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Supplementary Information for

Discrete TrkB-expressing neurons of the dorsomedial hypothalamus regulate feeding and thermogenesis.

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Supplementary Materials and Methods

Mice

Mice were maintained on a C57BL6/J background in a climate-controlled facility at 22°C on a 12h /12h light-dark cycle with *ad libitum* access to water and chow diet (Teklad Rodent Diet 2920) unless otherwise specified. The Ai9/+ mouse strain [Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J; stock no. 007909] was acquired from Jackson Laboratory. The *Ntrk2*$^{CreER/+}$ (also known as TrkB^{CreER}) mouse strain was generously provided by Dr. David Ginty (Harvard Medical School). Male and female mice aged 6-12 weeks old were used for initial investigations of chemogenetic activation or inhibition of global DMH^{TrkB} neurons. Both males and females showed similar phenotypes, thus only female mice were used for subsequent projection specific manipulation of DMH^{TrkB} neuronal activity. Nuclear localization of Cre recombinase in *Ntrk2*$^{CreER/+}$ mice was induced by tamoxifen (20 mg/ml in corn oil, Sigma-Aldrich T-5648) administration for 6 days [3 mg per animal per day, intraperitoneally (i.p.)] except where otherwise indicated. For DREADD experiments, mice were injected i.p. with CNO (1.5 mg/kg, Cayman Chemical 16882). All experiments were performed in accordance with relevant guidelines and regulations regarding the use of experimental animals. The Animal Care and Use Committees at The Scripps Research Institute Florida approved all animal procedures used in this study.

Immunohistochemistry

For Fos quantification in *Ntrk2*$^{CreER/+}$;Ai9/+ animals, labeling of TrkB+ neurons was induced with tamoxifen as described above. One week after the last tamoxifen injection, mice were individually placed in fresh cages in either warm (39°C), cold (10°C), or thermoneutral (30°C) ambient temperatures 2 hours prior to transcardial perfusion with 4% paraformaldehyde (PFA). Brains were dissected, post-fixed in 4%PFA overnight at 4°C, and cryoprotected in 30% sucrose for 3 days prior to collection of 40 µm floating coronal sections. For Fos staining to confirm activation of DMH^{TrkB} neurons expressing hM3, or projection-specific activation of DMH$^{TrkB\rightarrow RPa}$ neurons expressing hM3, mice were treated with CNO 2 hours prior to dissection and fixation of the brain. Sections were permeabilized in 1% Triton-X100 (T-X100), incubated in blocking buffer (10% horse serum, 1% bovine serum albumin (BSA), 0.1% T-X100 in PBS) for one hour at room temperature (RT) followed by incubation with primary antibody, rabbit anti-cFos (1:10,000, Synaptic System #226003) in 1% horse serum, 0.1% BSA, 0.1% T-X100 in PBS overnight at 4°C. The next day, sections were washed in PBS before incubation with secondary antibody (1:800, 488-donkey anti-rabbit, Jackson ImmunoResearch, 171-545-152) in blocking buffer for 1 hour at RT. Sections were washed again in PBS before staining for 20 minutes in DAPI (5 µg/mL, Roche 10236276001) in PBS. Sections were then mounted on Superfrost plus microscope slides (Fisher, 12-550-15) and sealed with mounting media (2.5% PVA-DABCO) and glass coverslips.

For analysis of anterograde tracing in BAT and heart tissues, mice were perfused with PBS followed by 4% PFA, and brown adipose tissue and heart were dissected and fixed overnight in 4% PFA. Tissues were cryoprotected in 30% sucrose, embedded in OCT, and frozen before collecting 20 µm sections with a cryostat. Sections were permeabilized in 1% T-X100 for 9 minutes, blocked in 5% horse serum/1% bovine serum albumin (BSA)/0.1% T-X100 for 1 hour, and incubated with primary antibodies (rabbit anti-UCP1, 1:750, Abcam ab10983; mouse anti-tyrosine hydroxylase, 1:1,500, Sigma-Aldrich T1299) in blocking buffer overnight at 4°C. Sections were washed in PBS and incubated with secondary antibodies (1:800, Jackson ImmunoResearch) in blocking buffer for 1 hour at RT. Sections were washed with PBS and stained with DAPI and sealed with mounting media (2.5% PVA-DAPCO) and glass coverslips.

Images representing 1-µm optical slices were taken using a Nikon C2+ confocal microscope equipped with 405, 488, 561, and 640nm coherent lasers. For Fos quantification of DMH^{TrkB} neurons, every third brain section was imaged bilaterally for a total of 7 sections spanning 520 µm of the DMH. Cell counts were performed using ImageJ Cell counter (NIH). Averages of TrkB+ and TrkB+/Fos+ neurons were taken for the first two sections representing the anterior DMH, the 3rd-5th sections representing the middle DMH, and the last two sections representing the posterior DMH.

Stereotaxic surgery

Ntrk2$^{CreER/+}$ mice at 6-8 weeks of age were anesthetized with isoflurane and mounted on a stereotaxic frame (Kopf Model 960). An incision through the skin along the midline of the head was made to expose the skull and the location of Bregma was determined as the reference point for the injection site coordinates [anterior-posterior (AP)/ mediolateral (ML)/ dorsoventral (DV)]: POA (AP, +0.60 mm/ ML, ±0.35 mm/ DV, -5.70 mm), PVH (AP, -0.50 mm/ ML, ±0.35 mm/ DV, -5.30 mm), DMH (AP, -1.50 mm/ ML, ±0.35 mm, DV, -5.70 mm), RPa (AP, -6.00 mm/ ML, 0.00 mm/ DV, -6.35 mm). A small hole was drilled through the skull at the injection site, the dura was gently peeled away, and a 2.5-µl syringe (Hamilton 7632-01) with 33G needle was slowly lowered into the brain. Virus was infused at a rate of 25 nl/min with a micropump and controller (World Precision Instruments, UMP3 pump and Micro4 controller) for a total of 250 nl (DMH) or 150 nl (POA, PVH, and RPa). Mice were allowed to recover for 1 week before induction

with tamoxifen as described above, and experiments were conducted 1 week following the last tamoxifen injection. CTB-488 and CTB-647 (Invitrogen) were stereotaxically injected into a brain site at 1 mg/ml.

For polysynaptic anterograde tracing with HSV129ΔTK-tt, *Ntrk2*$^{CreER/+}$ mice were given daily injections of tamoxifen (3mg/animal) starting 2 days prior to stereotaxic viral injection of HSV129ΔTK-TT (150nl, 2.2x10^{10} pfu/mL) unilaterally into the DMH, and continuing on postoperative days 1-4.

