SUPPLEMENTARY METHODS

Animals. All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Mice from a mixed background (129/C57/B6) were housed at 22°C–24°C with a 12 hr light/12 hr dark cycle with standard mouse chow (Teklad F6 Rodent Diet 8664; 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat; Harlan Teklad, Madison, WI) and water provided ad libitum. All diets were provided as pellets. Mice were euthanized by CO_2 narcosis. *AgRP-Ires-cre* were previously described (4).

Generation of AAV-hM3Dq-mCherry and AAV-hM4Di-mCherry vectors. The hM3Dq and hM4Di coding sequences were cloned into a mCherry vector (20) 5' of the mCherry sequence to generate C-terminal mCherry fusion proteins. Then, the hM3Dq-mCherry and hM4Di-mCherry coding sequences were amplified by PCR, and the amplicons and a cre-inducible AAV vector with a human *synapsin1* promoter (21,22; a generous gift from Karl Deisseroth, Howard Hughes Medical Institute, Department of Bioengineering and Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, California, USA.) were digested with *NheI* and *AscI*. The digestion products were ligated such that the coding regions for the fusion proteins were in a 3' to 5' orientation relative to the promoter. The final vectors were sequence-verified.

Packaging of Virus. AAV-hM3Dq-mCherry virus was packaged in serotype 8: 0.8 mL; 1.2 X 10^{13} genome copies per mL and was prepared and titered at the Harvard Gene Therapy Initiative (Boston, MA 02115). Virus was generated by tripartite transfection (AAV-rep/cap expression plasmid, adenovirus miniplasmid pHGTI-Adeno and AAV vector plasmid) into 293A cells and purified by iodixanol density gradient and Q sepharose column chromatography. The purified virus was dialyzed against PBS, concentrated by Amicon spin column and titered by dot blot hybridization. AAV-hM4Di-mCherry virus was packaged in serotype 8; 0.8 mL; 1.6 X 10^{13} genomes copies per ml and was prepared and titered at the University of North Carolina Gene Therapy Center (Chapel Hill, NC 27599-7352). Aliquots of virus were stored at -80°C before stereotaxic injection.

Stereotaxic AAV-DREADD-mCherry injections. Mice were anesthetized with 7% chloral hydrate diluted in saline (350 mg/kg) and placed into a stereotaxic apparatus (KOPF Model 963). After exposing the skull via small incision, a small hole was drilled for injection. A pulled-glass pipette with 20-40 μm tip diameter was inserted into the brain and 200 nL bilateral injections were made (coordinates, bregma: AP:-1.40mm, DV:-5.80mm, L:+/-0.30mm) by an air pressure system. A micromanipulator (Grass Technologies, Model S48 Stimulator) was used to control injection speed at 25 nL/min and the pipette was withdrawn 5 mins after injection. AAV-hM3Dq-mCherry, serotype 8 (titer 1.2 X 10^{13} genomes copies per ml) or AAV-hM4Di-mCherry, serotype 8 (titer 1.6 X 10^{13} genomes copies per ml) was injected bilaterally into the arcuate nucleus of 6 wk

old *AgRP-Ires-cre* mice and wildtype (non-cre-expressing littermates). For postoperative care, mice were injected intraperitoneally with meloxicam (0.5 mg/kg). Following injection, mice were singly housed, and body weights and food intake were assessed weekly. At the conclusion of the study, mice were perfused, and brains were processed for fluorescence. All injected *AgRP-Ires-cre* mice were positive for bilateral hits. Mice were allowed one week to recover and then acclimated to handling for one week before the start of any in vivo studies.

Food intake studies. Food intake studies on chow were performed as previously described (4). For the acute feeding studies in AAV-hM3Dq-mCherry injected mice, food intake was measured for 8 days in 10-12 weeks old male mice (chow diet). Briefly, food intake (0.5 hour, 1 hour, 2 hours and 4 hours) was monitored 3 hours after the start of the “lights on” cycle from 9am-1pm in either saline- or CNO- i.p. injected, ad lib fed male mice (n=12). These mice were singly housed for at least 1.5 weeks beforehand. Food intake was recorded from the study subjects after they received injections of saline (day 1-3), CNO (day 4), saline (day 5), CNO (day 6), saline (day 7) and CNO (day 8) at 9am. The food intake data from all 8 days was then combined for analysis. CNO was administered at 0.30 mg/kg of body weight. Saline was delivered at the same volume to maintain consistency in the studies. These crossover studies clearly demonstrate that the effects of the drug completely clear the system after 24 hrs. Previous publications suggest the duration of the drugs effect at the dosage used throughout the studies is approximately 8 hrs (11). Data shown is from male

mice.

