Supplementary Figures

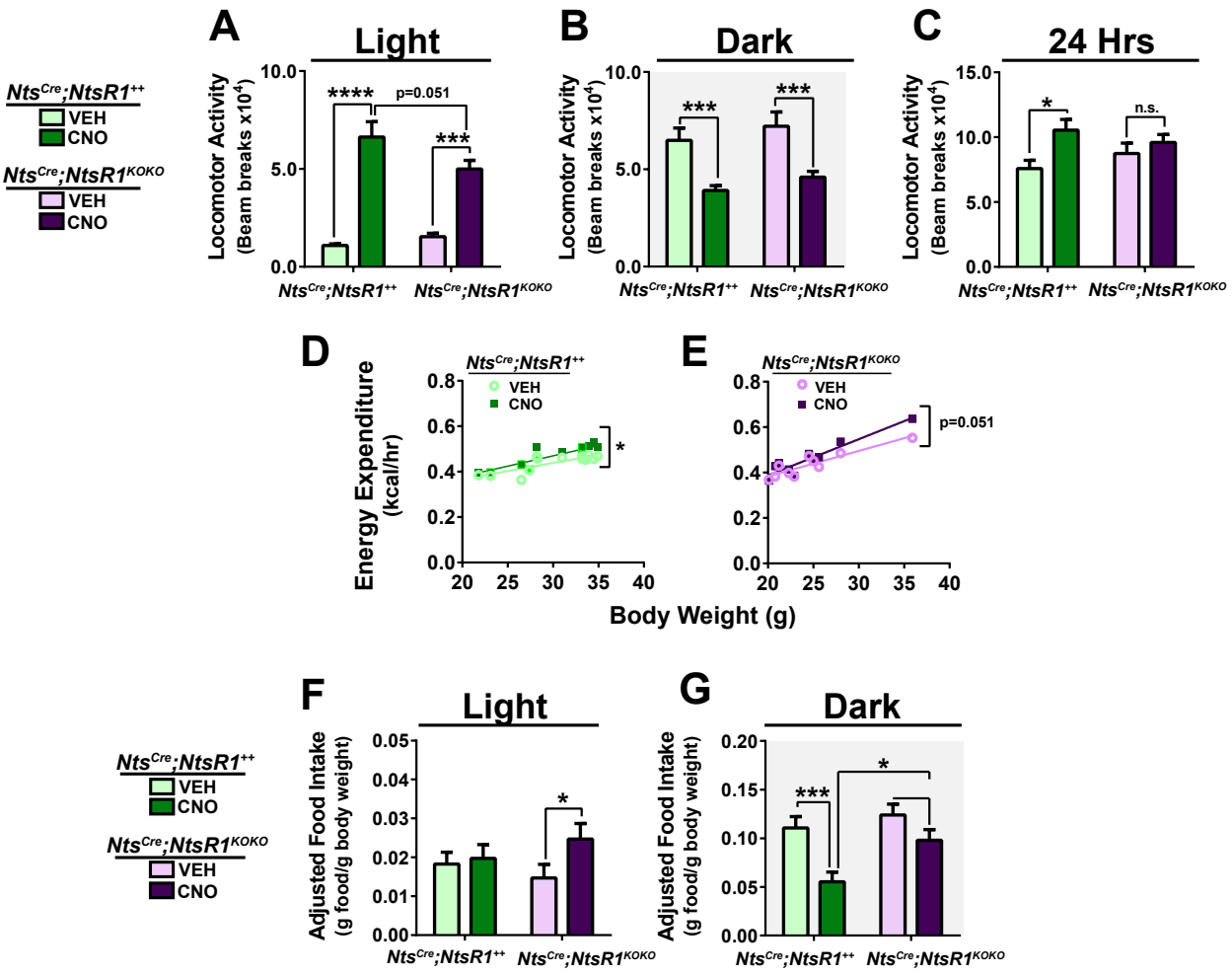

Figure S1 ***(related to Fig. 2)*** **Supplemental Acute Metabolic Data.** Light refers to the period 9 hours post VEH or CNO injection while dark refers to data collected during dark cycle (gray background). **A)** locomotor activity during the light cycle, **B)** dark cycle, and **C)** over 24 hours. **D-E)** average energy expenditure over 24 hours with VEH or CNO injection. **F)** weight-adjusted food intake during the light cycle and **G)** dark cycle. Total weight-adjusted food intake over 24 hours is shown in Figure 2J. Bar graphs were analyzed by repeated measures two-way ANOVA with Sidak post-tests, while energy expenditure