breakpoint effort for *Laser+Sucrose*. CeA ChR2 stimulation of CRF-containing neurons also caused ~200% increases in laser-paired breakpoint similarly at 10Hz ($t_6$= 4.992, $p$ = 0.002 n=7) or 40Hz ($t_4$ = 4.3981, $p$ = 0.012, n=5). BNST ChR2 excitation during PR testing showed comparable ~50% reductions in laser-paired breakpoint at10Hz ($t_7$= 6.178, $p$<0.001, n=8) and 40Hz ($t_3$ = 5.333, $p$ = 0.013, n=4). Means and SEM reported. n.s., nonsignificant, *p<0.05, **p<0.01, ***p<0.001.

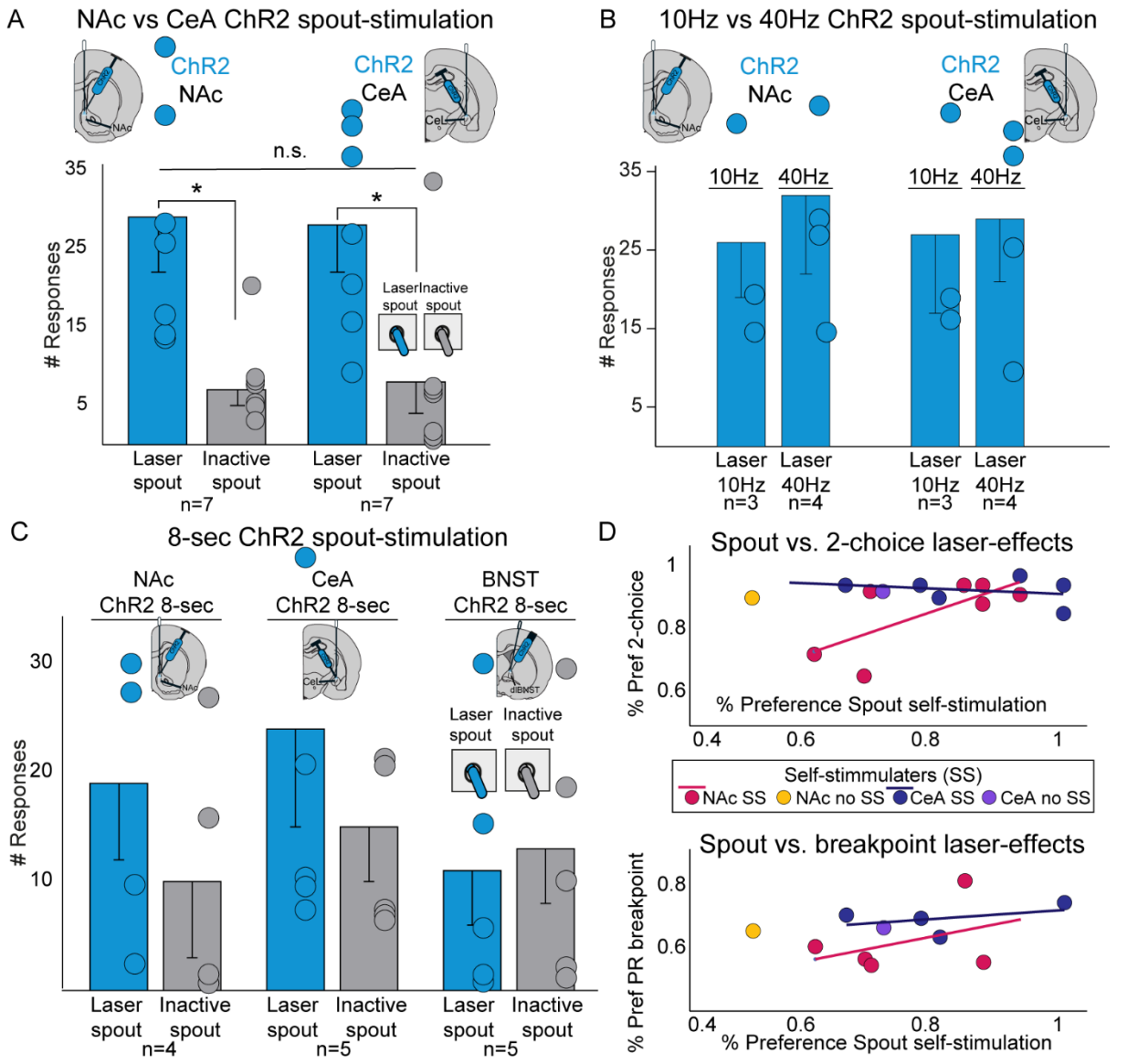


**Figure S4. Spout self-stimulation extended data. A)** There was no difference in the magnitude of self-stimulation between NAc (n=7) and CeA (n=7) ChR2 rats (days 2-3, 10Hz and 40Hz combined; laser x group interaction, $F_{1,12} = 0.002$, $p = 0.961$). **B)** Both 10Hz (NAc n=3; CeA n=3) and 40Hz (NAc n=4; CeA n=4) ChR2 excitation in NAc and CeA ChR2 self-stimulators caused similar self-stimulation for *Laser-spout* on average, though groups are underpowered to detect potential differences. **C)** A pilot experiment tested self-stimulation for 8-sec laser durations across ChR2 groups, though the present small pilot is not sufficiently powered to detect potential effects

(10Hz and 40Hz, 2-3mW, 8-sec bins). **D)** Correlations between percent preference for *Laser-spout* in spout self-stimulation task and percent preference or percent enhancements for *Laser+Sucrose* lever in two-choice and progressive ratio tasks. Correlations and lines depict data only from rats designated as self-stimulators, while non-self-stimulating individuals are depicted for comparison. Means and SEM reported. n.s., nonsignificant *p<0.05.