For the chronic feeding studies, food intake as well as body weight was measured every morning at 9am for 16 days in 10-12 week old female mice (chow diet). These mice were group-housed in threes at weaning age and remained so throughout the studies. Each mouse received 2 daily i.p. injections of saline (days 1-5), CNO (days 6-10) or saline (days 11-15) at 9am and 5 pm. Food intake and body weight was assessed from the study subjects every morning before the first daily injection at 9am. Importantly, unlike AgRP ablation studies (7,8), these neurons remain healthy as CNO-induced stimulation at the conclusion of the chronic experiment, i.e. 5 days after CNO treatment was terminated, potently stimulated food intake. Data shown is from female mice.

Body composition (fat mass) was analyzed using EchoMRI (EchoMRI-100™, Echo Medical Systems, LLC., Houston, TX. MRI was performed on mice at 8:00am before the daily injections of saline or CNO on days 6, 11 and 16 of the study.

Energy expenditure measurements. Energy expenditure was assessed by measuring oxygen consumption. Oxygen consumption was measured using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Mice were acclimatized in the chambers for 48 hrs prior to data collection. At 8:30am, each individually-housed male mouse was i.p.injected with

saline or CNO and immediately placed back into the metabolic chambers. CNO was administered at 0.30 mg/kg of body weight. Saline was delivered at the same volume to maintain consistency in the studies. The mice had free access to food and water during this study. Data shown is from male mice.

Nosepoke Assay. Operant conditioning sessions were conducted in Med Associates mouse operant conditioning chambers (Med Associates, St Albans, VT). Chambers are equipped with 2 nosepoke holes (above each is a cue light) on either side of a central feeder. When mice poke the holes, a beam is broken, and a response is recorded in the Med associates Med-PC software (Med Associates, St Albans, VT). In our experiments, one hole is designated the correct hole and the other the false hole. Pokes in both holes are recorded but only pokes in the correct hole will lead to delivery of reinforcements.

For 5 consecutive days ad libitum fed mice were injected with saline (i.p.) placed in chambers and acclimated to nosepoking for food pellets for a period of 60 mins (14mg chow precision pellets, Testdiet, Richmond, IN) under a fixed ratio 1 (FR1) operant paradigm where each correct poke leads to delivery of a food pellet (up to a maximum of 20 pellets). Animals that failed to learn the paradigm after 5 days (either did not reach 10 pellets on 2 consecutive days or did not have a 4 to 1 correct to false nosepoke ratio) were excluded from further testing.

Following completion of FR1 training, ad libitum fed mice were then exposed to a progressive ratio 3 (PR3) testing paradigm (60 min duration) for the following 6 days and were treated with saline or CNO (0.3 mg/kg) on alternate days. Sessions ended earlier for mice that failed to poke for 30 min. Data are presented as the breakpoint during the PR3 period i.e,. the highest number of presses for a single pellet attained during PR3.

Following the completion of drug treatments, mice were fasted for 24 hours and then tested again under PR3 conditions immediately following injection with saline as previously described. Data shown is from male mice.

Beambreak Assay. Locomotor activity (17) was assessed using an OptoM3 apparatus from Columbus Instruments (Columbus, Ohio). Briefly, individually-housed male mice were placed in their home cages, and ambulatory counts along the X-axis were recorded every min for 2 weeks using CI BUS software from Columbus Instruments (Columbus, Ohio). Animals were acclimated to the room for 48 hrs before the study began, and all of the mice showed normal circadian locomotor rhythms. At 10:00am, each ad lib fed, individually-housed male mouse was i.p. injected with saline or CNO and immediately placed back into the home cage. CNO was administered at (0.3 mg/kg of body weight). Saline was delivered at the same volume to maintain consistency in the studies. The study was divided into two parts. In the first section spanning 5 days (day

1=saline, day 2=saline, day 3=CNO, day 4=saline, day 5=CNO), X-ambulatory counts were measured for 5 hrs between 10:00am-3:00pm with food freely available to the animal (food +). In the second section spanning 5 days (day 1=saline, day 2=saline, day 3=CNO, day 4=saline, day 5=CNO), using the same cohort of mice, X-ambulatory counts were measured for 5 hrs between 10:00am-3:00pm when food was absent (food -). The average number of X-ambulatory counts were averaged for each genotype in 1 hr blocks. Data shown is from male mice.

