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Supplemental Information

A Neural Circuit Underlying the Generation of Hot Flushes

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Supplemental Experimental Procedures

Generation and maintenance of *Kiss1*^{Cre} (v2) mice

The previous line of *Kiss1*$^{Cre:Gfp}$ knock-in mice (v1) were prone to ectopic expression when genetically crossed to conditional reporter mice, and also retained a low level of *Kiss1* expression (Popa et al., 2013). Barring genetic crosses, this v1 line remains a useful tool for visualizing Kiss1 neurons for electrophysiological recordings with the Gfp-fused-Cre-reporter transgene. Also, viral injections into adult v1 mice, as opposed genetic crosses, can be used to gain control of those Kiss1 neurons and thereby avoid developmental ectopic recombination.

In this study we generated a second *Kiss1*^{Cre} line (v2) modified by: (a) removing the nuclear localization signal from Cre, (b) using a non-optimal initiation codon, (c) removing part of the N-terminal sequence of Cre, (d) adding a 3' untranslated region from the *Myc* gene that promotes a short mRNA half-life, (e) deleting part of exon 1 that includes the signal peptide, and (f) performing the gene targeting in C57Bl/6 ES cells. Fig. S1 legend contains a detailed description of the genetic construct that was used to engineer this transgenic mouse line. When crossed to a conditional reporter line, Ai14, v2 faithfully reports Kiss1 neurons in the rostral periventricular area, bed nucleus of the stria terminalis, arcuate nucleus and medial amygdala, with little to no ectopic expression (Fig. S1C). Genotyping was performed by 3-primer PCR. *(a) Kiss1* forward: 5'GACCTAGGCTCTGGTGAAGTACG; *(b) Kiss1* reverse: 5'AGCCTCCAGTGCTCACAGCAG; *(c)* Cre reverse: 5'CTTGCGAACCTCATCACTCGTTGC.

Viruses and stereotactic injections

Mice under 3 months of age received bilateral injections of either a control adeno-associated virus (AAV, serotype 1) vector encoding Cre-dependent mCherry fluorescent reporter (AAV1-EF1α-DIO-mCherry) or co-injections of AAV1 vectors encoding Cre-dependent ChR2:mCherry (AAV1-Ef1α-DIO-ChR2:mCherry) and Cre-dependent hM3Dq:YFP (AAV1-Ef1α-DIO-hM3Dq:YFP); 500 nl of virus (~10^9 particles per ml) was delivered to each targeted injection site. Viruses were prepared at the University of Washington as described (Gore et al., 2013). Surgeries were performed aseptically under constant infusion of isoflurane (2% isoflurane/O_2) while head-fixed to a stereotaxic frame. Targeted injections were either determined by manual placement on a stereotaxic frame (David Kopf Instruments) with glass pipette

delivery or using a Neurostar Drill and Injection Robot system (Tubingen, Germany) with a 33-Ga needle (NanoFil NF33BL). Viruses were injected bilaterally into the ARH (with respect to Bregma, anteroposterior (AP): −1.25 mm; mediolateral (ML): ± 0.30 mm; dorsoventral (DV): 5.80 mm) over 5 min, and injectors were left in place for 10 min before slow withdrawal. Fiber-optic cannulas were made in-house using 2.5-mm Ceramic Stick Ferrules (Precision Fiber Products) and 0.22 NA multimode fiber (Thor Labs) as described (Sparta et al., 2011), and were positioned such that the fiber tip was ~0.5 mm above the targeted cell population. ARH fibers were placed unilaterally (AP: -1.25; ML: +0.3; DV: 5.3), while the single rostral POA fiber was placed centrally (AP: +0.45; ML: 0.0; DV 4.25). Fiber optic cannulas were fixed in place using Metabond (Parkell) and dental cement. Animals included in the results of this manuscript were qualified by post-hoc immunohistochemistry to be either unilateral or bilateral hits for viral expression in the ARH.