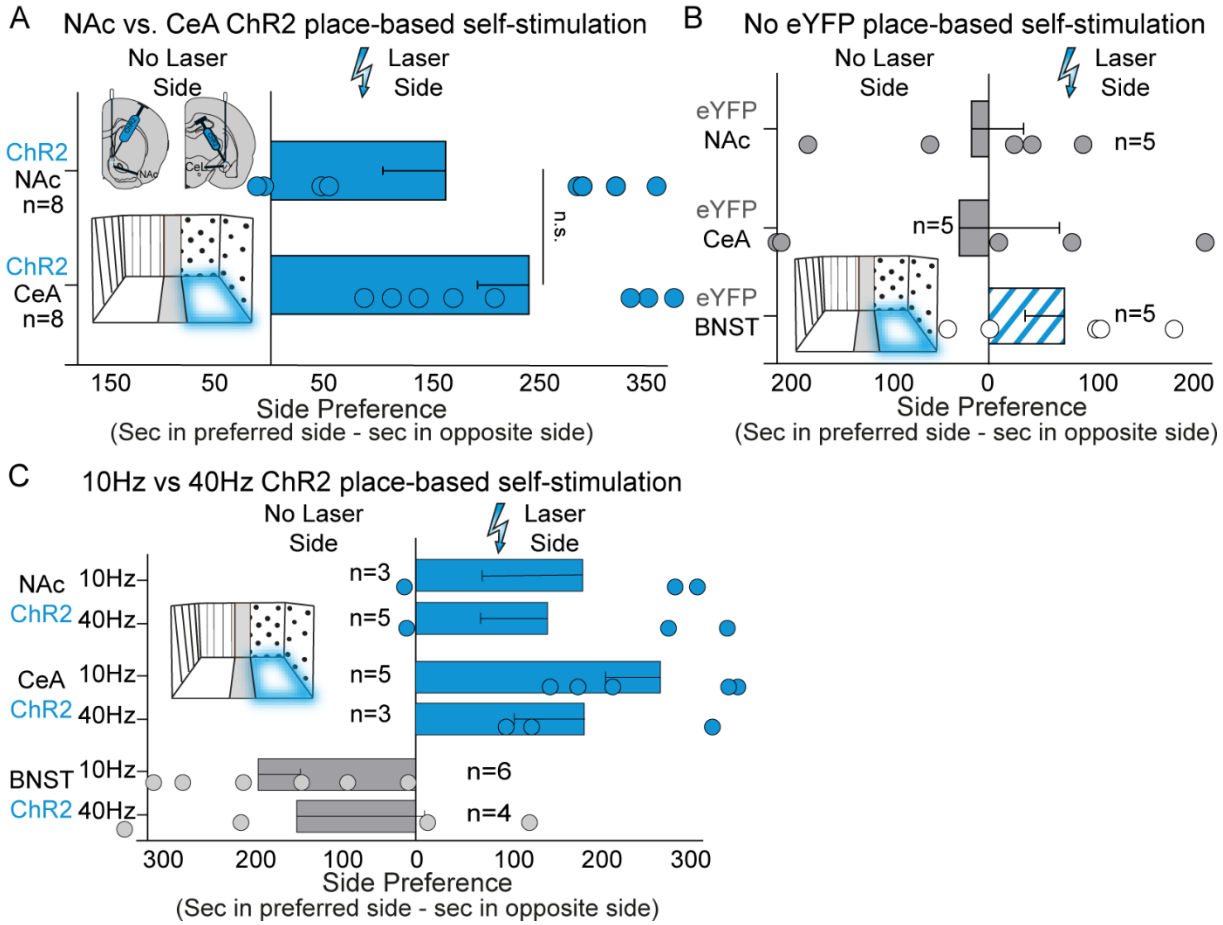


**Figure S5. Place-based self-stimulation extended data. A)** Both NAc ChR2 and CeA ChR2 sites supported comparable levels of place-based self-stimulation of CRF-containing neurons (laser x group, $F_{1,14} = 0.028$, $p = 0.871$). **B)** No significant laser-preference or avoidance was present for eYFP rats (main effect of laser, NAc: $F_{1,4} = 0.113$, $p = 0.754$, n=5; CeA: $F_{1,4} = 0.086$, $p = 0.784$,

n=5; BNST: $F_{1,4} = 3.726$, $p = 0.126$, n=5). **C)** Both 10Hz and 40Hz ChR2 excitation in NAc caused similar levels of self-stimulation, causing ~150% increases on average in time spent in the *Laser-delivering* chamber at 10Hz (n=3) and 40Hz (n=5). CeA ChR2 self-stimulation across frequencies was on average stronger at 10Hz frequency (n=5; 200±10% increase) than 40Hz (n=3; 150±25% increase), though groups are not properly powered to detect meaningful differences. *Laser-delivering* chamber avoidance was present in rats receiving BNST CRF-neuron excitation at both laser frequencies tested (n=6 10Hz, n=4 40Hz) causing ~50% decrease in time spent in *Laser-delivering* side on average in the current sample. Means and SEM reported. n.s., nonsignificant.

