SI Materials and Methods

Animals. POMC-Cre and Rosa26-CAG-stop^{flox}-ChR2(H134R)-eYFP (Ai32) mice were generated as previously described (16, 17). These two lines of mice were maintained in our laboratory by breeding to C57BL/6J mice and the animals have been backcrossed at least 5 generations. Mice were housed in a climate-controlled environment at 22°C with a 14:10 light/dark schedule of lights on between 0500 and 1900 and *ad libitum* access to standard lab pellet food (5L0D-PicoLab, Brentwood, MO) and water. Breeding Ai32 heterozygotes with POMC-Cre heterozygotes generated transgenic POMC-ChR2 (Tg POMC-ChR2) heterozygote experimental mice. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the Care and Use of Animals and were approved by the IACUC at University of Michigan.

Stereotactic Surgery and Virus Vector Injection. Tg POMC-ChR2 mice (8-12 weeks old) were anesthetized with isoflurane and placed into a stereotaxic apparatus (David Kopf, CA). After exposing the skull via a small incision, a small hole was drilled, and an optical fiber (200 µm core, NA 0.37, Doric lenses, Canada) was unilaterally placed above the right Arc (coordinates, Bregma: AP: -1.62 mm, DV: 5.60 mm, ML: 0.33 mm). Tg POMC-ChR2 mice were singly housed after surgery and allowed to recover for 3 weeks before experiments. For the stereotactic AAV injection study, POMC-Cre mice aged 8 to 12 weeks were anesthetized with isoflurane. 0.8 µl of Cre-dependent double-floxed deno-associated virus AAV5-EF1α-DIO-ChR2(H134R)-eYFP (50) (UNC vector core, Chapel hill, NC) was injected unilaterally in the right Arc (coordinates, Bregma: AP: -1.62 mm,

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DV: 5.70 mm, ML: 0.30 mm) using a microsyringe pump injector (model UMP3, World precision instruments, CA) that controlled a NanoFil syringe (World precision instruments) to generate an injection rate of 100 nl per min. Following injection, the syringe was slowly withdrawn after waiting 10 min. Immediately after withdrawal of the injection syringe, an optical fiber (200 μm core, NA 0.37, Doric lenses, Canada) was implanted over the Arc (AP: -1.62 mm, DV: 5.45 mm, ML: 0.30 mm). This mouse line of POMC-Cre with selectively expression of ChR2 in adulthood by viral-mediated method was referred to as viral POMC-ChR2. Viral POMC-ChR2 mice were also singly housed after surgery and allowed to recover for 3 weeks before experiments.

Slice Electrophysiology. Experimental animals were deeply anesthetized with isoflurane and then rapidly decapitated. The brain was removed and hemisected; each hemisphere was affixed to a stage submerged in ice-cold, oxygenated sucrose solution (in mM: 206 sucrose, 26 $NaHCO_3$, 10 D-glucose, 2.8 KCl, 3 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, and 0.4 ascorbic acid.). Coronal slices (300 μm) through the hypothalumus region were collected (Leica VT1200; Wetzlar, Germany) and stored in a holding chamber containing room temperature oxygenated ACSF (in mM: 125 NaCl, 25 $NaHCO_3$, 25 D-glucose, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 0.4 ascorbic acid) until used for recording. Slices were allowed to recover for at least an hour before use. For recording, individual slices were transferred to a nylon net and submerged in warmed (31–32°C) oxygenated ACSF, which was constantly perfused into the bath. Neurons were visually identified via differential interference contrast (DIC) optics with a 40x objective on an upright microscope (Scientifica, East Sussex, UK). Patch pipettes were fashioned from