Core body temperature measurements

Animals were allowed to acclimate for 6 days to thermoneutral (30°C) or cold (10°C) housing for excitatory hM3 or inhibitory hM4 DREADD experiments, respectively. During this time animals were also conditioned to mock i.p. injections, and temperature measurements with a digital thermometer (Fisher Traceable Type-K thermometer) with rodent rectal temperature probe (World Precision Instruments, RET-3). For experiments performed in a cold environment, animals were housed individually. On the day of the experiment, rectal temperature was recorded immediately prior to i.p. injection of vehicle. Rectal temperature was recorded every 20 minutes for 1 hour. After the collection of the last timepoint following vehicle injection, another baseline measurement was recorded immediately prior to injection with CNO, and rectal temperature was acquired every 20 minutes for 1 hour for mice housed at thermoneutrality and for 1.5 hours for mice housed at 10°C. This procedure was repeated for two additional days and the average of the measurements for three days was determined for each animal.

iBAT temperature measurements

Animals were allowed to acclimate to thermoneutral housing and handling 6 days prior to experimentation. The day before testing, mice were anesthetized and the hair on their backs and above their shoulders was shaved. On the day of experimentation, a thermal camera (FLIR E53sc) was mounted above a cage with mice expressing either control (mCherry) or excitatory (hM3) virus in DMH^{TrkB} neurons. Thermal images were collected 1 hour after i.p. injection of vehicle or CNO and regions above the shoulders (above interscapular BAT), lumbar area (body), and tail were analyzed using Research IR software (FLIR). The average of 3 days of testing was determined for each animal.

Physiological measurements

Locomotor activity and oxygen consumption in individual mice were measured using a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments). Mice were previously conditioned for at least 1 week to thermoneutrality (for excitatory DREADD experiments) and 10°C (for inhibitory DREADD experiments) and allowed to acclimate to metabolic chambers for at least 24 hours prior to experimentation. Mice received a vehicle i.p. injection in the morning (between 8-9 am) and a second i.p injection with CNO (between 12-1 pm). Physical activity and metabolic data were collected for 4 hours following treatment with either vehicle or CNO. This paradigm was repeated for a second day and the data for each animal was averaged for days 1 and 2.

Heart rate and blood pressure were recorded using a tail cuff system (MC4000, Hatteras Instruments). Animals were acclimated to handling and restraint in the tail cuffs for 5 days prior to experimentation. The platform was set to 35°C, with 10 preliminary measurement cycles were followed by 15 cycles of measurement. Animals were placed in the tail cuffs 30 minutes after i.p injection with vehicle or CNO. Vehicle and CNO measurements were collected on different days at the same time of day to minimize the effect of stress due to restraint in the tail cuffs.

Nocturnal food intake studies were performed as previously described (1). Mice were acclimated to handling and single housing for 3 days prior to experimentation. Food intake was monitored for 4 nights following treatment with either vehicle (nights 1 and 2) or CNO (nights 3 and 4). On the day of the experiment, food was removed from the cage 3 hours prior to lights-off, and animals were given an i.p. injection of vehicle or CNO 10 minutes before lights-off. At the onset of lights-off, two food pellets (Teklad Rodent Diet 2920X; 3.1 kcal/g energy density) were weighed (with 0.01g precision) and placed in the cage with the mouse. Food was weighed again every hour for 4 hours after lights-off. Food intake for the two nights following either vehicle or CNO injection were averaged for each animal.

Quantitative RT-PCR

For measurement of *Ucp1* levels in BAT following activation of DMH^{TrkB} neurons, BAT tissue was dissected 2 hours after injection of CNO and snap-frozen in liquid nitrogen. RNA was extracted from 15-20 mg of BAT with TRIzol (Invitrogen) and chloroform, and precipitated with isopropanol. RNA was reverse-transcribed with M-MuLV reverse transcriptase (New England Biolabs, M0253) and random hexomers (ThermoFisher N8080127), and cDNA was diluted (1:5) prior to quantitative PCR (qPCR) analysis. qPCR was carried out using SYBR green mix (Roche) in an StepOne cycler (Applied Biosystems), using the primers: UCP1-F, 5′-GATGGTGAACCCGACAACTT-3′; UCP1-R, 5′-CTGAAACTCCGGCTGAGAAG-3′; 18S rRNA-F, 5′-CGCCGCTAGAGGTGAAATTC-3′; 18S rRNA-R,

5′-TTGGCAAATGCTTTCGCTC-3′. Fold change in UCP-1 transcript levels was calculated using the −2ΔΔCt method, normalizing to 18S rRNA transcript.

Immunoblotting

Two hours following stimulation with CNO (1.5 mg/kg), BAT was dissected from the interscapular region of mice expressing mCherry or hM3 in DMH^{TrkB} neurons, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Samples of BAT (10-20 mg) were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 1 mM NaF) supplemented with PhosStop (Millipore #4906845001) and protease inhibitor cocktail (Roche, 11-836-170-001). Samples were then prepared in 1x Laemmli buffer and 10 µg protein was resolved by SDS-PAGE. Following transfer, PVDF membranes were blocked with 5% milk and incubated with primary antibodies (Cell Signaling Technology #4139, rabbit anti-phospho-HSL(Ser563), 1:1000, and Sigma-Aldrich #A5441, mouse anti-β-actin, 1:5000) overnight at 4°C, followed by washing and incubation with species-appropriate HRP-conjugated secondary antibodies (ThermoFisher Scientific #A16110, goat anti-rabbit-HRP, 1:5000, and #31430, goat anti-mouse-HRP, 1:5000). Immunoblots were then stripped and re-probed for total HSL levels (Cell Signaling Technology #4107, rabbit anti-HSL, 1:1000). All immunoblots were visualized with ECL Supersignal West Pico PLUS Detection Reagent (Thermo Scientific, 34580) and densitometric analysis of bands was performed using ImageJ software.

Glycerol measurement

Glycerol content of BAT from mice expressing either mCherry or hM3 in DMH^{TrkB} neurons and treated with CNO (1.5 mg/Kg) was determined using a Glycerol-Glo Assay (Promega, J3150) according to the manufacturer's instructions. Briefly, BAT tissue was dissected from the interscapular region of mice expressing mCherry or hM3 in DMH^{TrkB} neurons 2 hours following treatment with CNO, snap frozen in liquid nitrogen and stored at -80°C until further processing. 5-10 mg of tissue was homogenized in Glycerol Lysis buffer and incubated at 37°C for 30 minutes. Samples were further diluted in Glycerol Lysis Buffer and incubated with Glycerol Detection Reagent for 1 hour at room temperature before detection of luminescence using a plate reader (BMG Labtech, FLUOstar Omega).

***In situ* hybridization**

Brains from $DMH^{TrkB\rightarrow RPa}$:mCherry and $DMH^{TrkB\rightarrow PVH}$:mCherry mice were dissected and immediately frozen on dry ice and stored at -80°C. Prior to sectioning, samples were embedded in OCT (Tissue Tek). Fresh frozen sections (14 µm) were collected with a cryostat (Leica) onto Superfrost Plus slides (Fisherbrand, 12-550-15). Fluorescent *in situ* hybridization was carried out according to the manufacturer's instructions (ACD, RNA Scope Multiplex Fluorescent Detection Kit v2, 323110). Briefly, sections were postfixed for 15 minutes in 4% paraformaldehyde and dehydrated with ethanol (50%, 75%, 100%). Sections representing at least one anterior DMH position (~B = -1.6 mm) and one middle DMH position (~B = -1.9 mm) were outlined with a hydrophobic barrier and allowed to dry for 20 minutes. Sections were placed in a humidified chamber and treated with H_2O_2 for 10 minutes at room temperature, digested with Protease IV for 20 minutes at room temperature, and then hybridized with target probes (mCherry #431201-C4, mm-Brs3 #454111-C2, mm-Slc17a6 #319171-C3, and mm-Slc32a1 #319191-C2). Signal amplification was caried out by incubation with v2-Amp1, v2-Amp2, and v2-Amp3, followed by TSA Plus Fluoroscein/Rhodamine/Cyanine 5 (NEL754001KT, Akoya). Sections were counterstained with DAPI and 4-5 µm confocal z-stacks were acquired (Nikon C2+).

