Supplemental Materials and Methods

Drugs: Cocaine-HCl, norbinaltorphimine-HCl (norBNI), and ±U50488 were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD) and were dissolved in 0.9% saline. Sodium pentobarbital, Beuthanasia Special-D, and isoflurane were obtained from University of Washington Medical Center Drug Services. CP 94253-HCl, GR 127935-HCl, and GR 125487 were purchased from Tocris Bioscience and dissolved in artificial cerebrospinal fluid (ACSF).

Viral reagents: CAV2-DIO-ZsGreen was provided by Dr. Larry Zweifel (University of Washington). UNC Vector Core or Addgene provided: AAV5-DIO-EYFP (UNC/ Addgene #27056), AAV5-DIO-SwiChR$_{CA}$-EYFP (UNC), AAV5-DIO-ChR2-EYFP (UNC/ Addgene #20298), AAV5-EGFP (#105547), AAV5-Cre-EGFP (Addgene #105545), and AAVrg-DIO-EYFP (Addgene #27056). Viral suspensions were stored at -80°C until use and injected undiluted **(**2×10^{12} - 3×10^{13} vg/ml).

Animals: Adult (8-20wk) male C57BL/6 mice and transgenic strains on C57BL/6 genetic background were group housed (2-5/cage), given access to food pellets and water *ad libitum*, and maintained on a 12hr light:dark cycle (lights on at 7AM). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee and conformed to US National Institutes of Health guidelines. We obtained *Slc6a4*-Cre (SERT-Cre) mice from the GENSAT project (MMRRC:017260-UCD), *Oprk1*-Cre (KOR-Cre) mice from Dr. Sarah Ross (University of Pittsburgh) [1], *Pdyn*-IRES-Cre (Pdyn-Cre) and *Pdyn*-lox/lox (Pdyn-flx) mice from Dr. Richard Palmiter (University of Washington), *Slc17a8*-Cre (VGluT3-Cre**)** and *Oprk1*-lox/lox (KOR-flx) from Jackson Labs (MGI:5823257, MGI:5316477). KOR^{SERT} conditional knockout (cKO) mice were generated as previously described [2].

General behavioral methods: Mice were kept in the same housing facility in which behaviors were assayed for at least 1 week prior to experimentation. For all optogenetic experiments, controls were Cre^{+} mice injected with AAV-DIO-EYFP instead of the active opsin. Cage changes were conducted no less than 3 days prior to behavioral testing to minimize confounding effects of environmental stress exposure. Mice were habituated to handling daily for 3 days prior to the initiation of each experiment. All experiments were conducted on mice naïve to prior treatment, except for the optical stimulation during rFSS and social approach assays, which were conducted 2 weeks after the completion of cocaine CPP. EthoVision Software (Version 3.0 & 11.0, Noldus Information Technology) was used to assess movement and generate path heatmap graphics. Experiments were conducted in sound-attenuating behavioral rooms with medium-intensity lighting.

Stereotaxic Surgery: For aseptic surgery, mice were anesthetized in an induction chamber with 4% isoflurane before placement into a stereotaxic frame (David Kopf Instruments Model 1900) where they received 1-2% isoflurane as described previously [2]. Viral injections were performed using Hamilton Neuros syringe (Sigma-Aldrich) at a rate of 100nl/min (500nl for all behavioral studies, 750nl for tracing studies). The syringe was left in place for 5min following the injection. Injection sites were as follows: DRN (AP -4.35, ML 0, DV-2.7; 20° angle) or NAc (AP+1.35, ML +-0.7, DV -4.6) and optic fibers (Doric) were placed 0.5mm above the target site. All NAc viral

injections for behavioral studies and optical stimulation were bilateral and unilateral for retrograde tracing. For drug microinfusion, guide cannula (Plastics One #C235G/SPC-1.4mm) were placed above the NAc (AP +1.35, ML +-0.7, DV -4.1), with internal cannula projecting 0.5mm past the guide cannula. Implants were secured using Metabond (Parkell) and dental cement (Stoelting). Following surgeries, mice were given carpofen for 5 days to reduce inflammation and pain and allowed time for recovery and viral expression (10 days for infusion studies, 4 weeks for somatic optical stimulation, and 5 weeks for terminal optical stimulation and anatomical tracing studies).