was analyzed by ANCOVA, *p < 0.05, ***p < 0.001, ****p<0.0001. *Nts^{Cre};NtsR1^{++}* n=10-11; *Nts^{Cre};NtsR1^{KOKO}* n=9-10, .

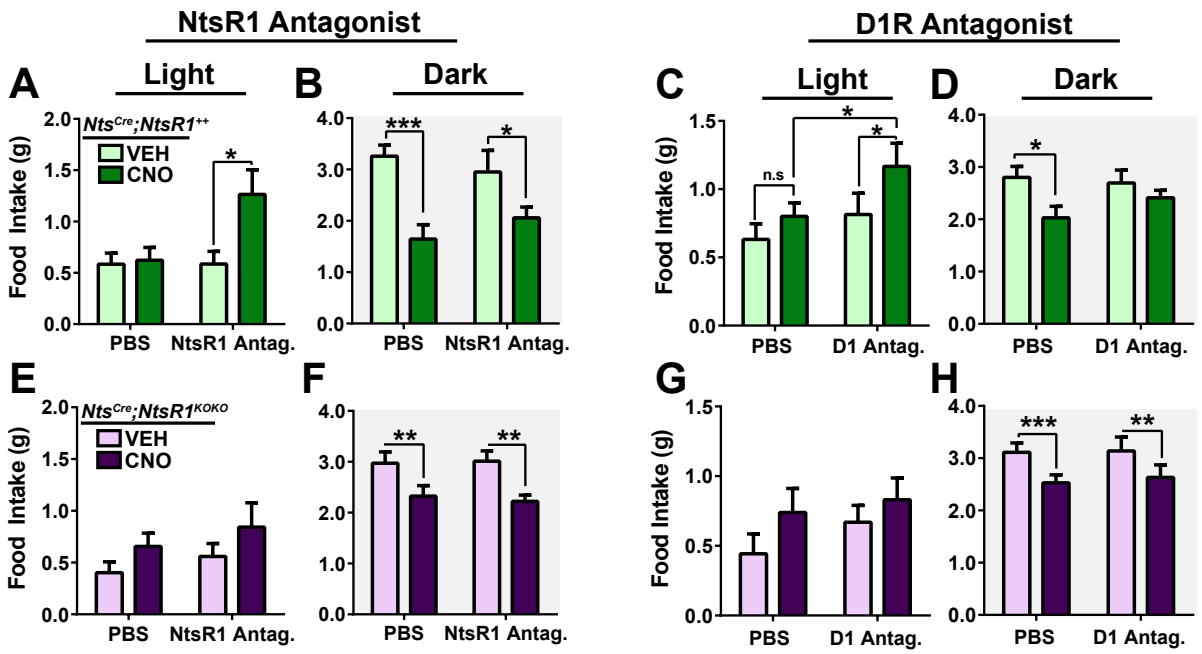

Figure S2 (*related to Fig. 3*). Food intake separated by circadian period with NtsR1 or D1R antagonists. A, B) food intake in $Nts^{Cre};NtsR1^{++}$ mice during light or dark phases with NtsR1 antagonist pre-treatment or **C, D)** D1 receptor pre-treatment. **E, F)** food intake in $Nts^{Cre};NtsR1^{KOKO}$ mice during light or dark with NtsR1 antagonist pre-treatment or **G, H)** D1R antagonist pre-treatment. Data were analyzed by repeated measures two-way ANOVA with Sidak post-tests, *p < 0.05, **p<0.01, ***p < 0.001.