Stimulation of Kiss1^{ARH} neurons

CNO (1.0 mg/kg) or saline (10 μl/g BW) was administered intraperitoneally immediately following baseline temperature recordings at ZT 13:00. For optogenetic experiments, mice were patched to tethers for ≥ 6 h before recording. Laser emission with 473-nm light (calibrated to 10 mW at the fiber tip) was delivered at 2 Hz with 10-ms pulse width as described (Qiu et al., 2016).

Pharmacological blockade of neurokinin receptors in the POA

Guide cannulas were stereotaxically positioned at, AP: +0.5; ML: 0.0. The ventral tip of the guide or injection cannula (4.75 mm or 4.25 mm, respectively) was implanted 4 mm from the dura and cemented in place. Mice were handled daily for two weeks prior to testing. During this habituation period, mice were regularly restrained, tethered to injection tubing, and acclimated to intraperitoneal saline injections.

On test day, mice were tethered to injection tubing at ZT = 12:30 and treated with 300 nl of either ACSF or a cocktail of NkB receptor antagonists: SDZ-NKT 343, 167 pmol (NK1R, Tocris cat. #2394); GR 94800, 167 pmol (NK2R; Tocris cat. #1667); and SB222200, 500 pmol (NK3R; Tocris cat. #1393). At ZT = 13:00 the first tail-skin recording was taken immediately prior to CNO injection (1 mg/kg). This study was performed as a crossover design with randomized starts.

Determining effect of ovariectomy

Mice were randomly assigned concentrations of 0.1, 0.3, 0.5, and 1.0 mg/kg CNO and their tail-skin temperatures were recorded after administration. Allowing at least two days for recovery in between each trial, mice were then re-assigned a different concentration until, over the course of 5 experimental days, each mouse had received each concentration once. Mice were then ovariectomised, allowed to recover for 2 weeks, and then re-tested with the 0.3 mg/kg dose that gave approximately half-maximal response in the prior dose-response experiment.

Ovariectomy: Animals were anesthetized with 2% isoflurane/O_2, fur was removed from a ~5 mm area of the flank on both sides. The skin was sterilized and a small incision was made to expose the peritoneum. The ovary was clamped with a hemostat and removed. Dissolvable sutures were used to close the fascia. For the validation of the new *Kiss1*^{Cre} line (Supplemental Figure 2), during the same surgery a small perforated tube was also implanted in the subscapular region of the back, which contained either sesame oil (20 µl) or 17β–estradiol, E_2 dissolved in oil (20 µl, 1 mg/ml); animals were euthanized and prepared for histology one week after surgery.

Physiological recordings

Tail-skin temperature (T-sk) was recorded using an infrared camera (FLIR E4; FLIR Instruments). Images were uploaded using the software provided (FLIR Tools) and a custom Python script was used to extract temperature values taken from the base of the tail. Core-body temperature (T-b) and locomotor activity were recorded using an implantable telemetry system (G2 E-Mitters, Starr Life Sciences) and analyzed using the software provided (VitalView).

Immunohistochemistry

Mice were given Beuthanasia-D Special (effective dose 320 mg/kg pentobarbital) and transcardially perfused by flushing the circulation with cold saline, followed by administration of 40 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PFA). Brains were rapidly dissected and post-fixed in PFA at 4°C overnight, followed by subsequent cryoprotection in 30% sucrose. Brains were then rapidly frozen at -

30°C in a 2-methylbutane/Dry Ice slurry and stored at -80°C until processed. Floating 30-µm coronal sections were incubated in PBS and processed within 1 week of sectioning. For immunohistochemistry, sections were first blocked for 1 hour at room temperature (RT) in PBS with 0.2% Triton X-100, and 3% normal donkey serum. Sections were incubated overnight with the primary antibody at 4°C and the next day they were exposed to a corresponding secondary antibody for 2 hours at RT. Primary antibodies: rabbit anti-DsRed (Clontech 632496; diluted 1:1000) or goat anti-Fos (Santa Cruz SC-52-G; diluted 1:300). Processed sections were mounted onto glass slides and coverslipped with DAPI Fluoromount-G (Southern Biotech, 0100-290). Images were acquired with an Olympus FV1200 confocal microscope.