Forced Swim Stress: Mice were subjected to a modified Porsolt forced swim stress (rFSS) as described previously [3]. All swim sessions were conducted in 31±1ºC water. On day 1, mice received a 15min initial swim, followed 22hr later by four 6min swims, each separated by 6min. After each swim, mice were removed from the water, towel dried, and returned to their home cage.

Optogenetic stimulation during rFSS: Mice were connected to the optical tether 1min prior to swim sessions on day 1 and 2, and they remained tethered throughout the swim session. Mice were visually monitored during swim sessions. Mice that submerged due to impaired swimming were removed from the water and excluded from subsequent analysis (3 EYFP-injected and 2 ChR2-injected mice were excluded). Optical stimulation was delivered 1min prior to the initial swim and 6min prior to the second and fourth swim on the day 2.

Cocaine conditioned place preference: Mice were assayed in a balanced place conditioning apparatus with distinct visual and tactile cues in each chamber as previously described [2,4]. On day 1, an initial preference test was conducted for place preference bias. Conditioning occurred on days 2 and 3, consisting of cocaine administration and 30min confinement to the drug-paired chamber in the morning (15mg/kg, IP) and saline administration (10ml/kg, IP) and confinement to the other chamber 4hr later. On day 4, mice were allowed to freely explore the apparatus for a postconditioning assessment in the absence of drug. Preference tests and conditioning sessions lasted for 30min and were conducted in sound attenuating chambers.

Manipulations prior to cocaine conditioning: Repeated forced swim stress prior to cocaine conditioning: mice were subjected to rFSS (as described above) 30min after the initial preference test on day 1 and before cocaine conditioning on day 2, terminating 10min prior to cocaine administration.

Optogenetic inhibition of DRN subtypes prior to cocaine conditioning: VGluT3-Cre and SERT-Cre mice expressing EYFP or SwiChR were tethered to fiber optic cables coupled to a 473-nm laser in an empty cage bottom and received optical stimulation (0.33 Hz, 15ms pulse duration) for 30min on each conditioning day. Following optical stimulation, mice were returned to their home-cage 30min prior to each cocaine conditioning session.

Optogenetic excitation of $SERT^{DRN\text{-}NAc}$ terminals during U50488 pretreatment: mice received the selective KOR agonist U50488 (5mg/kg, IP) 1hr prior to cocaine conditioning and were immediately tethered to optical fibers providing 473-nm stimulation (15Hz, 10ms pulse

duration) in an empty cage bottom. Mice were untethered 5min before each cocaine conditioning session.

Local infusion of 5-HT receptor antagonists prior to cocaine conditioning: Wild-type (WT) mice with guide cannula placed in the NAc received infusions of the 5-HT_{1B} antagonist GR 127935 or 5-HT_4 antagonist GR 125487 (1µg/0.2µl in ACSF, 0.1µl/min) 135min and/or 75min prior to each cocaine conditioning session. Following infusions, mice were returned to their home cage.

Conditioned place aversion: Cannulated SERT-Cre mice expressing EYFP or SwiChR were assayed in a balanced place conditioning apparatus with distinct visual and tactile cues as previously described [2]. An initial preference test was performed on day 1 to assess baseline preference. On days 2 & 3 optogenetic conditioning was performed, comprising a tethering session with confinement to the less-preferred chamber in the morning and optical stimulation (0.33 Hz, 15ms pulse duration) with confinement to the more-preferred chamber 4hr later. On the 4^{th} day, mice were allowed to freely explore the apparatus for a final preference test in the absence optical stimulation.

Social approach: Social interaction was assessed as described previously using a three chambered apparatus with two clear internal partitions [5]. The day prior to the experiment, age-matched target mice were habituated to confinement in an inverted pencil cup (Spectrum Diversified Designs) for 1hr. WT mice with cannula placed in the NAc received infusions of 5-HT_{1B} antagonist GR 127935 (1µg/0.2µl in ACSF; 0.1 µl/min), and 125min later were allowed to freely explore the social interaction apparatus for a 10min habituation period. The mouse was then briefly removed to a holding cage, and two inverted pencil cups were placed in the far corners of the apparatus, with one cup containing a target mouse. The experimental mouse was then reintroduced and allowed to explore for an additional 10min. Time spent in an interaction zone adjacent to each cup was recorded.