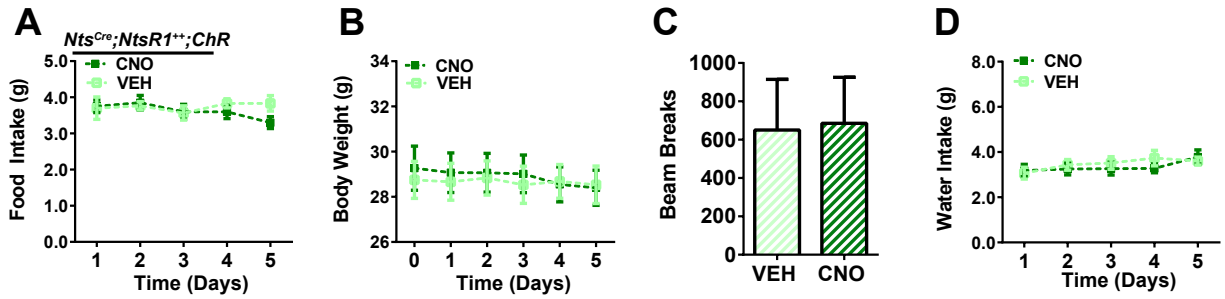

Figure S3 (*related to Fig. 4*). Chronic VEH or CNO injection in Nts^{ChR} controls. A) chow intake, **B)** body weight, **C)** 1 hr locomotor activity, and **D)** water intake in Nts^{ChR} controls (n=6). Data were analyzed by repeated-measures two-way ANOVA, except for locomotor activity which was analyzed by paired t-test.