borosilicate glass (OD 1.2 mm, ID 0.8 mm; Warner Instruments, Hamden, CT) on a Flaming-Brown P-97 pipette puller (Sutter Instruments, Novato, CA) to an open-tip resistance of 4–8 MΩ. Internal solution consisted of (in mM) 115 K-gluconate, 20 KCl, 10 HEPES, 10 Tris-phosphocreatine, 2 Na_2-ATP, 2 $MgCl_2$, 0.3 Tris-GTP, and 0.1% biocytin (for subsequent morphological evaluation). Intracellular solution had a pH of 7.3 (obtained using 1 M KOH) and an osmolarity of 300–310 mOsm. With the amplifier in voltage-clamp mode, a micromanipulator (Scientifica) was used to slowly advance the patch pipette towards the cell until a sharp increase in tip resistance was observed. Negative pressure was applied to form a gigaohm seal, after which brief mouth-suction pulses were used to break into the cell. After whole-cell configuration was achieved, the amplifier was switched to current-clamp mode. Series resistance and capacitance compensation were monitored and adjusted throughout the duration of the experiment. Data were sampled at 50 kHz by using pClamp 10 software (Molecular Devices, Sunnyvale, CA) with a MultiClamp 700B amplifier (Molecular Devices), filtered at 5 kHz, and stored via a Digidata 1400 analog-to-digital board (Molecular Devices) on a personal computer. The health and responsiveness of each cell was initially characterized by a series of 500-ms step current injections, ranging from -200 pA to +400 pA. Neurons were accepted for recording if the resting membrane potential was less than -60 mV and action potentials overshot 0 mV. Slices were stimulated optogenetically using an upright IR-DIC/fluorescence optical platform (Scientifica) outfitted with a Texas Instruments DLP® light source containing a digital micro-mirror device that permits targeted, patterned stimulation of individual neurons (Polygon 400; Mightex, Toronto, Ontario Canada).

Immunohistochemistry. Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) dissolved in PBS. The brains were removed and post-fixed in PBS containing 4% PFA for 8 hours and subsequently immersed in a cryoprotectant consisting of PBS containing 20% sucrose for 24-48 hours. Brains were subsequently snap-frozen in 2-methylbutane and stored at −80°C. Coronal sections 15 µm were cut on a cryostat. Brain sections were processed for immunostaining then coverslipped using ProLong Gold Antifade Reagent (Life Technologies, CA). Slides were imaged on an Olympus Fluoview FV1000 confocal microscope with a multiline argon laser. Microscope control and image acquisition was achieved using Olympus FV10 software.

To determine the specificity of ChR2-eYFP expression in POMC neurons of viral POMC-ChR2 mice, primary antibodies anti-GFP (1:1000, chicken, Aves labs) and anti-POMC (1:200, rabbit, Phoenix Pharmaceuticals) were used in conjunction with fluorophore-conjugated secondary antibodies AlexaFluor488 goat anti-chicken and AlexaFluor568 goat anti-rabbit (1:200, Life Technologies) respectively. To characterize the ChR2-eYFP expression in the Arc of Tg POMC-ChR2 mice, primary antibodies anti-GFP (1:1000, chicken, Aves labs) and anti-POMC (1:200, rabbit, Phoenix Pharmaceuticals) were used for POMC neurons; primary antibodies anti-GFP (1:1000, chicken, Aves labs) and anti-AgRP (1:500, goat, Neuronomics) were used for AgRP neurons. Secondary antibodies were AlexaFluor488 goat anti-chicken and AlexaFluor568 goat anti-rabbit (1:200, Life Technologies) respectively, or AlexaFluor488 donkey anti-chicken (1:250, Jackson ImmunoResearch) and AlexaFluor594 donkey anti-goat (1:200, Life Technologies).

For all cFos protein induction experiments, mice were handled and habituated to an open field chamber (36 X 36 cm) for 4 consecutive days before optogenetic stimulation. Mice received 6 min of blue laser (473 nm, Vortran Laser Technology, CA) stimulation (10 Hz, 15 ms pulses, 4.3 mW on the tip of fiber) in the same open field chamber, then returned to their home cage. Animals were perfused 90 min after the onset of light stimulation. For viral POMC-ChR2 mice, primary antibodies anti-GFP (1:1000, chicken, Aves labs) and anti-cFos (1:200, rabbit, Santa Cruz) were used. Secondary antibodies were AlexaFluor488 goat anti-chicken and AlexaFluor568 goat anti-rabbit (1:200, Life Technologies) respectively. For Tg POMC-ChR2 mice, primary antibodies anti-GFP (1:1000, chicken, Aves labs), anti-β-endorphin (1:100, goat, Santa Cruz), and anti-cFos (1:200, rabbit, Santa Cruz) were used for detection of cFos activation in POMC-positive neurons of the Arc; primary antibodies anti-GFP (1:1000, chicken, Aves labs), anti-AgRP (1:500, goat, Neuronomics) and anti-cFos (1:200, rabbit, Santa Cruz) were used for detection of cFos activation in AgRP-positive neurons of the Arc. Secondary antibodies were AlexaFluor488 donkey anti-chicken (1:250, Jackson ImmunoResearch), AlexaFluor594 donkey anti-goat (1:200, Life Technologies), and AlexaFluor647 donkey anti-rabbit (1:200, Life Technologies).