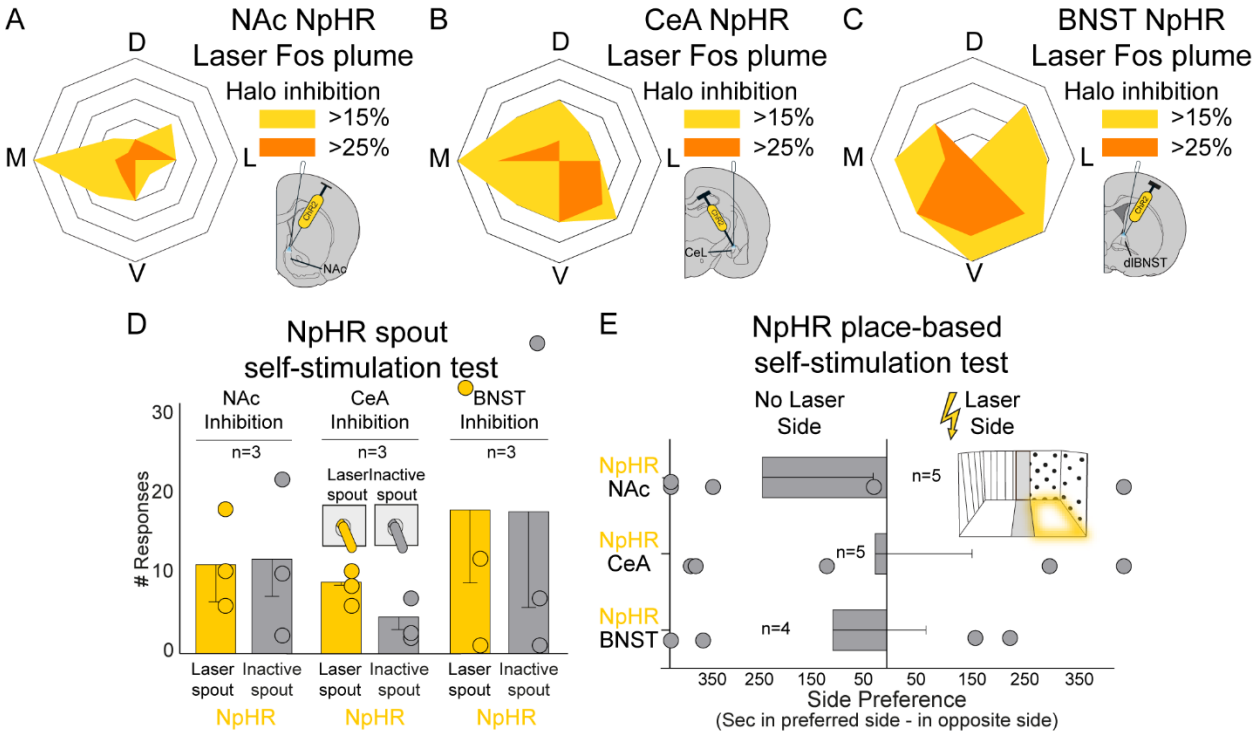


**Figure S6. Halorhodopsin pilot data. A)** Average Fos plume in *Crh*-Cre+ NpHR rats after laser inhibition targeting NAc CRF-expressing neurons (plumes of 0.11-0.22mm radius from fiber tip), **B)** CeA CRF-expressing neurons (plumes 0.08-0.16mm radius), and **C)** BNST CRF-expressing neurons (0.09-0.14mm radius; >15% suppression from eYFP control baseline: yellow; >25% suppression from eYFP: orange). D: dorsal, M: medial, L: lateral, V: ventral. **D)** NAc, CeA, and BNST NpHR pilot rats in the spout self-stimulation test responded minimally and equally between *Laser-spout* (8-10mW; constant; 8-sec) and *Inactive-spout*, though groups are underpowered to properly detect laser-effects (NAc n=3; CeA n=3; BNST n=3). **E)** Inhibition of CRF-expressing neurons in NAc (n=5), CeA (n=5), or BNST (n=4) NpHR rats does not support place-based self-stimulation or avoidance, at least in these current pilot groups. Means and SEM reported.