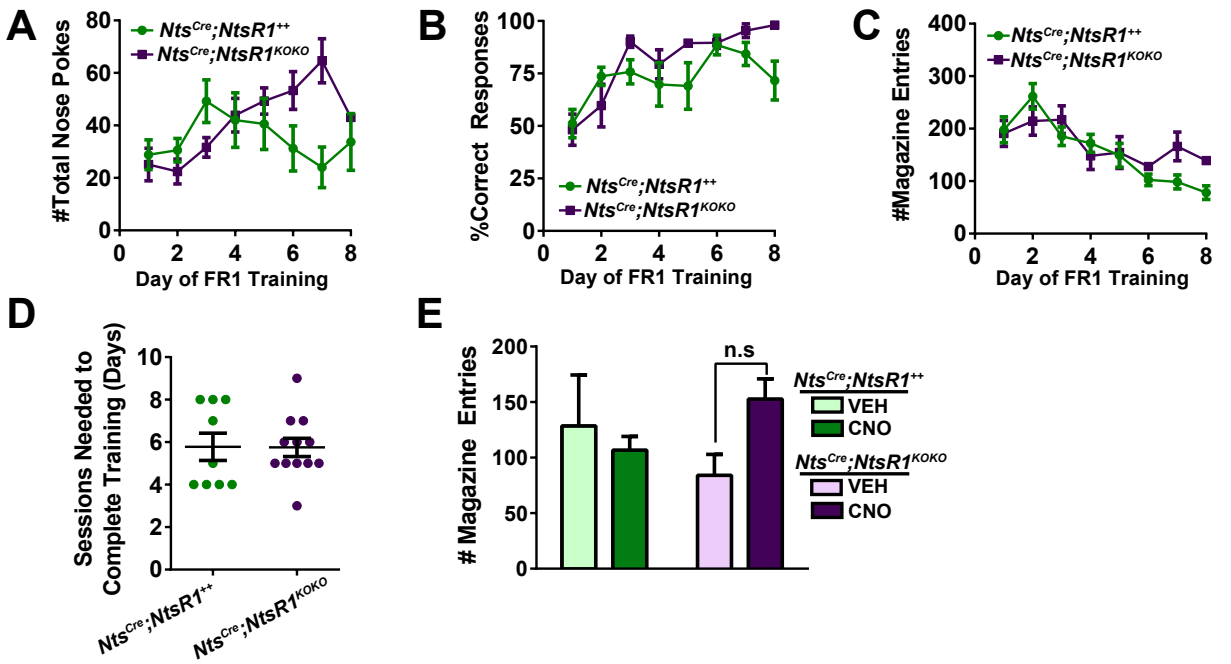

Figure S4 (*related to Fig. 7*) Supplemental operant training data. *Nts^{Cre};NtsR1^{++}* and *Nts^{Cre};NtsR1^{KOKO}* mice were trained to nose-poke for sucrose pellets on an FR1 schedule until they earned >20 rewards with 75% accuracy for 3 consecutive days. **A, B, C)** No differences were detected in total number of active and inactive nose pokes, accuracy, and number of magazine during FR1 training. **D)** *Nts^{Cre};NtsR1^{++}* and *Nts^{Cre};NtsR1^{KOKO}* mice required a similar number of training sessions to learn the task. **E)** CNO treatment did not significantly increase the number of magazine entries during PR testing. *Nts^{Cre};NtsR1^{++}* n=9; *Nts^{Cre};NtsR1^{KOKO}* n=12. Data were analyzed by repeated-measures two-way ANOVA **(A, B, C, E)** or unpaired t-test **(D)**.

Supplementary Methods:

Reagents
CNO was obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the NINDS. 40x CNO stock solutions were made by diluting with PBS/10% beta-cyclodextrin (Sigma). VEH was PBS. SR48690 was purchased from Sigma and 20x stock solutions were made in 1% TWEEN. SCH23390 was also purchased from Sigma and 50x stock solutions were made in PBS. All stock solutions were aliquoted and stored at -20˚C until use.

Generation of *NtsR1$^{IRES\text{-}Cre}$* Knock-In Mice
An *NtsR1$^{IRES\text{-}Cre}$* targeting vector was generated by inserting an IRES-Cre sequence between the stop codon and the polyadenylation site of the sequence encoding the 3' end of the mouse *Ntsr1* gene, with an *frt*-flanked Neo cassette inserted upstream of the IRES-Cre. The linearized targeting vector was electroporated into mouse R1 embryonic stem (ES) cells (129sv background) and cells were selected with G418. DNA from ES cell clones was analyzed via qPCR for loss of homozygosity using Taqman primer and probes for the genomic *Ntsr1* insertion site (Forward: TCT GAT GTT GGA CTT GGG TTC, Reverse: TCT GAT GTT GGA CTT GGG TTC, Probe: TCT GAT GTT GGA CTT GGG TTC). *NGF* was used as a copy number control[1]. Putative positive ES clones were expanded, confirmed for homologous recombination by Southern blot and injected into mouse C57BL/6 blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 females (Jackson Laboratory), and germline transmission was determined initially via progeny coat color, then confirmed via conventional PCR for IRES-Cre.

Breeding and Genotyping
To generate breeders, heterozygous *Nts^{cre}* mice[2] (Jackson stock #017525) were mated to *NtsR1^{KO+}* (Jackson stock #005826) mice, and progeny with the genotypes *Nts^{Cre};NtsR1^{KO+}* and *++; NtsR1^{KO+}* were subsequently mated to generate *Nts^{Cre};++* and *Nts^{Cre};NtsR1^{KOKO}* study animals. To maximize animal usage, study animals also arose from the following crosses: *Nts^{Cre}:++* X *Nts^{Cre};NtsR1^{++}* or *Nts^{Cre};NtsR1^{KOKO}* X *++;NtsR1^{KOKO}*. To visualize NtsR1 neurons, *NtsR1$^{IRES\text{-}Cre}$* mice were crossed to homozygous *Rosa26$^{EGFP\text{-}L10a}$* mice and progeny heterozygous for both alleles were studied. Genotyping was performed with standard PCR using the following primer sequences: *Nts^{cre}:* common forward: 5' ATA GGC TGC TGA ACC AGG AA, cre reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26$^{EGFP\text{-}L10a}$*: mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC. *NtsR1^{KO}:* Forward: CTC TAA TGT GCC ACA GCT CAG AGA G, common: CAG CAA CCT GGA CGT GAA CAC TGA C, Reverse: CCA AGC GGC TTC GGC CAG TAA CGT T *NtsR1$^{IRES\text{-}Cre}$:* Forward: GGA CGT GGT TTT CCT TTG AA, Reverse: AGG CAA ATT TTG GTG TACG G.