Local infusion of 5-HT_{1B} ligands prior to histology: WT mice with guide cannula placed in the NAc received norBNI (10mg/kg, IP) 24hr prior to the experiment to minimize the effects of infusion-induced stress on pERK-IR [6]. Mice received control infusions (ACSF, 0.2µl) in the right hemisphere and drug infusions (GR 127935 or 5 CP94253; 1µg/0.2µl, 0.1µl/min) in the left hemisphere. Drug infusions consisted of CP 94253 alone or following infusions of GR127935 135min or 75min prior. 15min after infusion of CP 94253, mice were deeply anesthetized, transcardially perfused, and brains were prepared for histology as described below.

Immunohistochemistry: Mice were transcardially perfused with 4% paraformaldehyde in 0.1M phospho-buffered saline (PBS) as reported previously [7]. Brains were then dissected, cryoprotected with 30% sucrose at 4°C overnight, frozen, cut into 40µm sections (Leica microtome, SM200R), and stored in 0.1M PBS containing 0.1% sodium azide at 4°C until further processing. Standard immunohistochemical procedures were used to stain NAc sections as described previously [6]. Briefly: floating sections were washed 3x5min in PBS, then blocked for 1hr in 5% normal goat serum (Vector Labs), 0.3% Triton-X in PBS before 24hr at room temperature incubation with primary antibodies: 1:400 rabbit anti-pERK antibody (CS4370, Cell Signaling) for phospho-ERK detection or 1:1000 Chicken anti-GFP (AB12970, Abcam) to

enhance detection of anterograde and retrograde tracing. Sections were washed again 4x5min in PBS before incubation with the 1:500 goat anti-rabbit 488 or goat anti-chicken 488 (Life Technologies) for 2hr at room temperature. Lastly, sections were washed 4x5 in PBS, then once with 0.5X PBS before mounting on Fisher Superfrost slides (Sigma-Aldrich) and coverslipped using Vectashield (Vector Laboratories).

Fluorescent in situ hybridization (ISH) using RNAscope: Brains were rapidly dissected and flash frozen on dry ice. For stress experiments, brain dissection was performed either 30min or 24hr following the last swim session of the rFSS protocol or unhandled (no rFSS) controls. Thin (14µm) coronal sections containing the NAc or DRN were collected and mounted onto Superfrost plus slides using a cryostat (Leica CM 1850) maintained at -20ºC. RNAscope ISH was performed according to the Advanced Cell Diagnostics as previously reported [8]. Each set of staining included a negative control, in which probes were omitted from the process. Probes were discriminated using tyramide signal amplification (TSA) fluorophores (NEL744001, NEL745001, NEL741001; Akoya Biosciences).

Characterization of DRN subpopulations: Probes for *Oprk1 (mm-Oprk1), Slc6a4 (mm-Slc6a4), and Slc17a8 (mm-Slc17a8)* were used to label tissue from the central DRN (AP +4.3-4.5) of unstressed mice*. NAc Htr1b distribution:* sections containing the central NAc (AP 1.1-1.3) were obtained from stressed and unstressed mice, and separate sets of tissue were stained with probes to *Htr1b(mm-Htr1b)*/*Chat (mm-Chat)* and *Htr1b/Pdyn (mm-Pdyn)/Adora2a (mm-Adora2a).*

Microscopy and Image Quantification: All images used for quantitation were taken using a confocal microscope (SP8X, Leica Microsystems), except for initial determination of expression in retrograde tracing studies and confirmation of viral expression in behavioral studies, in which a scanning widefield microscope was used (DMI6000, Leica Microsystems).

Anterograde tracing: Two brain sections containing the NAc (AP +1.3 and AP +1.0) were imaged at 10x magnification for each $SERT^{DRN}$ ChR2 and $VGluT3^{DRN}$ ChR2 mouse. Boundaries of the mNAc were determined using the Paxinos atlas [9]. Terminal density was determined using Image J software (NIH) by binarizing the images and calculating the density of positive pixels within the mNAc as described previously [10].

Retrograde tracing: For KOR retrograde tracing from the mNAc, every sixth section within the DRN (AP -4.0 to AP -5.0) was mounted and imaged at 10x magnification following anti-GFP staining to enhance detection of labeled cell bodies. For Pdyn retrograde tracing from the mNAc, every 12^{th} section throughout the collected tissue (AP +2.5 to AP -5.5) was mounted to survey across all brain regions and imaged at 10x on a scanning widefield microscope. These were visually inspected for signal by an observer blind to treatment. Subsequently, confocal images were taken of the medial NAc at 10x magnification.