| **NAc CRF neurons** | **Fos+ count** | | **ChR2 vs. eYFP Unpaired t-test, p-value** | | **Confidence Interval** | **Effect size** |
|---|---|---|---|---|---|---|
| Region | NAc ChR2 (n=6) | NAc eYFP (n=5) | *t* | *p* | 95% CI | *d* |
| IF | 58.5 ± 6.4 | 47.8 ± 2.7 | 0.68 | *0.52* | | |
| OFC | 64.2 ± 1.8 | 54.2 ± 9.4 | 0.37 | *0.72* | | |
| NAcC | 87.7 ± 3.8 | 49.4 ± 2.7 | 7.89 | *<0.001** | 27, 49 | *5.06* |
| aVP | 62.7 ± 1.4 | 36.4 ± 3.6 | 7.29 | *<0.001** | 18, 34 | *4.61* |
| pVP | 68.6± 1.7 | 28.0 ± 2.4 | 13.56 | *<0.001** | 34, 48 | *8.73* |
| aBNST | 60.0 ± 1.9 | 36.6 ± 2.7 | 6.66 | *<0.001* | 15, 31 | *4.25* |
| pBNST | 84.6 ± 3.4 | 39.4 ± 2.7 | 10.48 | *<0.001** | 35, 55 | *6.45* |
| aLH | 65.7 ± 2.9 | 36.2 ± 1.7 | 8.37 | *<0.001** | 21, 37 | *5.46* |
| pLH | 67.2 ± 3.3 | 30.4 ± 2.5 | 8.62 | *<0.001** | 27, 46 | *5.41* |
| PVN | 58.5 ± 3.5 | 49.6 ± 3.1 | 1.87 | *0.10* | | |
| MeA | 66.8 ± 4.2 | 34.8 ± 3.8 | 5.58 | *<0.001** | 19, 45 | *3.42* |
| CeA | 65.2 ± 3.3 | 30.6 ± 1.3 | 9.00 | *<0.001** | 26, 43 | *6.29* |
| BLA | 44.8 ± 4.6 | 40.7 ± 4.3 | 0.34 | *0.74* | | |
| VTA | 56.0 ± 1.2 | 27.8 ± 3.0 | 9.27 | *<0.001** | 21, 35 | *5.76* |
| SN | 32.2 ± 3.2 | 25.6 ± 5.0 | 1.15 | *0.28* | | |
| PAG | 52.3 ± 3.6 | 40.6 ± 4.9 | 1.97 | *0.08* | | |
| **CeA CRF neurons** | **Fos+ count** | | **ChR2 vs. eYFP Unpaired t-test, p-value** | | **Confidence Interval** | **Effect size** |
| Region | CeA ChR2 (n=6) | CeA eYFP (n=5) | *t* | *p* | 95% CI | *d* |
| IF | 52.0 ± 4.9 | 39.4 ± 2.2 | 2.18 | *0.057* | | |
| OFC | 71.2 ± 4.6 | 46.4 ± 2.1 | 4.52 | *0.001* | 12, 37 | *3.12* |
| aNAcSh | 63.3 ± 3.5 | 37.2 ± 1.7 | 6.28 | *<0.001** | 17, 36 | *4.21* |
| pNAcSh | 90.0 ± 8.7 | 35.2 ± 4.2 | 5.28 | *0.001** | 31, 78 | *3.56* |
| NAcC | 95.8 ± 9.6 | 25.8 ± 6.8 | 5.72 | *<0.001** | 43, 98 | *3.62* |