Behavioral Experiments. All behavioral tests were conducted at least 3-week post-surgery during the same circadian period (0900 – 1600). Mice were handled and attached to the optical fiber without photostimulation for 4 days before behavioral tests. For the hot-plate test, applied heat (IITC Life Science, CA) was used at a constant temperature of 51.2 – 51.4°C. The latency (in seconds) for the mice to lick their paws was measured

(cut-off time 60 sec). On three consecutive testing days, mice were connected to the optical fiber and placed in an empty mouse cage for 5 min (day 1, blue laser off; day 2, blue laser on; day 3, blue laser off). Immediately following this 5-minute period, mice were then tested on the hot-plate. Laser stimulation (15 ms pulses, 10 Hz, 4.3 mW on the tip of fiber) on day 2 continued until the completion of the test. For naloxone pretreatment studies, mice were injected intraperitoneally with 0.9% saline or naloxone HCl (10 mg/kg, Sigma) 15 min prior to laser stimulation.

For the regular food intake test, mice had *ad libitum* access to standard lab pellet food in the home cage before and after tests. Each mouse was tested at the same time each day. Mice were placed in an empty mouse cage for 30 min before installation of optical fiber. Mice were connected to the optical fiber and transferred to a test cage (29 cm × 17 cm) for 30 min to measure food intake. Food consumption was measured for three consecutive days (day 1, blue laser off; day 2, blue laser on; day 3, blue laser off). The laser stimulation parameters used on day 2 were 15 ms light pulses at 10 Hz for 30 s every minute for 30 min with 4.3 mW light power on the tip of fiber. For naloxone pretreatment in Tg POMC-ChR2 mouse line, animals were injected intraperitoneally with 0.9% saline or naloxone HCl (2 mg/kg; 10 mg/kg) 15 min prior to laser stimulation. For food intake test in viral POMC-ChR2 mice, animals were food-deprived for 4 hours with water freely available prior to regular food intake test each day. The laser stimulation on day 2 was the same as described above.

3D Visualization. For 3D imaging of immunofluorescence in viral POMC-ChR2 mice using CLARITY procedures (19-21), animals were perfused with 4% PFA in PBS. Whole

brains were extracted, post-fixed in PFA for 18 hours, rinsed in PBS overnight, and further crosslinked with A4P0 (4% monomer acrylamide and 0.25% VA-044 enzyme) similar to that previously described (21). Brains were placed in a stainless steel brain matrix, and dissected to obtain Bregma levels from approximately 0.98 to -3.08 mm. Briefly, PFA-fixed brains were immersed in A4P0 for 2 days at 4°C, degassed in a vacuum chamber for 30 minutes, and polymerized at 37°C for 3 hours. Samples were rinsed in PBS+0.01% Triton X-100 overnight, dissected as described above, and lipids were removed with 4% sodium dodecyl sulfate in 0.2M boric acid using an X-CLARITY™ tissue clearing system (Logos Biosystems, Inc., South Korea) set at 1.5 A, 37°C, and 32 rpm pump speed for 12 hours. After rinsing overnight in 0.02M boric acid + 0.1% Triton X-100 (BTX), immunofluorescence was performed using similar published protocols (19, 20). Brain tissue was incubated in BTX with primary rabbit anti-GFP antibody conjugated to Alexa Fluor™ 555 (1:100; Sigma) at 37°C for 5 days and then rinsed 3 days in BTX before being placed in 88% Histodenz refractive index matching solution (21) overnight at 37°C.