Surgery
At 8-12 weeks of age, male *Nts^{Cre};++* and *Nts^{Cre}:NtsR1^{KOKO}* mice received a pre-surgical injection of carprofen (5mg/kg *s.c.*) and were anesthetized with 3-4% isoflurane/O_2 in an induction chamber before being placed in a stereotaxic frame (Kopf). Under 1-2% isoflurane, access holes were drilled in the skull allowing a guide cannula with stylet (PlasticsOne) to be lowered into the brain target area. Mice were injected bilaterally with either 300 uL of AAV-hM3Dq-mCherry or AAV-ChR-mCherry (UNC vector core) in the LHA (AP: -1.34, ML: -1.05, DV: -5.2). After 5 min to allow for virus absorption, the injector and cannula were removed from the skull and the incision was closed using Vet Bond. Analysis began 1-2 weeks after recovery. Mice were only included in the final study data if injections were confined to the LHA on both sides. Approximately 90% of animals included in the study were bilaterally targeted; however in 10% of cases, animals with robust unilateral targeting were included in the study if CNO injection induced >1mL of water consumption, as analysis of several cohorts revealed this as a reliable indicator of LHA Nts targeting. To visualize NtsR1 neurons, adult male and female *NtsR1^{Cre};GFP* mice were bilaterally injected with 1uL FlpO adenovirus (Vector Biolabs) into the lateral ventricles (A/P: -0.22, M/L: +/- 1.0, D/V: -2.0). Mice were perfused 10 days after surgery to permit sufficient time for FlpO-mediated excision of the *frt*-flanked Neo cassette and GFP expression.

Gene Expression
Male 12-16wk old *Nts^{Cre};NtsR1^{++}* and *Nts^{Cre};NtsR1^{KOKO}* mice were deeply anesthetized with sodium pentobarbital and tissue from the VTA was microdissected and immediately snap frozen on dry ice and stored at -80˚C (n=10-11 per group). RNA was extracted using Trizol (Invitrogen) and 200 ng samples were converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Sample cDNAs were analyzed in triplicate via

quantitative RT-PCR for gene expression using TaqMan reagents and an ABI 7500 (Applied Biosystems). With *GAPDH* expression as an internal control, relative mRNA expression values are calculated by the $2^{-\Delta\Delta Ct}$ method. *NtsR1* expression levels in 10 of 11 $Nts^{Cre};NtsR1^{KOKO}$ mice were undetectable and had GAPDH Ct values comparable to controls, thus the fold change was reported as 0 for those samples.

Operant Testing

Based on methods by Fulton *et al*.[3], mice were food restricted to 90% of their body weight and trained on a fixed ratio-1 (FR1) schedule until they achieved 75% response accuracy with ≥20 rewards earned on 3 consecutive days. Training sessions were terminated after 1 hr or when the animal had earned 50 rewards. Mice achieving these criteria were then switched to *ad lib* food and trained on an FR5 schedule for 3 consecutive days. On test days, mice were subject to a progressive ratio (PR) schedule were $PR=[5e^{(R*0.2)}]-5$ with R=number food rewards earned+1. Thus, the number of correct responses needed to earn a sucrose reward increases as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 *etc*. The PR breakpoint was recorded as the highest ratio completed for each 1 hr test session. Mice were tested until they achieved stable PR which was defined as <10% variation in rewards earned over 3 consecutive sessions.