Supplementary Citations

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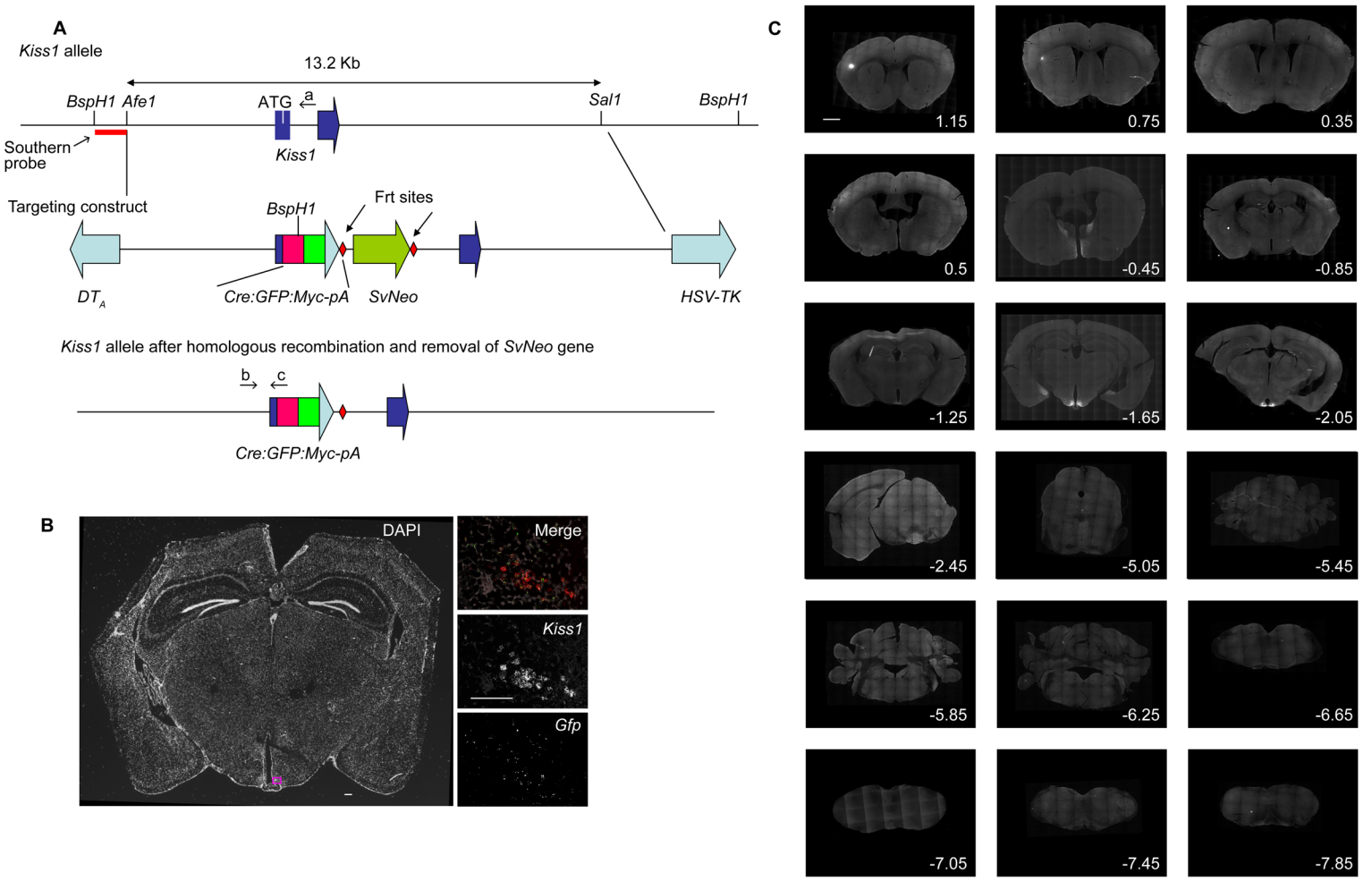