For 3D imaging of immunofluorescence in Tg POMC-ChR2 mice we used the iDISCO method (22). Animals were perfused with 4% PFA in PBS, whole brains were subsequently extracted and post-fixed for 18 hours. Following extensive rinsing in PBS, brains were dissected as described above for CLARITY. The original iDISCO protocol (22) was followed with slight modification including implementation of ethanol as a substitute for methanol during dehydration steps. Briefly, brain samples were placed in ascending alcohol concentrations to reach 100% ethanol, bleached overnight in 75% ethanol/20% DMSO/5% hydrogen peroxide at 4°C, and rehydrated to PBS. Brain tissue was then sequentially incubated at 37°C in a permeabilization solution of PBS/0.2% Triton

X-100/20% DMSO/0.3 M glycine, blocked in PBS/0.2% Triton X-100/10% DMSO/6% goat serum, and rinsed PBS/0.2% Tween-20 with 10 μg/ml heparin (PTH) for 18 hours each. Following overnight rinsing in PTH, samples were incubated in chicken anti-GFP primary antibody (1:200; Aves Labs Inc., Tigard, Oregon) in PTH/5% DMSO/3% goat serum for 4 days, rinsed for 2 days in PTH before 4-day incubation in CF™ goat anti-chicken 555 Fab-fragment secondary antibody (1:200; Sigma). After rinsing in PTH, samples were successively placed in 50%, 70%, 80% (in distilled H_2O) and 100% tetrahydrofuran (THF; Sigma) then incubated in dibenzyl ether (DBE; Sigma) prior to imaging.

CLARITY optimized light sheet microscope (COLM) was used to acquire the images (20). Tiles of acquired image stacks were stitched together using TeraStitcher (Integrated Research Centre of University Campus Bio-Medico of Rome, Italy) and subsequently visualized in 2D through orthoslice mode and in 3D through maximum intensity projection volume rendering mode using Amira 3D software (FEI, Thermo Fisher Scientific).

cFos mRNA Expression Following Optogenetic Stimulation. For all cFos mRNA response experiments, mice were handled and habituated to an open field chamber (36 X 36 cm) for 4 consecutive days before laser stimulation. Mice received 6 min of either blue laser stimulation (10 Hz, 15 ms pulses, 4.3 mW on the tip of fiber) or no laser stimulation in the same open field chamber, then returned to their home cage. Mice were killed by rapid decapitation 30 min after the onset of laser stimulation and their brains were removed, snap-frozen in 2-methylbutane, and stored at −80°C until sectioned. For

naloxone pretreatment in Tg POMC-ChR2 mouse line, animals were injected intraperitoneally with naloxone HCl (10 mg/kg) 15 min prior to laser stimulation.

In situ hybridization (ISH) methodology was performed as previously described (51). The cFos probe was a 667-bp fragment directed against the mouse cFos mRNA. cFos cDNA segment was extracted, subcloned in Bluescript SK (Stratagene), and confirmed by nucleotide sequencing. The probe was labelled in a reaction mixture of 1 µg of linearized plasmid, 1× transcription buffer (Epicentre Technologies), 125 µCi of ^{35}S-labeled UTP, 125 µCi of ^{35}S-labeled CTP, 150 µM ATP and GTP, 12.5 mM DTT, 1 µL RNase inhibitor, and 1.5 µL RNA polymerase. After hybridizing the labelled probe to the sectioned tissue, sections were thoroughly washed and exposed to Kodak BioMax MR Scientific Imaging Film (Sigma Aldrich). Radioactive signals were quantified using computer-assisted optical densitometry software ImageJ (National Institutes of Health).