Conditioned Place Preference (CPP)

CPP boxes (San Diego Instruments) consisted of a box divided into two chambers with different visual and tactile cues (gray wall and smooth floor or striped wall and rough floor) separated by a small center chamber. *Day 1 (Pre-test):* Mice were allowed to roam freely between chambers for 15 min. After pre-test data were collected, an unbiased, counterbalanced strategy was then used to assign which chamber was paired with either VEH or CNO injection such that approximately half the mice received CNO pairing in the preferred side and half received CNO in the non-preferred side. *Days 2-5 (conditioning):* each morning, mice received an injection of VEH and were immediately placed in the VEH-paired side for 30 min. After the session, mice were returned to their housing environment to facilitate memory consolidation and 4 hours later, they were conditioned with CNO for 30 minutes in the opposite chamber. *Day 6 (post-test):* Mice were again allowed two roam freely amongst both chambers for 15 minutes. Time-spent and locomotor activity in each side of the box was assessed by laser beam breaks and data were gathered using the manufacturer's software. One $Nts^{Cre};NtsR1^{KOKO}$ animal was excluded for displaying side-preference in the pre-test (>75% of time spent on that side).

Nestlet-Shredding

Immediately after receiving VEH or CNO injection, mice were given a white cotton pillow (Ancare) in their home cages, which was weighed prior to placement in the cage. After 90 min, any intact remnants of the original pillow were removed from the cage, air-dried overnight and weighed the following day.

Elevated Plus Maze (EPM)

Mice were assessed for anxiety-like behavior using an elevated plus maze (EPM) as previously described[4]. Briefly, the EPM apparatus was custom-built based on plans from ANY-maze (www.anymaze.com, Stoelting Co.) and mice were given free access to the open and closed arms for 5 minutes. Their behavior was recorded using a digital CCD camera and the percentages of time spent in the open and closed arms were analyzed using Topscan automated video tracking software (Clever Sys).

Immunohistochemstry

Mice were treated with a lethal dose of *ip* pentobarbital followed by transcardial perfusion with 10% neutral-buffered formalin (Fisher Scientific). Brains were removed, post-fixed in 10% formalin overnight at 4˚C, dehydrated with 30% sucrose in PBS for 2-3 days, and sectioned into 30 µm slices using a sliding microtome (Leica). Brain sections were then analyzed by immunofluorescence or immunohistochemistry as previously described[2,5]. For characterization of NtsR1 expression, sections from $NtsR1^{Cre};GFP$ mice were stained with chicken anti-GFP (1:2000, Abcam) followed by donkey anti-chicken conjugated to AlexaFluor 488 (Jackson ImmunoResearch). For DREADD studies, $Nts^{Cre};NtsR1^{++}$ and $Nts^{Cre};NtsR1^{KOKO}$ mice were treated with VEH or CNO 90 minutes prior to perfusion, and brain sections were stained for cFos (1:500, goat, Santa Cruz) with secondary detection via DAB (Life Technologies), followed by immunofluorescent detection of dsRed (1:1000, Clontech) as described above. Brains were analyzed using an Olympus BX53 fluorescence microscope outfitted with transmitted light to analyze DAB-labeled tissue, as well as FITC and Texas Red filters. Microscope images

were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe).

Supplemental References

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3 Sharma, S., Hryhorczuk, C. & Fulton, S. Progressive-ratio responding for palatable high-fat and high-sugar food in mice. *J Vis Exp*, e3754, doi:10.3791/3754 (2012).

4 Eagle, A. L. *et al.* Experience-Dependent Induction of Hippocampal DeltaFosB Controls Learning. *J Neurosci* **35**, 13773-13783, doi:10.1523/JNEUROSCI.2083-15.2015 (2015).

5 Opland, D. *et al.* Loss of neurotensin receptor-1 disrupts the control of the mesolimbic dopamine system by leptin and promotes hedonic feeding and obesity. *Molecular metabolism* **2**, 423-434, doi:10.1016/j.molmet.2013.07.008 (2013).