SI References

1. G. Y. Liao, C. E. Kinney, J. J. An, B. Xu, TrkB-expressing neurons in the dorsomedial hypothalamus are necessary and sufficient to suppress homeostatic feeding. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 3256-3261 (2019).

Supplementary Figures

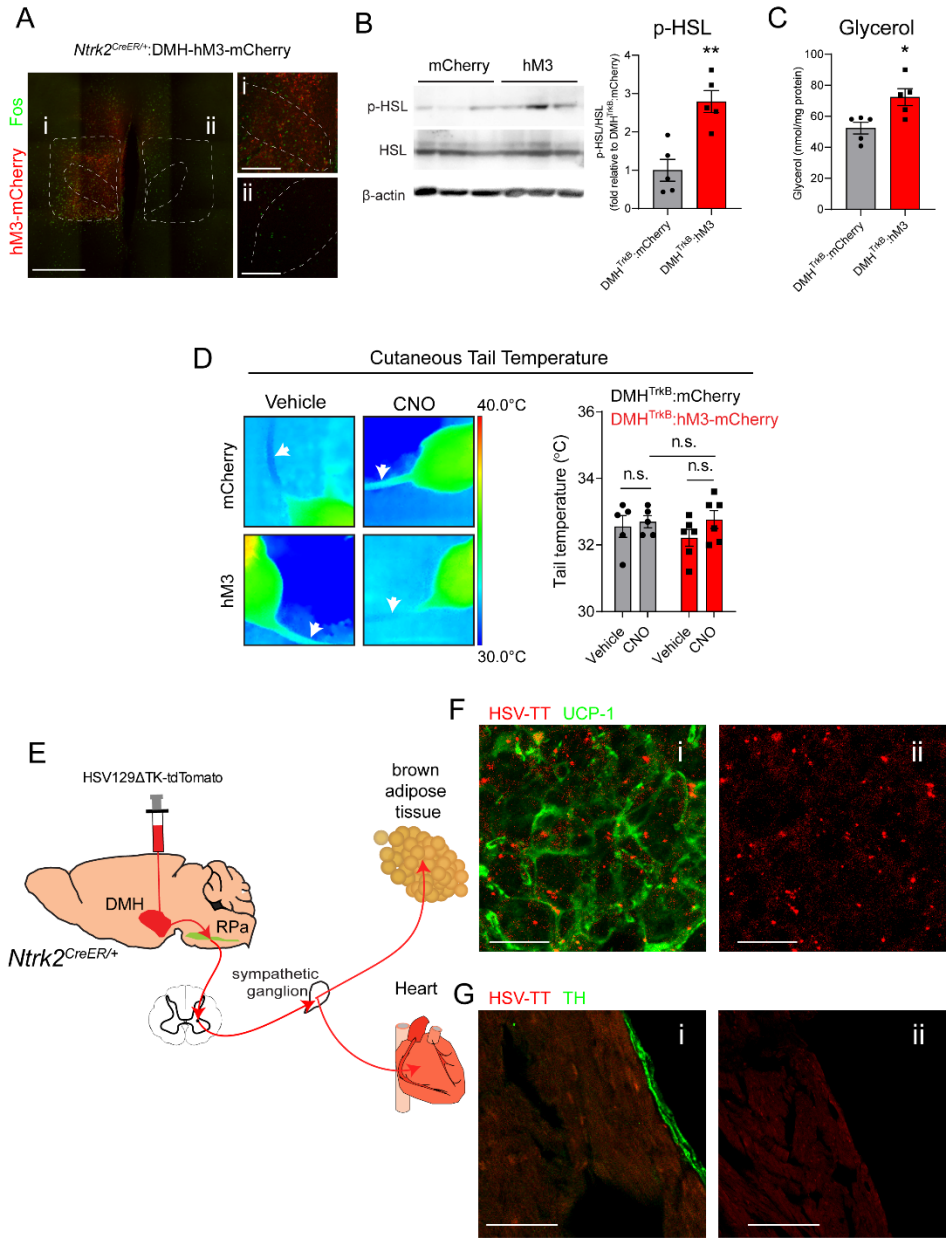

Figure S1. DMH^{TrkB} neurons do not influence vasoconstriction and form polysynaptic connections with BAT but not heart. (**A**) Expression of Fos (green) after unilateral stimulation of DMH^{TrkB} neurons expressing hM3-mCherry (**Ai, red**) with CNO, and (**Aii**) the non-injected contralateral side. Scale bars = 500 µm (**A**), 200µm (**Ai, ii**). Representative images are from *n* = 3 animals. (**B, C**) Level of phosphorylated hormone sensitive lipase (p-HSL) and glycerol in brown adipose tissue from DMH^{TrkB}:mCherry (*n* = 5) or DMH^{TrkB}:hM3 (*n* = 5) mice following treatment with CNO. *p<0.05 and **p<0.01 by T test. (**D**) Activation of DMH^{TrkB} neurons did not alter cutaneous tail temperature (indicator of vasoconstriction or vasodilation) as measured by thermal imaging (*n* = 5 mCherry, 6 hM3). Two-way RM ANOVA: mCherry vs. hM3, $F_{(1, 18)}$ = 0.2741, p=0.6070. (**E**) Diagram of polysynaptic anterograde tracing of DMH^{TrkB} neurons in *Ntrk2$^{CreER/+}$* mice. Following induction with tamoxifen, stereotactic delivery of Cre-dependent HSV129ΔTK virus expressing tdTomato into the DMH of *Ntrk2$^{CreER/+}$* mice allows for labeling of multiple orders of DMH^{TrkB} neuron targets 4-5 days post-injection. (**F**) Analysis of interscapular brown adipose tissue, (**Fi**) labeled by UCP-1, showed significant (**Fii**) deposits of tdTomato indicating robust anterograde labeling from DMH^{TrkB} neurons that drive thermogenesis. (**G**) Analysis of the heart revealed (**Gi**) labeling of sympathetic fibers by tyrosine hydroxylase (TH), but no detectable (**Gii**) tdTomato labeling (HSV-TT) from DMH^{TrkB} neurons. Values represent mean ± s.e.m., Sidak post-test (n.s. = not significant). (**F, G**) Representative images are from *n* = 4 animals. Scale bars = 20 µm (**Fi-ii**), 50 µm (**Gi-ii**).