Effects of 5-HT_{1B} drug infusion on pERK in the NAc: The left and right medial NAc of two sections (AP +1.3 and AP +1.0) were imaged for each animal. Z-stacks (5µm thick, 7 steps) were taken at 60x magnification and an average projection was generated using LASX software (Leica Microsystems). A detection threshold for each section was set according to the brightest

5% of pixels in the ACSF image and positive cells (more than half of cell above threshold) for each image were quantified manually by an observer blind to treatment using ImageJ software (NIH). Average counts of two sections for the ACSF and drug treated hemispheres were taken for each animal. Percent increase of NAc pERK-IR^{+} cells in the drug-treated hemisphere was calculated as ($pERK_{Drug}$-$pERK_{ACSF}$)/ $pERK_{ACSF}$.

Fluorescent ISH: DRN subpopulations: The entire DRN was imaged at 20x magnification from one section (AP 4.4) of each subject. Images were taken during the same imaging session, and capture settings were adjusted such that no signal was observed in negative controls and kept constant for all subsequent images. For DRN subregion analysis, an observer blind to staining conditions determined subregion boundaries and selected a rectangular region within each DRN subregion for colocalization analysis. Cells that contained signal brighter than threshold were counted as positive or overlapping. *NAc Htr1b distribution:* a region immediately medial and ventral to the anterior commissure (ac) was imaged at 20x magnification, with capture settings adjusted to ensure no signal in the negative controls. Two bilateral images from two sections were taken. Images were processed using custom MATLAB scripts (available upon request) for positive cells, co-expressing cells, and levels of RNA detected per cell; these values were averaged for each subject animal and then group averages were calculated. Cells that contained signal brighter than threshold were counted as positive or overlapping. Within positive cells, RNA levels were calculated by determining the percent of area within positive cells that was above threshold. For all analyses, total cells were determined by the number of DAPI-stained nuclei.

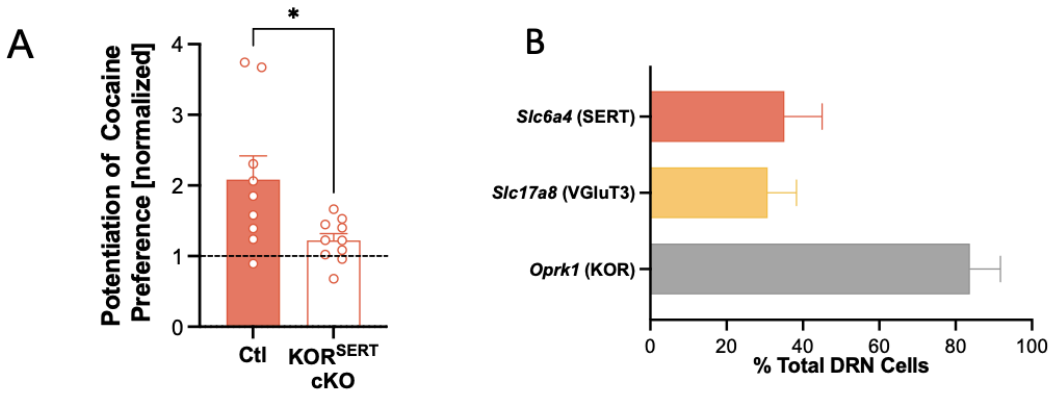
Data Analysis: Sample sizes were based on prior studies but were not predetermined by statistical methods. All aspects of histology and histological analysis were performed by an experimenter blind to genotype and treatment. Prior to further analysis, outliers in data sets were excluded using Grubb's Test for statistical outliers. The assumption of normal distribution was tested for each data set and was statistically corrected for (welch's correction) when this criterion was not met. *Post-hoc* tests used were Sidak's, except for the analysis of antagonist pretreatment on cocaine CPP, where Dunnet's T3 was used due to statistical correction. Alpha was set at 0.05 for all analyses.

Supplemental Results

(Supplemental to Text Figure 1)

We found that KOR transcript was expressed in the majority of DRN cells (84±8.0%) (Figure S1B). *Slc6a4* and *Slc17a8* were present in a roughly equal percentage of DRN neurons (35±9.9% and 31±7.5%, respectively).