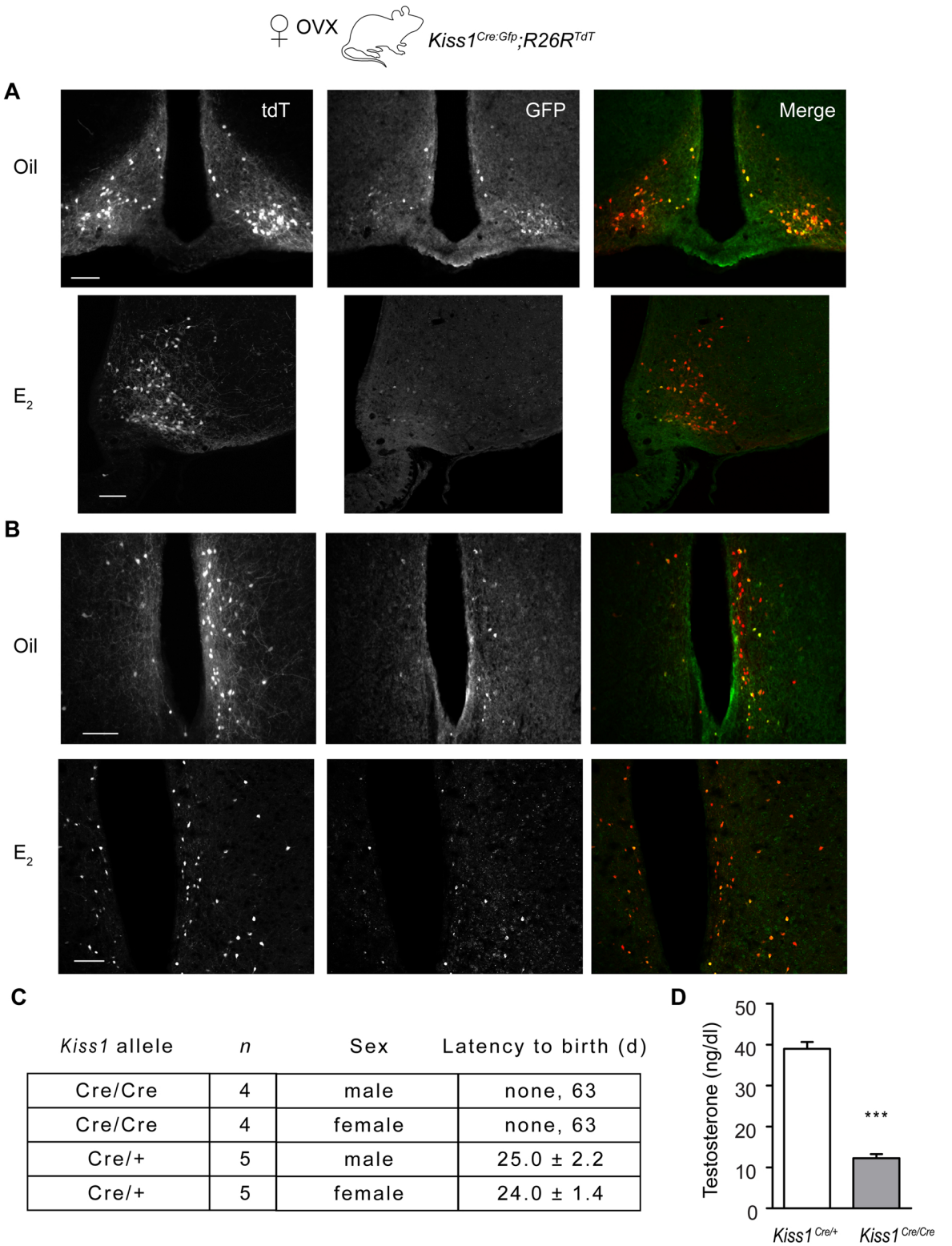
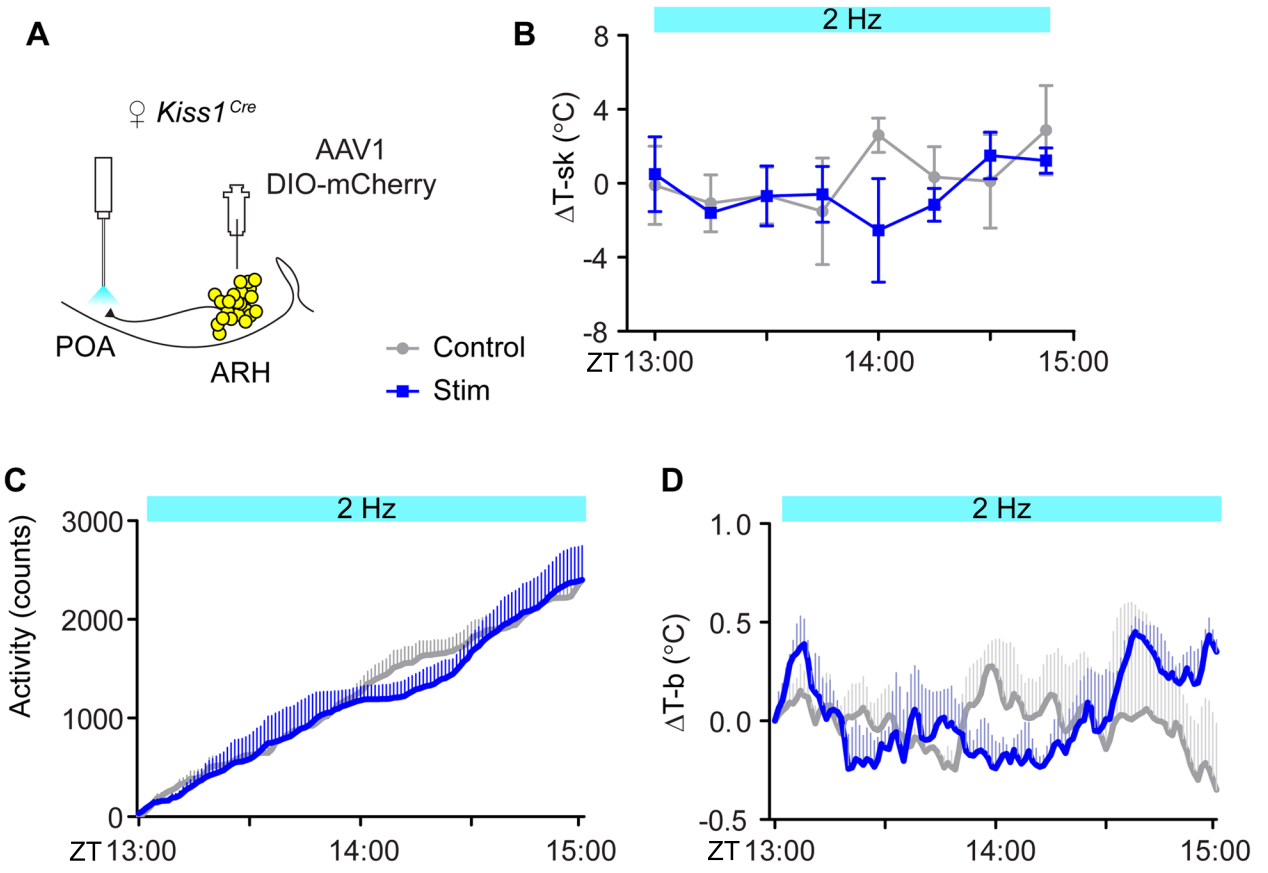