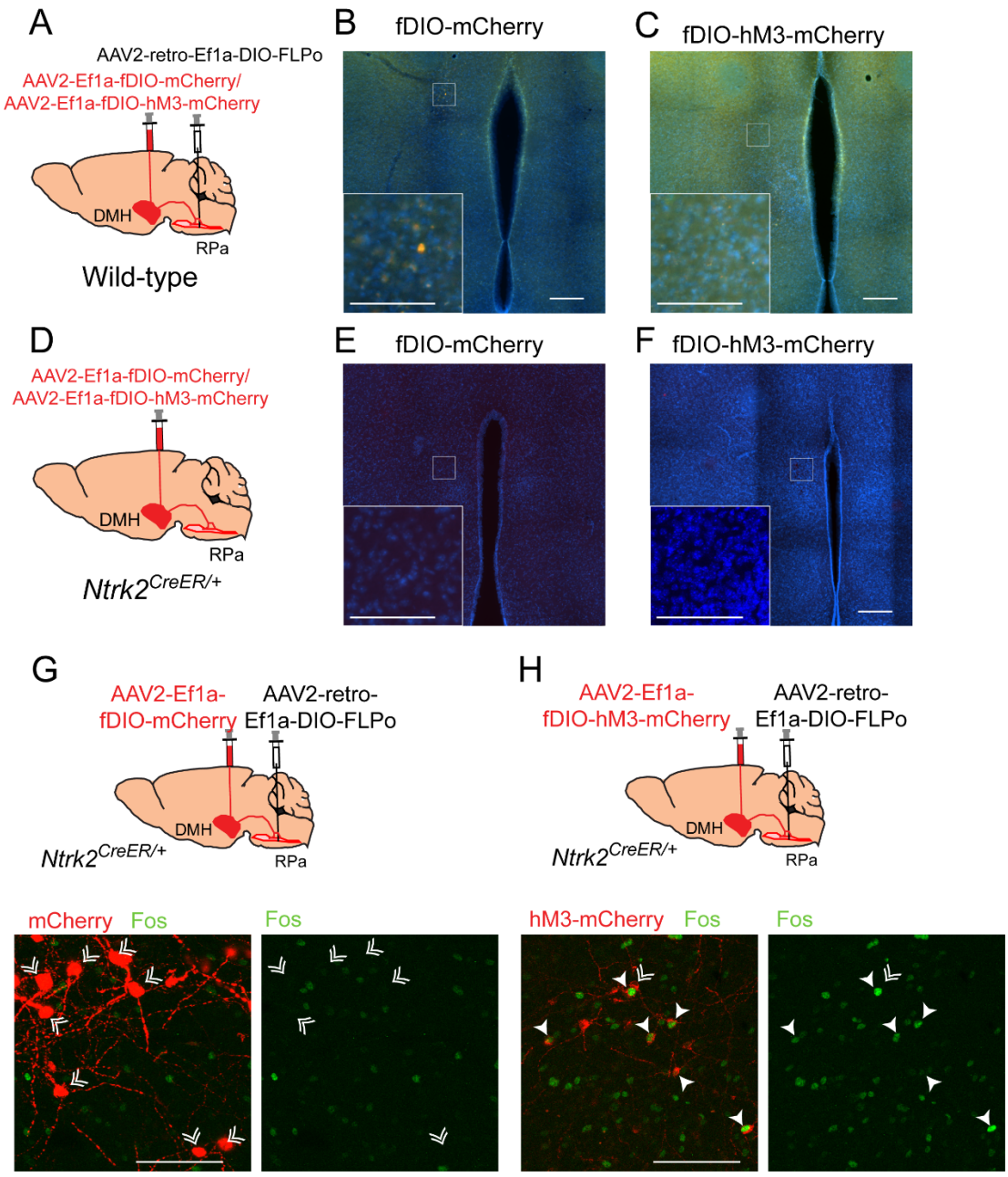

Figure S2. Validation of viral strategy for projection-specific expression and chemogenetic excitation of DMH^{TrkB} neurons. (**A**) Schematic of stereotactic delivery of retrograde AAV expressing Cre-dependent FLPo (AAV2-retro-Ef1a-DIO-FLPo) and AAV2-CMV-GFP to the RPa, and FLP-dependent (fDIO) mCherry or hM3-mCherry expressing virus to the DMH in wild-type mice. (**B, C**) No expression of mCherry or hM3-mCherry was detectable within the DMH of wild-type mice. (**D-F**) Injection of *Ntrk2$^{CreER/+}$* with AAV expressing FLP-depended (**E**) mCherry or (**F**) hM3-mCherry does not result in expression without the additional injection of retrograde AAV expressing Cre-dependent FLP into a DMH^{TrkB} projection target. Scale bars = 200 µm, inset scale bars = 100 µm. (**G, H**) Expression of Fos after treatment with CNO in mice expressing (**G**) mCherry or (**H**) hM3-mCherry in DMH^{TrkB} neurons. Double arrowheads indicate Fos^{-} DMH^{TrkB} neurons. Solid arrowheads indicate Fos^{+} DMH^{TrkB} neurons. Scale bars = 100 µm.