Table S1. Distributions and relative abundance of immunoreactive fibers and terminals originating from Arc POMC neurons in the viral POMC-ChR2 mice

Anatomical sites	Intensity of immunoreactivity	
	Ipsilateral	Contralateral
Septal nucleus (lateral & medial)	+++	+
Lateral habenular nucleus	++	-
Medial thalamic nucleus	++	+
Lateral posterior thalamic nucleus	++	+
Bed nucleus of the stria terminalis	+	+
Nucleus of the vertical limb of the diagonal band	++	+
Nucleus of the horizontal limb of the diagonal band	+	-
Nucleus accumbens core	+	+
Medial nucleus accumbens shell	+	+
Preoptic area	+	+
Periventricular thalamic nucleus	++	+
Posteromedial/lateral thalamic nucleus	++	+
Laterodorsal thalamic nucleus	++	+
Tectal nucleus	+	-
Periaqueductal gray	+++	+
Anterior hypothalamic nucleus	++++	+
Dorsomedial hypothalamic nucleus	++++	+
Zona incerta	++++	+
A13 dopamine cells	+++	+
Posterior hypothalamic area	+++	+
Periventricular hypothalamic nucleus	++++	+
Lateral hypothalamic nucleus	++++	+
Arcuate nucleus	+++++	+++
Median eminence	++	++
Amygdala (BMA/CeL/CeC/MePD)	++	-

The intensity of immunoreactivity is estimated and indicated as: -, undetectable immunoreactivity; +, light density; ++, moderate; +++, dense; ++++, heavy; +++++, compact. *n* = 3 mice.

Table S2. Optical density for cFos mRNA in brain regions following optogenetic stimulation in the Arc of the viral POMC-ChR2 mice

Brain regions	Off	On
Cerebral cortex	0.0866 ± 0.0063	0.1758 ± 0.0155**
NAcc	0.0496 ± 0.0027	0.0476 ± 0.0034
L septum	0.0932 ± 0.0059	0.1525 ± 0.0110**
PVN	0.0710 ± 0.0138	0.1193 ± 0.0055**
LH	0.0627 ± 0.0055	0.0691 ± 0.0042
DM hypothalamus	0.0652 ± 0.0060	0.1014 ± 0.0079*
Arc	0.0576 ± 0.0005	0.0863 ± 0.0070*
Arc, medial posterior	0.0606 ± 0.0029	0.1034 ± 0.0119*

Results are expressed as mean ± SEM with n = 3 mice for the no stimulation group (light off) and n = 6 mice for the stimulation group (light on). Unpaired two-tailed t-test: cerebral cortex, $t(7) = 3.87$, $^{**}P = 0.006$; NAcc, $t(7) = 0.39$, $P = 0.71$; L septum, $t(7) = 3.59$, $^{**}P = 0.009$; PVN, $t(7) = 3.99$, $^{**}P = 0.005$; L hypothalamus, $t(7) = 0.89$, $P = 0.4$; DM hypothalamus, $t(7) = 2.96$, $^{*}P = 0.02$; Arc, $t(7) = 2.79$, $^{*}P = 0.03$; medial posterior part of Arc, $t(7) = 2.44$, $^{*}P = 0.04$. NAcc, nucleus accumbens; L septum, lateral septum; PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamus; DM hypothalamus, dorsomedial hypothalamus; Arc, arcuate nucleus.

Table S3. Optical density for cFos mRNA in brain regions following optogenetic stimulation in the Arc of the Tg POMC-ChR2 mice

Brain regions	Off	On
Cerebral cortex	0.3178 ± 0.0219	0.9297 ± 0.1629**
PFC	0.4835 ± 0.0440	1.678 ± 0.2192***
NAcc	0.1228 ± 0.0074	0.2045 ± 0.0151***
CPu	0.1699 ± 0.0255	0.2437 ± 0.0208*
Septum	0.4529 ± 0.0389	0.6948 ± 0.0469**
PVN	0.4127 ± 0.0442	0.7872 ± 0.0839**
L thalamus	0.2716 ± 0.0316	0.4413 ± 0.0477*
Amy	0.2529 ± 0.0201	0.3201 ± 0.0310
LH	0.1888 ± 0.0117	0.3952 ± 0.0473**
DM hypothalamus	0.2504 ± 0.0269	0.5045 ± 0.0500**
Arc	0.2043 ± 0.0169	0.9454 ± 0.1544**