Supplemental Figure S1

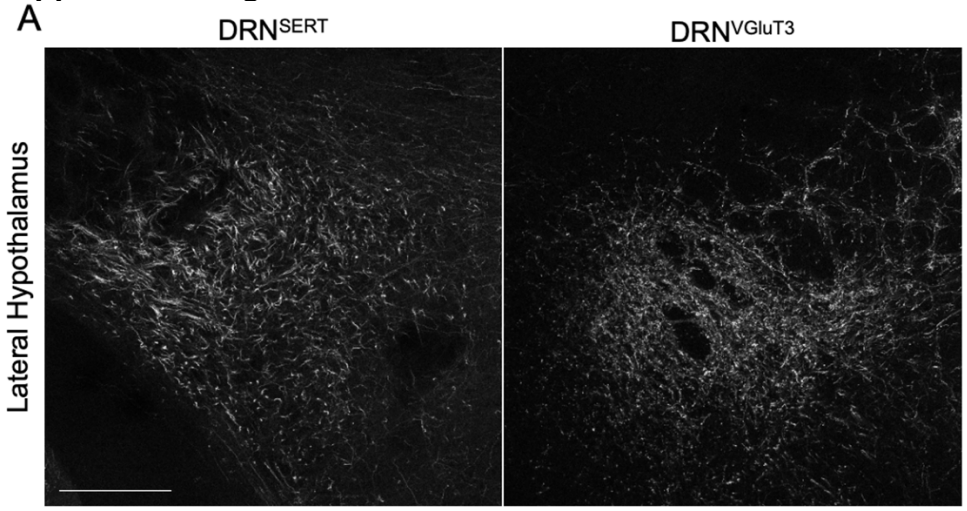


(Legend) (A) Cocaine preference scores with each genotype normalized to its unstressed control (n=9-10). (B) Quantitation of cells expressing transcripts for SERT, VGluT3, KOR, expressed as percentage of total DRN cells (n=3).

(Supplemental to Text Figure 2)

In contrast to differences observed in terminal density within the mNAc, we observed that the terminal density in the lateral hypothalamus was similar for $SERT^{+}$ and $VGluT3^{+}$ projections.

Supplemental Figure S2



(Legend) (A) Expression of EYFP labeled terminals in lateral hypothalamus following injection of AAV5-DIO-ChR2-EYFP in VGluT3-Cre or SERT-Cre mouse. Scale bar= 200μm.

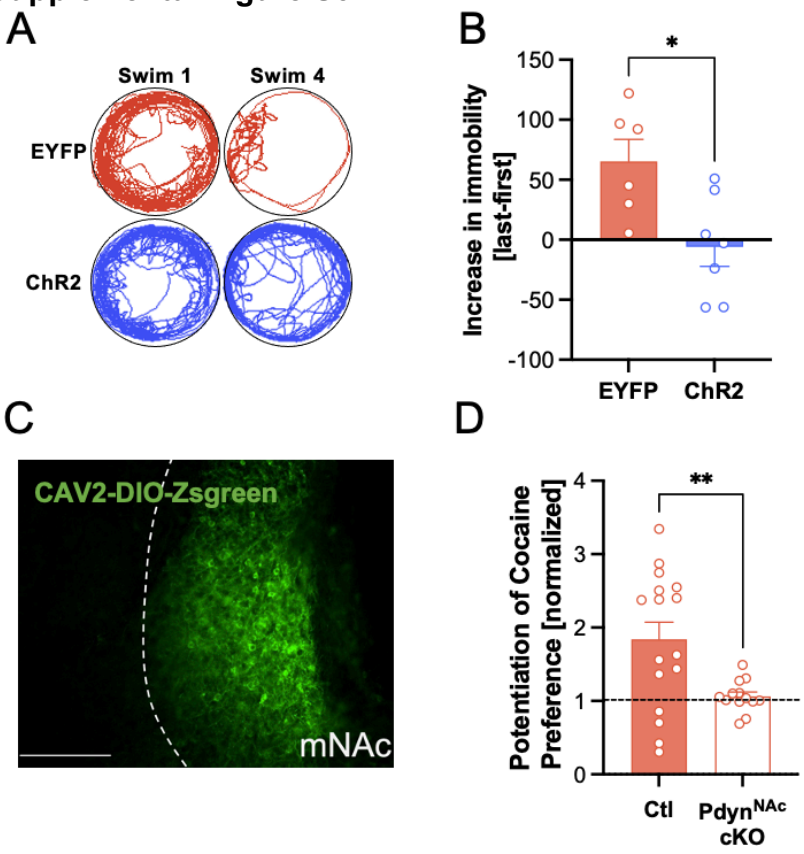
(Supplemental to Text Figure 3)

Movement was tracked during swim sessions and indicated a decrease in movement in the EYFP group that was not apparent in $SERT^{DRN\text{-}NAc}$ ChR2 group (Figure S3A). To calculate the

escalation of immobility in mice of each group, time immobile during first swim was subtracted from the last swim of day 2 (Figure S3B). The results indicate that stimulation of serotonergic terminals in the NAc prevents escalation of immobility within subjects (t test, P= 0.014).