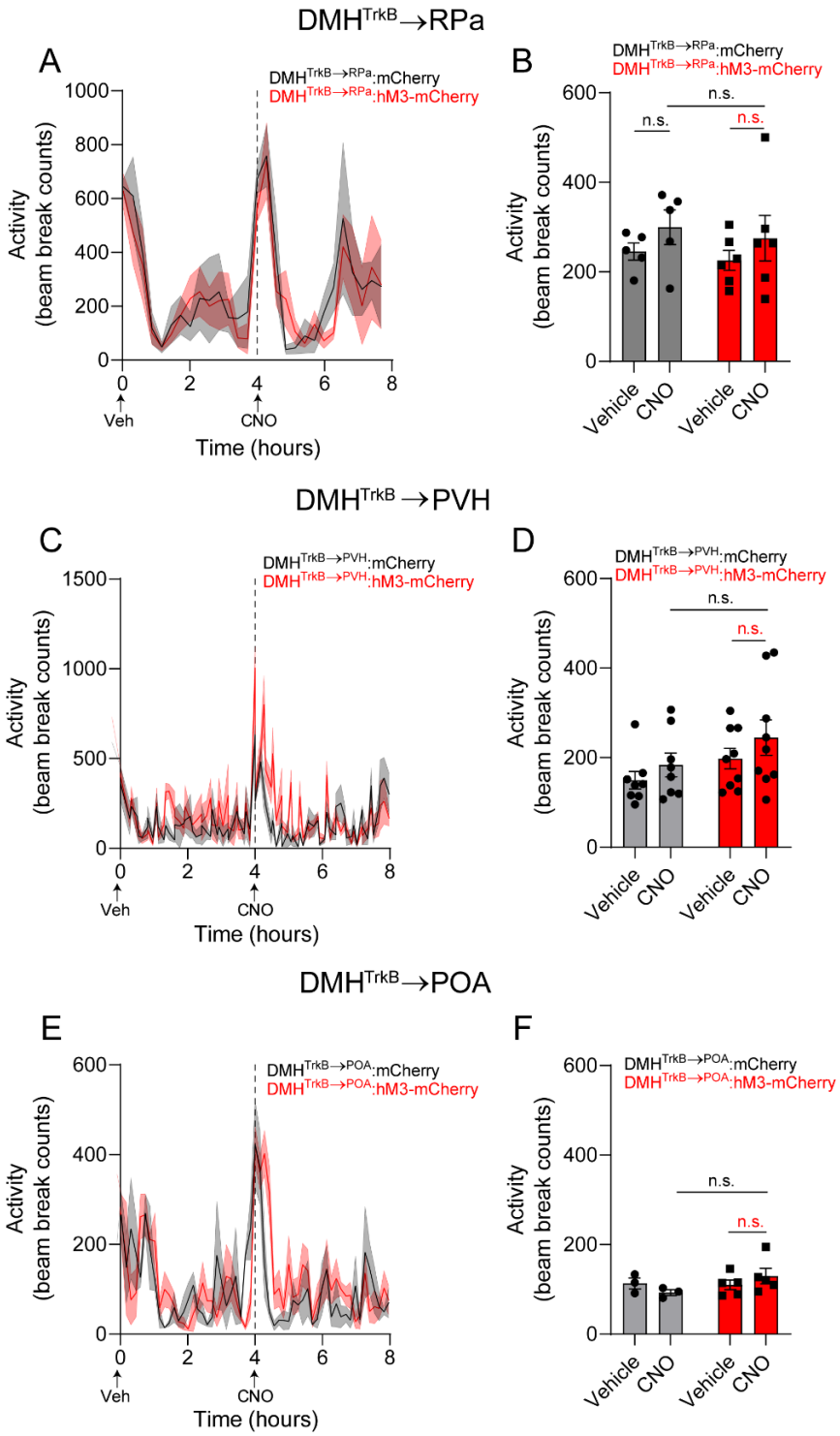

Figure S3. PVH-, POA-, and RPa-projecting DMH^{TrkB} neurons do not regulate locomotor activity. Mice housed at thermoneutrality were treated with vehicle (Veh) and then CNO during the light cycle. Activation of (**A**, **B**) DMH$^{TrkB→RPa}$ neurons (*n* = 5 mCherry mice, 6 hM3 mice), (**C**, **D**) DMH$^{TrkB→PVH}$ neurons (*n* = 8 mCherry, 9 hM3), or (**E**, **F**) DMH$^{TrkB→POA}$ neurons (*n* = 3 mCherry, 5 hM3) did not alter physical activity. (**A**, **C**, **E**) Locomotor activity after vehicle administration (hour 0-4) followed by CNO (hour 4-8). Two-way RM ANOVA: mCherry vs. hM3 (post-CNO), (**A**) $F_{(1, 9)}$ = 0.06638, p=0.8025; (**C**) Mixed effects model: mCherry vs hM3, $F_{(1, 15)}$ = 1.478, p=0.2429; (**E**) Two-way RM ANOVA: $F_{(1, 6)}$ = 2.533, p=0.1626. (**B**, **D**, **F**) Average locomotor activity for the duration of 4 hours following either vehicle or CNO treatment. Two way RM ANOVA: mCherry vs. hM3, (**B**) $F_{(1, 9)}$ = 0.4086, p=0.5386, (**D**) $F_{(1, 15)}$ = 2.111, p=0.1669, (**F**) $F_{(1, 6)}$ = 3.189, p=0.1244. Values represent mean ± s.e.m., with Sidak post-test (n.s. = not significant).

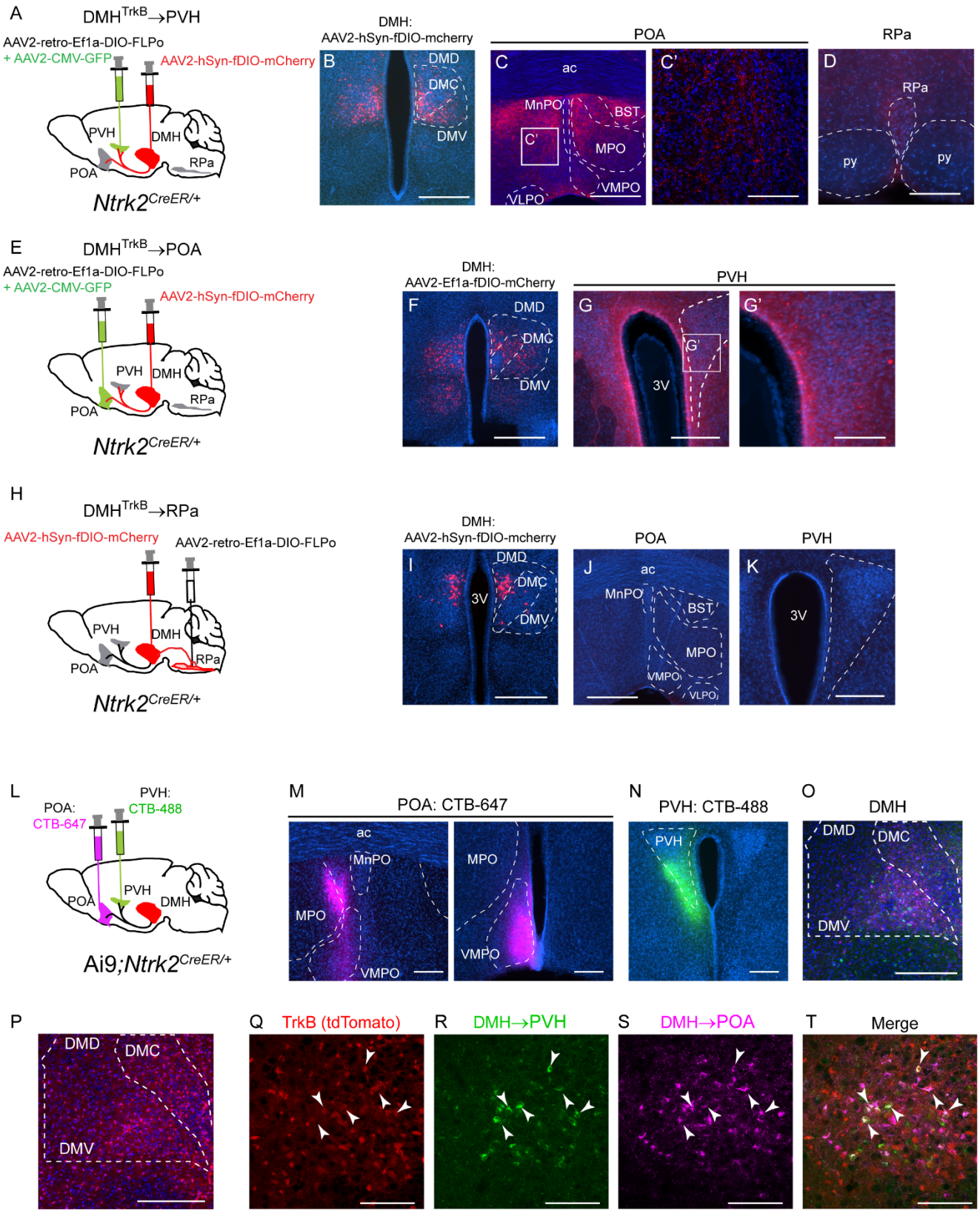

Figure S4. DMH$^{TrkB→PVH}$ neurons send collaterals to the POA. (**A-D**) Selective viral expression of mCherry in PVH-projecting DMH^{TrkB} neurons reveals collaterals of these neurons in the POA, but not in the RPa. (**E-G**) Selective viral expression of mCherry in POA-projecting DMH^{TrkB} neurons reveals collaterals of these neurons in the PVH. (**H-K**) Selective viral expression of mCherry in DMH$^{TrkB→RPa}$ neurons shows that these neurons do not innervate the POA or the PVH. (**L**) Diagram of simultaneous retrograde tracing from both the POA and the PVH in Ai9;*Ntrk2*$^{CreER/+}$