Statistics. Statistical analyses were performed using KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD *post-hoc* test. A P value of < 0.05 was considered significant in these studies.

Electrophysiology. The protocols of slice preparation and whole cell recording are previously described (24). 5-7 week-old mice injected with AAV 10-14 days previously were used. In brief, mice were anesthetized with isoflurane before decapitation and removal of the entire brain. Brains were immediately submerged in ice-cold, carbogen-saturated (95% O_2, 5% CO_2) high sucrose solution (238 mM sucrose, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.0 mM NaH_2PO_4, 5.0 mM $MgCl_2$, 10.0 mM $CaCl_2$, 11 mM d-glucose). Then, 200-μM thick coronal sections were cut with a Leica VT1000S Vibratome and incubated in oxygenated recording aCSP (126 mM NaCl, 21.4 mM $NaHCO_3$, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM

$MgCl_2$, 2.4 mM $CaCl_2$, 10 mM d-glucose) at room temperature (21-24°C) for at least 1 hr before recording. The hypothalamic slice was put in a recording chamber and bathed in oxygenated aCSF heated to approximately 30°C at a flow rate of approximately 2mL/min. AgRP neurons were clearly distinguished from adjacent, nonfluorescent neurons with an Axioskop FS2 Plus microscope (Zeiss). Whole-cell recordings were then made on identified neurons under IR-DIC optics by using a MultiClamp 700B Amplifier (Axon Instrument) with the software pClamp 10.2 (Axon Instrument). Recording electrodes had resistances of 3.5-5 MΩ when filled with the K-gluconate internal solution, which contained (in mM) K-gluconate 128, HEPES 10, EGTA 1, KCl 10, MgCl2 1, CaCl2 0.3, Mg-ATP 3, and Na-GTP 0.3 (pH 7.35 with KOH). Clozapine-N-oxide (CNO) was applied to bath solution through perfusion. After acquisition of stable whole-cell recordings for 2-5 min, aCSF solution containing 5uM CNO was perfused. In Figure 1d and 2f, the bar above the recording trace indicates the duration of CNO application.

c-fos Studies. *AgRP-Ires-cre* and wildtype mice were handled for 12 consecutive days beginning 1 week after bilateral injection of AAV-hM3Dq-mCherry to minimize stress. On the last day, animals were injected with either saline of CNO (0.3 mg/kg; i.p.), and food and water were removed for 90 mins, at which point the animals were sacrificed with 7% chloral hydrate diluted in saline (350 mg/kg).

Tissue Collection for Histology Studies. Animals were deeply anesthetized by

i.p. injection of chloral hydrate (500 mg/kg) and subsequently perfused transcardially with diethylpyrocarbonate (DEPC)-treated, 0.9% phosphate buffered saline (PBS) followed by 10% neutral buffered formalin. Brains were removed, stored in the same fixative for 4-6 hrs at 4°C, transferred into 20% sucrose in DEPC-treated PBS, pH 7 at 4°C overnight, and cut into 25-µm coronal sections on a freezing microtome.

Immunohistochemistry. This procedure was performed as previously described (24). Briefly, sections were washed in PBS, pH 7.4, pre-treated with 0.3% H_2O_2 in PBS, pH 7.4 for 30 mins at room temperature and then incubated in 3% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) with 0.25% Triton X-100 in PBS (PBT-Azide) for 2 hrs. For the colocalization studies, the slides were subsequently incubated overnight at room temperature using rabbit anti-dsRed antiserum (Clonetech; 1:2,500) and chicken anti-GFP antiserum (Abcam; 1:1000). After several washes in PBS, sections were incubated in the secondary antibodies used were Alexa Fluor 594 goat anti-rabbit Ig (H+L) (Invitrogen; 1:200) and Alexa Fluor 488 goat anti-chicken Ig (H+L) (Invitrogen; 1:200) at room temperature, followed by 30 min wash in PBS. For the c-fos studies, we used rabbit anti-c-fos (Calbiochem; 1:25,000) as primary and biotin-SP-conjugated affinipure donkey anti-rabbit Ig (H+L) (Jackson Immuno Research; 1:1000).