Supplemental Figure S1. Generation of *Kiss1*$^{Cre:GFP}$(v2) Mice and Expression Analysis. Related to Figure 1. (**A**) The targeting construct for *Kiss1* included ~5.0 kb DNA 5' of the translation start site and ~8 kb on the 3' side. The targeting construct contained a Cre:GFP fusion protein (the nucleotide sequence at the encoding the N-terminus of Cre was modified to be: CCCC ATG GCT GCA TTA CCG GTC) followed by the 3' untranslated region and poly-A addition sequence from the *Myc* gene. This cassette was inserted just 5' to the *Kiss1* initiation codon. A frt-flanked *SvNeo* gene (for positive selection) was inserted at the end of the fist exon, such that the sequence between the start codon and the end of that exon was deleted. The construct also contained *Pgk*-DTA and HSV-TK genes for negative selection. C57Bl/6 ES cells were electroporated and 7 positive clones were identified by digesting DNA with *BspH*1 and performing a Southern blot using a 350 bp probe located just outside the 5' boundary of the targeting construct. Positive clones were injected into C57Bl/6 blastocysts and high-percentage chimeras were bred to achieve germline transfer. The frt-Neo gene was removed by breeding the heterozygous mice with Gt(ROSA)26Sor-FLP recombinase; mice were subsequently bred with c57Bl/6. (**B**) *In situ* hybridization demon strating the relationship between endogenous Kiss1 and GFP in the arcuate hypothalamus of an ovariectomized *Kiss1*$^{Cre:GFP}$ female. Scale bar, 100 µm (**C**) *Kiss1*$^{Cre:GFP}$*:: Ai14, Gt(ROSA)26Sor*$^{tm14(CAG-td-Tomato)}$ bisgenic mice were used to reveal the lineage of *Kiss1*$^{Cre:GFP}$-expressing cells. tdT expression is shown in serial coronal sections from rostral to caudal (Bregma coordinates indicated). Scale bar, 1 mm.