reporter mice. (**M**) Injection of cholera toxin subunit B Alexa Fluor 647 conjugate (CTB-647) into the POA. (**N**) Injection of CTB-488 into the PVH. **(O)** Retrograde labeling of neurons in the DMH that project to the POA (magenta) or the PVH (green). (**P**) Cre-dependent labeling of TrkB-expressing neurons in the DMH with tdTomato (red). (**Q**-**T**) Some neurons in the DMH express TrkB (red) are labeled by both CTB-488 injected into the PVH (green) and CTB-647 injected into the POA (magenta), as indicated by arrowheads. Scale bars = 500 μm in (**B**, **C**, **F**, **I**, **J**), 200 μm in (**D**, **G**, **K**, **M**-**P**), and 100 μm in (**C'**, **G'**, **Q**-**T).** Abbreviations: ac, anterior commissure; BST, bed nucleus stria terminalis; DMD, dorsal part of dorsomedial hypothalamus; DMC, central part of dorsomedial hypothalamus; DMV, ventral part of dorsomedial hypothalamus; MPO, medial preoptic area; MnPO, median preoptic area; PVH, paraventricular hypothalamus; VLPO, ventrolateral preotic area; VMPO, ventromedial preoptic area; 3V, third ventricle.

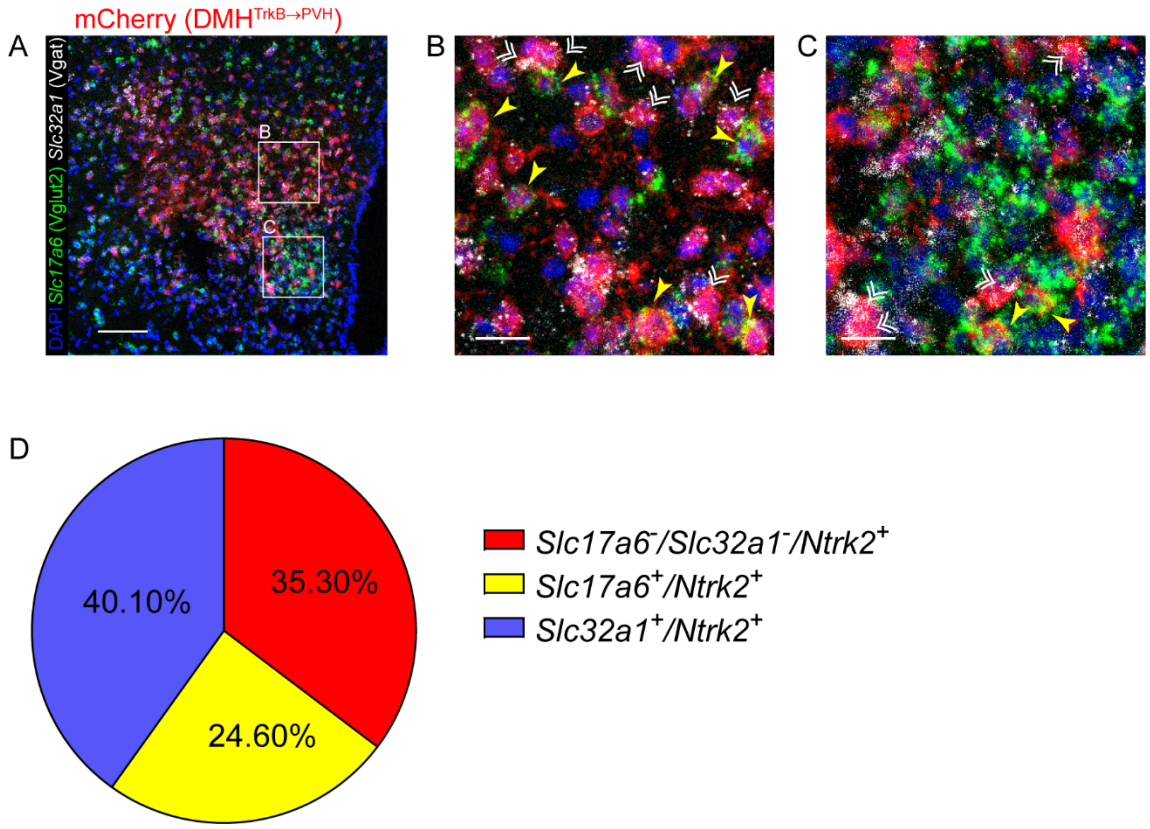

Figure S5. DMH$^{TrkB→PVH}$ neurons are heterogeneous and comprised of both inhibitory and excitatory neuron populations. (**A**) *In situ* detection of *Slc17a6* (Vglut2, green) and *Slc32a1* (Vgat, white) in the middle-dorsal (**B**) and middle-ventral (**C**) DMH of DMH$^{TrkB→PVH}$:mCherry mice. Yellow arrowheads indicate Vglut$^+$ DMH$^{TrkB→PVH}$ neurons. Double white arrowheads indicate Vgat$^+$ DMH$^{TrkB→PVH}$ neurons. (**D**) Quantification of percent DMH$^{TrkB→PVH}$ neurons that express *Slc17a6* (Vglut2) or *Slc32a1* (Vgat). Scale bars = 100 μm (**A**) and 20 μm (**B, C**). Representative images are from *n* = 2 animals.