Retrograde tracing utilizing a second virus (CAV2-DIO-ZsGreen) injected into the mNAc of a Pdyn-cre mouse also found expression that was restricted to the mNAc. These findings are likely not due to technical limits of the virus or transgenic line, as a recent report using the same virus and transgenic line noted expression in several regions following viral injection into the VTA [11].

Supplemental Figure S3



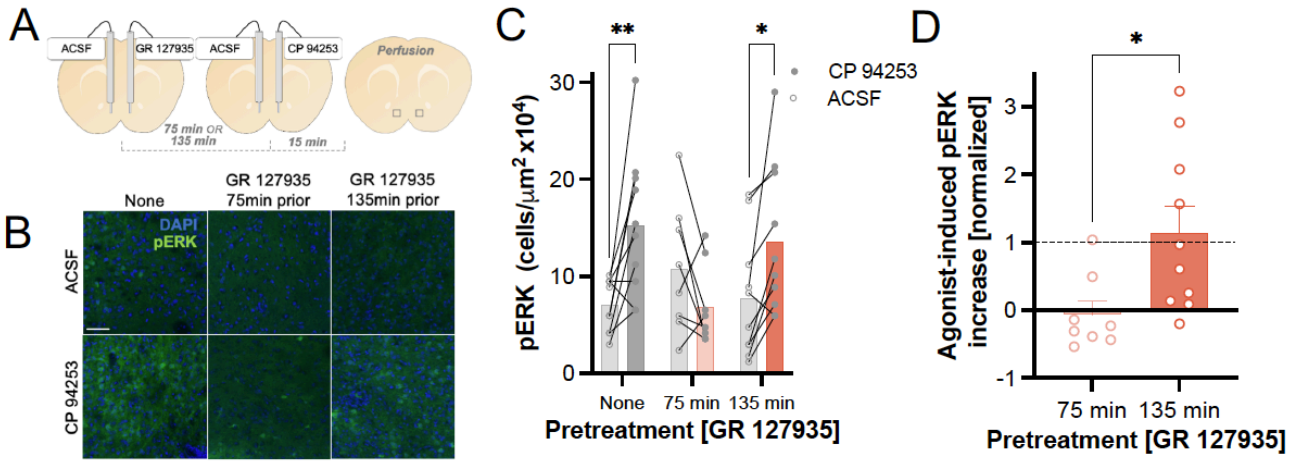
(Legend) (A) Representative track traces during first and last swims on day 2. (B) Change in immobility from first to last swim on day 2 (n=6-7). (C) Representative image of ZsGreen expression in NAc of a Pdyn-Cre mouse. (D) Cocaine preference scores with each group normalized to its unstressed controls (n=13-16).

(Supplemental to Text Figure 4)

Two time points were selected to evaluate the duration of antagonist-mediated blockade of the receptor. Injection cannula were placed in the mNAc of WT mice and 5-HT_{1B} antagonist GR 127935 was infused unilaterally (Figure S4A). This was followed either 75min or 135min later by