Kiss1 allele	*n*	Sex	Latency to birth (d)
Cre/Cre	4	male	none, 63
Cre/Cre	4	female	none, 63
Cre/+	5	male	25.0 ± 2.2
Cre/+	5	female	24.0 ± 1.4

Supplemental Figure S2. Analysis of *Kiss1*$^{Cre:GFP}$ (v2) Mice. Related to Figure 1. (**A, B**) Immunohistochemistry of ovariectomized *Kiss1*$^{Cre:GFP}$*::Ai14* mice treated with either oil or 17β-estradiol, E2. GFP reveals active *Kiss1/Cre* expression, while tdTomato reports all cells that have ever expressed *Kiss1*$^{Cre:GFP}$. (**A**) Sections from the ARH or (**B**) rostral periventricular area (R3PV). Scale bars, 100 µm. (**C**) *Kiss1*$^{Cre/Cre}$ homozygous mice, males and females, did not yield litters following 63 days of exposure to fertile mates, whereas *Kiss1*$^{Cre/+}$ heterozygous mice became pregnant/sired litters within the first 5 d of exposure to a fertile mate and had an average litter size of 8.20 ± 0.42 with a male to female ratio of 1.35 ± 0.26. (**D**) Homozygous *Kiss1*$^{Cre/Cre}$ male mice had diminished testosterone levels compared to heterozygous controls; Cre/+ (n = 8) M = 39.00 ng/dl, SD = 4.76; Cre/Cre (n = 8) M = 12.28 ng/dl SD = 2.81; $t(14) = 13.68$; $p < 0.0001$ (***).



Supplemental Figure S3. Control Experiments with only AAV-DIO-mCherry Expression in POA. Related to Figure 3. (**A**) Schematic representation of the targeted viral injection into the ARH and fiber placement 0.5 mm above the POA. (**B** to **D**) Mice were tethered to a patch cord, a baseline measurement was taken at ZT 13:00, then they were either laser stimulated (blue line, 2 Hz) or not (grey line, controls) for 2 h; paired 2-way ANOVA comparisons, $n = 3$. Continuous light-stimulation in the POA did not change (**B**) tail-skin temperature (T-sk); $F(1,16) = 1.17$; $p = 0.30$, (**C**) locomotor activity counts or (**D**) core-body temperature (T-b) with respect to controls; $F(1,242) = 1.48$; $p = 0.23$.