Supplementary Table

Supplementary Table 1: Summary of Statistical Analyses			
Figure	**Sample size**	**Statistical test**	**P value**
1D	4 mice	two-way ANOVA	position: F (1.880, 16.92) = 7.181, P = 0.2225
	per condition	Factor 1: anterior-posterior position	temperature F (2, 9) = 18.68, P = 0.0062
		Factor 2: temperature	interaction F (4, 18) = 1.580, P= 0.0006
		Dunnett's multiple comparison test	subject F (9, 18) = 5.079, P = 0.0017
2B		RM two-way ANOVA	time: F (1.402, 22.43) = 7.879, P = 0.0056
		Factor 1: time (post CNO)	viral expression: F (1, 16) = 10.11, P = 0.0058
		Factor 2: viral expression (mcherry, hM3)	interaction: F (3, 48) = 34.69, P < 0.0001
		Sidak's multiple comparison test	subject: F (16, 48) = 20.38, P<0.0001
2C		RM two-way ANOVA	treatment: F (1, 16) = 133.3, P<0.0001
		Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 16) = 36.55, P< 0.0001
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 16) = 151.7, P <0.0001
		Sidak's multiple comparison test	subject: F (16, 16) = 10.96, P<0.0001
2E	5 mCherry	RM two-way ANOVA	treatment: F (1, 10) = 4.824, P = 0.0528
	7 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 10) = 6.35, P = 0.0304
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 10) = 5.893, P = 0.0356
		Sidak's multiple comparison test	subject: F (11, 11) = 73.71, P < 0.0001
2F	9 mCherry 13 hM3	unpaired t test (two-tailed)	P = 0.0207
2G	9 mCherry	Mixed-effects model (REML)	time: F (48, 327) = 3.818, P<0.0001
	9 hM3	Factor 1: time (post CNO)	viral expression: F (1, 16) = 26.89, P<0.0001
		Factor 2: viral expression (mcherry, hM3)	interaction: F (48, 327) = 4.433, P < 0.0001
2H	9 mCherry	RM two-way ANOVA	treatment: F (1, 16) = 85.88, P<0.0001
	9 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 16) = 17.64, P = 0.0007
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 16) = 56.72, P <0.0001
		Sidak's multiple comparison test	subject: F (16, 16) = 3.262, P<0.0117
2I	9 mCherry	Mixed-effects model (REML)	time: F (41, 278) = 1.467, P = 0.0398
	9 hM3	Factor 1: time (post CNO)	viral expression: F (1, 16) = 16.65, P = 0.0009
		Factor 2: viral expression (mcherry, hM3)	interaction: F (41, 278) = 2.484, P < 0.0001
2J	9 mCherry	RM two-way ANOVA	treatment: F (1, 16) = 0.002426, P=0.0003
	9 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 16) = 5.072, P = 0.0387
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 16) = 20.88, P <0.0001
		Sidak's multiple comparison test	subject: F (16, 16) = 2.187, P = 0.0640
2K	9 mCherry	Mixed-effects model (REML)	time: F (41, 278) =4.667, P<0.0001
	9 hM3	Factor 1: time (post CNO)	viral expression: F (1, 16) = 39.78, P<0.0001
		Factor 2: viral expression (mcherry, hM3)	interaction: F (41, 278) = 4.134, P < 0.0001
2L	9 mCherry	RM two-way ANOVA	treatment: F (1, 16) = 57.24, P<0.0001
	9 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 16) = 30.86, P<0.0001
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 16) = 55.79, P <0.0001
		Sidak's multiple comparison test	subject: F (16, 16) = 1.765, P = 0.1332
2M	8 mCherry	RM two-way ANOVA	treatment: F (1, 18) = 0.04648, P = 0.8317
	12 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 18) = 0.6447, P = 0.4325
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 18) = 6.618, P = 0.0192

		Sidak's multiple comparison test	subject: F (18, 18) = 2.407, P = 0.0352
2N	8 mCherry	RM two-way ANOVA	treatment: F (1, 18) = 0.4427, P = 0.5143
	12 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 18) = 0.01210, P = 0.9136
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 18) = 1.586, P = 0.2240
		Sidak's multiple comparison test	subject: F (18, 18) = 7.885, P<0.0001
3B	6 mCherry	RM two-way ANOVA	time: F (2.993, 32.92) = 78.61, P<0.0001
	7 hM4	Factor 1: time (post CNO)	viral expression: F (1, 11) = 20.40, P = 0.0009
		Factor 2: viral expression (mcherry, hM4)	interaction: F (4, 44) = 8.696, P < 0.0001
		Sidak's multiple comparison test	subject: F (11, 44) = 7.509, P<0.0001
3C	6 mCherry	RM two-way ANOVA	treatment: F (1, 11) = 35.51, P<0.0001
	7 hM4	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 11) = 7.577, P = 0.0188
		Factor 2: viral expression (mcherry, hM4)	interaction: F (1, 11) = 10.49, P = 0.0079
		Sidak's multiple comparison test	subject: F (11, 11) = 2.388, P = 0.0822
3D	6 mCherry	Mixed-effects model (REML)	time: F (64, 282) = 3.046, P<0.0001
	7 hM4	Factor 1: time (post CNO)	viral expression: F (1, 11) = 3.066, P = 0.1078
		Factor 2: viral expression (mcherry, hM4)	interaction: F (64, 282) = 1.007, P= 0.4695
3E	6 mCherry	RM two-way ANOVA	treatment: F (1, 11) = 8.574, P = 0.0137
	7 hM4	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 11) = 0.1502, P = 0.7058
		Factor 2: viral expression (mcherry, hM4)	interaction: F (1, 11) = 16.66, P = 0.0018
		Sidak's multiple comparison test	subject: F (11, 11) = 7.934, P = 0.0009
3F	6 mCherry	Mixed-effects model (REML)	time: F (60, 266) = 2.602, P<0.0001
	7 hM4	Factor 1: time (post CNO)	viral expression: F (1, 11) = 39.28, P<0.0001
		Factor 2: viral expression (mcherry, hM4)	interaction: F (60, 266) = 2.202, P<0.0001
3G	6 mCherry	RM two-way ANOVA	treatment: F (1, 11) = 76.99, P<0.0001
	7 hM4	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 11) = 8.633, P = 0.0135
		Factor 2: viral expression (mcherry, hM4)	interaction: F (1, 11) = 29.00, P = 0.0002
		Sidak's multiple comparison test	subject: F (11, 11) = 3.15, P = 0.0403
4J	5 mCherry	RM two-way ANOVA	treatment: F (1, 9) = 0.008375, P = 0.9291
	6 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 9) = 29.24, P = 0.0004
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 19) = 39.34, P = 0.0001
		Sidak's multiple comparison test	subject: F (9, 9) = 2.126, P = 0.1383
4K	5 mCherry 5 hM3	unpaired t test (two-tailed)	P<0.0001
4L	5 mCherry	RM two-way ANOVA	time: F (3.565, 32.08) = 4.980, P = 0.0041
	6 hM3	Factor 1: time (post CNO)	viral expression: F (1, 9) = 4.695, P = 0.0584
		Factor 2: viral expression (mcherry, hM3)	interaction: F (12, 108) = 2.566, P = 0.0050
			subject: F (9, 108) = 18.80, P<0.0001
4M	5 mCherry	RM two-way ANOVA	treatment: F (1, 9) = 7.220, P = 0.0249
	6 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 9) = 1.227, P = 0.2967
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 9) = 10.55, P = 0.0100
		Sidak's multiple comparison test	subject: F (9, 9) = 4.916, P = 0.0133
4N	5 mCherry	RM two-way ANOVA	time: F (2.547, 22.92) = 2.704, P = 0.0769