Results are expressed as mean ± SEM with $n = 6$ mice for the no stimulation group (light off) and $n = 8$ mice for the stimulation group (light on). Unpaired two-tailed t-test: cerebral cortex, $t(12) = 3.2$, $^{**}P = 0.008$; PFC, $t(12) = 4.62$, $^{***}P = 0.0006$; NAcc, $t(12) = 4.36$, $^{***}P = 0.0009$; CPu, $t(12) = 2.27$, $^{*}P = 0.04$; septum, $t(12) = 3.78$, $^{**}P = 0.003$; PVN, $t(12) = 3.57$, $^{**}P = 0.004$; L thalamus, $t(12) = 2.75$, $^{*}P = 0.02$; Amy, $t(12) = 1.68$, $P = 0.12$; LH, $t(12) = 3.69$, $^{**}P = 0.003$; DM hypothalamus, $t(12) = 4.05$, $^{**}P = 0.002$; Arc, $t(12) = 4.1$, $^{**}P = 0.002$. PFC, prefrontal cortex; CPu, caudate-putamen; L thalamus, lateral thalamus; Amy, amygdala.

Table S4. Optical density for cFos mRNA in brain regions following optogenetic stimulation in the Arc of the Tg POMC-ChR2 mice by pretreatment with naloxone

Brain regions	Off	On
Cerebral cortex	0.3100 ± 0.0501	0.3317 ± 0.0184
PFC	0.3254 ± 0.0684	0.3966 ± 0.0302
NAcc	0.1087 ± 0.0118	0.1147 ± 0.0093
CPu	0.1165 ± 0.0110	0.1342 ± 0.0139
Septum	0.2557 ± 0.0515	0.3797 ± 0.0478
PVN	0.6691 ± 0.2048	1.0940 ± 0.2328
L thalamus	0.0872 ± 0.0084	0.1014 ± 0.0061
Amy	0.5673 ± 0.0704	0.5383 ± 0.0571
LH	0.1331 ± 0.0119	0.2316 ± 0.0267**
DM hypothalamus	0.1738 ± 0.0302	0.3675 ± 0.0462**
Arc	0.1692 ± 0.0229	0.4398 ± 0.0661**

Results are expressed as mean ± SEM with n = 6 mice for the no light stimulation group (light off) and n = 7 mice for the stimulation group (light on). Naloxone, 10 mg/kg. Unpaired two-tailed t-test: cerebral cortex, $t(11) = 0.43$, $P = 0.67$; PFC, $t(11) = 1$, $P = 0.34$; NAcc, $t(11) = 0.4$, $P = 0.7$; CPu, $t(11) = 0.98$, $P = 0.35$; septum, $t(11) = 1.77$, $P = 0.11$; PVN, $t(11) = 1.35$, $P = 0.2$; L thalamus, $t(11) = 1.39$, $P = 0.19$; Amy, $t(11) = 0.32$, $P = 0.75$; LH, $t(11) = 3.18$, $^{**}P = 0.009$; DM hypothalamus, $t(11) = 3.38$, $^{**}P = 0.006$; Arc, $t(11) = 3.62$, $^{**}P = 0.004$.

Movie S1. eYFP-expressing Arc POMC neurons and their connectivity shown as a series of *xy*-plane images in the viral POMC-ChR2 mouse brain. This movie shows optical cross-sections moving along the posterior-anterior axis of the brain.

Movie S2. Maximum intensity projected 3D visualization of eYFP-expressing Arc POMC neurons and their connectivity in 4mm-thick CLARITY processed brain tissue from a viral POMC-ChR2 mouse. This representative volume-rendered movie shows prominent ipsilateral arcuate connectivity with the paraventricular hypothalamus, zona incerta, periaqueductal grey and lateral septum in the viral POMC-ChR2 mouse brain.

Movie S3. Labeled Arc neurons derived from POMC-expressing progenitors and their connectivity shown as a series of *xy*-plane images in a Tg POMC-ChR2 mouse brain. This movie shows optical cross-sections moving along posterior-anterior axis.

Movie S4. Maximum intensity projected 3D visualization of labeled Arc neurons derived from POMC-expressing progenitors and their connectivity. 2 mm slices of the Tg POMC-ChR2 mouse brain were processed through the iDISCO method. Immunoreactivity in this representative volume-rendered video shows prominent arcuate connectivity with the dorsomedial, paraventricular, and lateral hypothalamic nuclei in the Tg POMC-ChR2 mouse brain.