unilateral infusion of 5-HT$_{1B}$ agonist CP 93129 and perfusion of the brain 15min later. ACSF was infused in the opposite hemisphere during each drug infusion to control for nonspecific effects of the infusion procedure on ERK phosphorylation. Coronal sections containing the mNAc were probed for pERK-immunoreactivity (IR), a consequence of 5-HT$_{1B}$ activation (Figure S4B) [12]. Comparison of treatments showed a significant main effect of agonist treatment on agonist-stimulated pERK-IR and a significant interaction (two-way ANOVA, agonist main effect, $F_{1,25}$ = 7.12, P= 0.013; pretreatment X agonist interaction, $F_{2,25}$= 8.04, P= 0.002). *Post-hoc* comparisons indicated that treatment with CP 93129 induced a significant increase in pERK-IR cells as compared to the ACSF control hemisphere in the absence of antagonist pretreatment (Sidak *post-hoc, P*= 0.002). This result confirmed that local infusion of the 5-HT$_{1B}$ agonist induces pERK-IR within mNAc cell bodies. Pretreatment with the antagonist GR 127935 75 min prior to CP 93129 infusion blocked the agonist-induced increase in pERK-IR (Sidak *post-hoc*, P= 0.293), whereas pretreatment 135min prior did not *(*Sidak *post-hoc*, P= 0.033) (Figure S4C). To further interrogate antagonist duration, the number of pERK+ cells were normalized to the ACSF hemisphere for each subject, and this value was expressed as a fraction of the effect from agonist treatment alone (Figure S4D). This direct comparison of antagonist pretreatment timing illustrates the transient block of pERK-IR caused by GR 127935 infused 75min before CP 93129, but not 135min before agonist (t test, P= 0.014).

Supplemental Figure S4

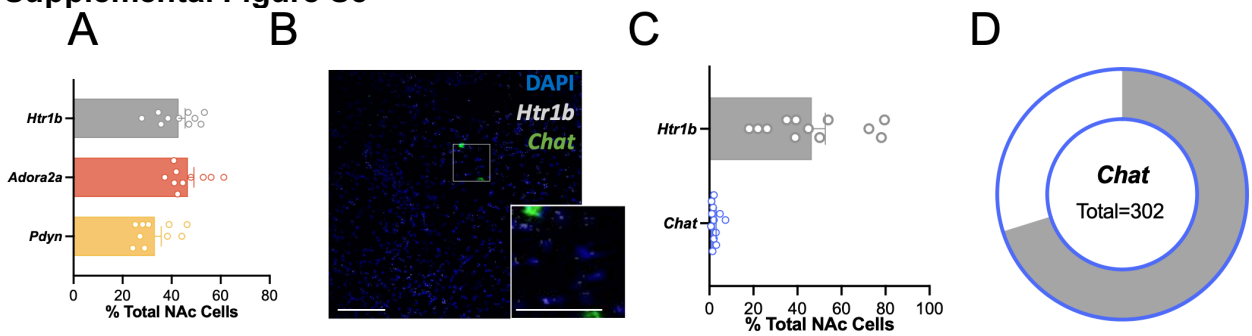


(Legend) (A) Schematic of unilateral NAc infusion of 5-HT$_{1B}$ antagonist GR 127935, followed 75 or 135min later by infusion of 5-HT$_{1B}$ agonist CP 94253 and perfusion. Rectangles show imaging field. (B) Representative images showing immunofluorescent lab phospho-ERK1/2 (pERK) in ACSF (control) hemisphere and hemisphere receiving 5-HT$_{1B}$ ligand infusions. Scale bar= 50μm. (C) Quantification of pERK+ cells in control hemisphere and agonist-infused hemispheres following NAc infusion of GR 127935 prior to CP 94253 (n=8-10). (D) Quantification of pERK+ cells following GR 127935 pretreatment, expressed as percentage of agonist-induced increase in pERK compared to ACSF and normalized to agonist-induced increase without pretreatment (n=8-10).

(Supplemental to Text Figure 5)

Staining and imaging were performed to assess the colocalization of *Htr1b* with *Pdyn* and *Adora2a*. Results indicate that nearly half (43±2.7%) of the neurons within the mNAc express *Htr1b* (Figure S5A). Staining for other markers show that *Pdyn* and *Adora2a* were detected in a sizeable fraction of neurons (33±2.5% and 45±2.5%, respectively). A second experiment was performed to establish *Htr1b* colocalization with *Chat* (Figure S5B, S5C). *Chat* was expressed sparsely (2.5±0.5%), in line with prior findings, and *Htr1b* was present in a majority of these neurons (Figure S5C, S5D)[13].

Supplemental Figure S5



(Legend) (A) Quantification of percent of total cells in the medial NAc positive for *Htr1b*, *Pdyn*, and *Adora2a* (n=6). (B) Representative image showing *in-situ* hybridization labeling *and Htr1b* and *Chat* transcripts in the medial NAc. Inset: higher magnification of rectangular region. Scale bar=100μm, 25μm. (C) Quantification of percent of total cells in the medial NAc positive for *Htr1b* and *Chat* (n=6). (D) Fraction of *Chat* cells expressing *Htr1b*.

Supplemental References

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