| | | | | | | |
|---|---|---|---|---|---|---|
| aVP | 61.0 ± 2.3 | 28.2 ± 2.0 | 10.65 | *<0.001** | 36, 40 | *6.62* |
| pVP | 62.0 ± 2.4 | 29.2 ± 2.1 | 10.02 | *<0.001** | 25, 40 | *6.19* |
| aBNST | 85.7 ± 6.1 | 28.8 ± 2.0 | 8.17 | *<0.001** | 41, 73 | *5.90* |
| pBNST | 104.3± 7.0 | 41.6 ± 5.0 | 6.97 | *<0.001** | 42, 83 | *4.41* |
| aLH | 55.3 ± 2.7 | 27.6 ± 4.1 | 5.86 | *<0.001** | 17, 38 | *4.20* |
| pLH | 53.7 ± 2.0 | 30.6 ± 0.6 | 9.93 | *<0.001** | 18, 28 | *7.33* |
| PVN | 65.2± 10.3 | 51.4 ± 6.1 | 1.09 | *0.30* | | |
| MeA | 60.5 ± 2.6 | 32.2 ± 3.3 | 6.82 | *<0.001** | 19, 38 | *4.13* |
| BLA | 54.5 ± 3.1 | 37.0 ± 6.3 | 2.65 | *0.027** | 3, 32 | *1.62* |
| VTA | 55.2 ± 7.0 | 33.6 ± 2.4 | 2.72 | *0.023** | 4, 40 | *1.94* |
| SN | 34.2 ± 4.2 | 29.6 ± 2.3 | 0.90 | *0.39* | | |
| PAG | 47.7 ± 3.1 | 47.6 ± 2.0 | 0.02 | *0.99* | | |
| **BNST CRF neurons** | **Fos+ count** | | **ChR2 vs. eYFP Unpaired t-test, p-value** | | **Confidence Interval** | **Effect size** |
| Region | BNST ChR2 (n=5) | BNST eYFP (n=4) | *t* | *p* | 95% CI | *d* |
| IF | 53.6 ± 6.2 | 47.5 ± 3.1 | 0.81 | *0.45* | | |
| OFC | 64.0 ± 7.7 | 49.5 ± 4.9 | 1.49 | *0.18* | | |
| aNAcSh | 65.0 ± 5.0 | 47.5 ± 5.9 | 2.28 | *0.056* | | |
| pNAcSh | 68.6 ± 7.2 | 47.8 ± 1.9 | 2.50 | *0.041** | 1, 41 | *2.08* |
| NAcC | 74.6 ± 5.6 | 43.3 ± 2.5 | 4.69 | *0.002** | 16, 47 | *3.58* |
| aVP | 47.8 ± 5.8 | 33.8 ± 4.6 | 1.69 | *0.13* | | |
| pVP | 63.2 ± 2.5 | 37.8 ± 0.86 | 8.752 | *<0.001** | 19, 32 | *7.06* |
| aLH | 67.8 ± 1.2 | 34.3 ± 1.5 | 17.44 | *<0.001** | 29, 38 | *11.6* |
| pLH | 65.4 ± 2.5 | 34.5 ± 2,6 | 8.49 | *<0.001** | 22, 40 | *5.72* |
| PVN | 73.8 ± 2.8 | 36.5 ± 1.6 | 10.74 | *<0.001** | 29, 46 | *7.94* |
| MeA | 72.4 ± 6.3 | 45.0 ± 4.0 | 3.45 | *0.011** | 9, 46 | *2.49* |
| CeA | 42.6 ± 3.9 | 29.0 ± 2.5 | 2.79 | *0.03** | 2, 25 | *1.95* |
| BLA | 72.0 ± 2.8 | 38.3 ± 2.1 | 9.26 | *<0.001** | 25, 42 | *6.48* |
| VTA | 43.8 ± 5.4 | 41.8 ± 3.6 | 0.30 | *0.77* | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SN | 41.0 ± 1.9 | 34.5 ± 2.5 | 2.07 | *0.077* | | |
| PAG | 71.0 ± 2.4 | 40.8 ± 5.0 | 5.84 | *0.001** | 18, 42 | *3.92* |

**Table S1. Brain-wide Fos activation following CRF-containing neuron excitation in NAc, CeA, or BNST.** Table shows Fos+ protein quantification in mesocorticolimbic regions after final exposure to ChR2 excitation in NAc (top; n=3 female, n=3 male ChR2 group), CeA (middle; n=3 female, n=3 male ChR2 group), or BNST (below; n=2 female, n=3 male ChR2 group). Fos+ protein quantification in mesocorticolimbic regions (left columns), for ChR2 rats and eYFP rats. "Fos+ Count" reflects mean of each group at each site ± standard error (SEM). Two-sided unpaired t-tests between ChR2 and eYFP rats were performed for each target group (NAc, CeA, or BNST). Also see Fig. 4. IF, infralimbic cortex; OFC, orbitofrontal cortex; aNAcSh, anterior nucleus accumbens shell; pNAcSh, posterior nucleus accumbens shell; NAcC, nucleus accumbens core; aVP, anterior ventral pallidum; pVP, posterior ventral pallidum; aBNST, anterior bed nucleus of stria terminalis; pBNST, posterior bed nucleus of stria terminalis; aLH, anterior lateral hypothalamus; pLH, posterior lateral hypothalamus; PVN, hypothalamic paraventricular nucleus; MeA, medial amygdala; CeA, central amygdala; BLA, basolateral amygdala; VTA, ventral tegmentum; SN, substantia nigra; PAG, midbrain periaqueductal gray. *p<0.05, **p<0.01, ***p<0.001

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