	6 hM3	Factor 1: time (post CNO)	viral expression: F (1, 9) = 0.2985, P = 0.5981
		Factor 2: viral expression (mcherry, hM3)	interaction: F (12, 108) = 0.3523, P = 0.9766
			subject: F (9, 108) = 13.05, P< 0.0001
4O	5 mCherry	RM two-way ANOVA	treatment: F (1, 9) = 0.3504, P = 0.5685
	6 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 9) = 0.03257, P = 0.8608
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 9) = 2.870, P = 0.1245
		Sidak's multiple comparison test	subject: F (9, 9) = 2.476, P = 0.0965
4P	5 mCherry	RM two-way ANOVA	time: F (2.319, 41.74) = 966.6, P <0.0001
	6 hM3	Factor 1: time	treatment/viral expression: F (3, 18) = 0.6880, P=0.5711
		Factor 2: treatment/viral expression	interaction: F (12, 72) = 0.8902, P = 0.5606
		Sidak's multiple comparison test	subject: F (18, 72) = 5.658, P< 0.0001
5D	5 mCherry	RM two-way ANOVA	treatment: F (1, 17) = 2.219, P = 0.1547
	6 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 17) = 3.178, P = 0.0925
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 17) = 6.715, P = 0.0190
		Sidak's multiple comparison test	subject: F (17, 17) = 8.078, P<0.0001
5E	5 mCherry	Mixed-effects model (REML)	time: F (40, 275) = 8.738, P< 0.0001
	6 hM3	Factor 1: time (post CNO)	viral expression: F (1, 17) = 0.4561, P = 0.5085
		Factor 2: viral expression (mcherry, hM3)	interaction: F (40, 275) = 2.847, P < 0.0001
5F	10 mCherry	RM two-way ANOVA	treatment: F (1, 17) = 7.898, P=0.0120
	9 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 17) = 1.252, P = 0.2787
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 17) = 3.971, P = 0.0626
		Sidak's multiple comparison test	subject: F (17, 17) = 12.56, P<0.0001
5G	10 mCherry	Mixed-effects model (REML)	time: F (38, 253) = 2.475, P< 0.0001
	9 hM3	Factor 1: time (post CNO)	viral expression: F (1, 17) = 5.311, P = 0.0341
		Factor 2: viral expression (mcherry, hM3)	interaction: F (38, 253) = 1.627, P < 0.0156
5H	10 mCherry	RM two-way ANOVA	treatment: F (1, 17) = 0.002755, P=0.9588
	9 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 17) = 1.614, P = 0.2211
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 17) = 17.49, P = 0.0006
		Sidak's multiple comparison test	subject: F (17, 17) = 9.047, P<0.0001
5I	10 mCherry	RM two-way ANOVA	time: F (1.153, 39.19) = 215.6, P <0.0001
	9 hM3	Factor 1: time	treatment/viral expression: F (3, 34) = 7.027, P = 0.0008
		Factor 2: treatment/viral expression	interaction: F (12, 136) = 5.185, P<0.0001
		Sidak's multiple comparison test	subject: F (34, 136) = 7.339, P<0.0001
5M	3 mCherry	RM two-way ANOVA	treatment: F (1, 6) = 1.756, P = 0.2334
	5 hM3	Factor 1: treatment (vehicle, CNO)	viral expression: F (1, 6) = 5.031, P = 0.0661
		Factor 2: viral expression (mcherry, hM3)	interaction: F (1, 6) = 1.004, P = 0.3551
		Sidak's multiple comparison test	subject: F (6, 6) = 1.362, P = 0.3585
5N	3 mCherry	RM two-way ANOVA	time: F (4.734, 28.41) = 6.860, P = 0.0003
	5 hM3	Factor 1: time (post CNO)	viral expression: F (1, 6) =3.557, P = 0.1083
		Factor 2: viral expression (mcherry, hM3)	interaction: F (28, 168) = 2.271, P = 0.0007
			subject: F (6, 168) = 4.619, P = 0.0002

5O	3 mCherry 5 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 6) = 0.03281, P = 0.8622 viral expression: F (1, 6) = 1.475, P = 0.2701 interaction: F (1, 6) = 0.1649, P = 0.6988 subject: F (6, 6) = 1.921, P = 0.2234
5P	3 mCherry 5 hM3	RM two-way ANOVA Factor 1: time (post CNO) Factor 2: viral expression (mcherry, hM3)	time: F (2.916, 17.50) = 1.330, P = 0.2965 viral expression: F (1, 6) =2.985, P = 0.1348 interaction: F (27, 162) = 3.425, P <0.0001 subject: F (6, 132) = 20.42, P<0.0001
5Q	3 mCherry 5 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 6) = 0.2211, P = 0.6548 viral expression: F (1, 6) = 0.2390, P = 0.6423 interaction: F (1, 6) = 9.596, P = 0.0212 subject: F (6, 6) = 4.742, P = 0.0400
5R	3 mCherry 5 hM3	RM two-way ANOVA Factor 1: time Factor 2: treatment/viral expression Sidak's multiple comparison test	time: F (2.906, 34.87) = 405.5, P <0.0001 treatment/viral expression: F (3, 12) = 28.53, P<0.0001 interaction: F (12, 48) = 13.07, P<0.0001 subject: F (6, 132) = 20.42, P<0.0001
S1B	5 mCherry 5 hM3	unpaired t test (two-tailed)	P = 0.0022
S1C	5 mCherry 5 hM3	unpaired t test (two-tailed)	P = 0.0170
S1D	5 mCherry 6 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 9) = 2.099, P = 0.1813 viral expression: F (1, 9) = 0.2308, P = 0.6424 interaction: F (12, 48) = 5.758, P<0.0001 subject: F (9, 9) = 1.462, P = 0.2902
S3A	5 mCherry 6 hM3	RM two-way ANOVA Factor 1: time (post CNO) Factor 2: viral expression (mcherry, hM3)	time: F (4.061, 36.55) = 5.784, P = 0.0010 viral expression: F (1, 9) = 0.06638, P = 0.8025 interaction: F (12, 108) = 0.3768, P = 0.9692 subject: F (9, 108) = 2.455, P = 0.0139
S3B	5 mCherry 6 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 9) = 1.859, P = 0.2059 viral expression: F (1, 9) = 0.4086, P = 0.5386 interaction: F (1, 9) = 0.003788, P = 0.9523 subject: F (9, 9) = 0.8222, P = 0.6123
S3C	8 mCherry 9 hM3	Mixed-effects model (REML) Factor 1: time (post CNO) Factor 2: viral expression (mcherry, hM3)	time: F (38, 225) = 5.370, P <0.0001 viral expression: F (1, 15) = 1.478, P = 0.2429 interaction: F (38, 225) = 2.233, P = 0.0002
S3D	8 mCherry 9 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 15) = 6.964, P = 0.0186 viral expression: F (1, 15) = 2.111, P = 0.1669 interaction: F (1, 15) = 0.1738, P = 0.6827 subject: F (15, 15) = 5.994, P = 0.0006
S3E	3 mCherry	RM two-way ANOVA	time: F (4.949, 29.69) = 6.543, P = 0.0003

	5 hM3	Factor 1: time (post CNO) Factor 2: viral expression (mcherry, hM3)	viral expression: F (1, 6) = 2.533, P = 0.1626 interaction: F (27, 162) =1.874, P = 0.0091 subject: F (6, 162) = 4.966, P<0.0001
S3F	3 mCherry 5 hM3	RM two-way ANOVA Factor 1: treatment (vehicle, CNO) Factor 2: viral expression (mcherry, hM3) Sidak's multiple comparison test	treatment: F (1, 6) = 0.0007374, P = 0.9792 viral expression: F (1, 6) = 3.189, P = 0.1244 interaction: F (1, 6) = 1.213, P = 0.3129 subject: F (6, 6) = 0.2720, P = 0.9309