

Supplementary Information for

Control of food approach and eating by a GABAergic projection from lateral hypothalamus to dorsal pons

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Supplementary text

Figures S1 to S9

Legend for Movie S1

SI References

Other supplementary materials for this manuscript include the following:

Movie S1

Supplementary Information Text

Material and methods

Subjects

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH Animal Care and Use Committee guidelines. Experiments involved male and female adult mice (10 to 20 weeks old at the time of surgery) maintained under a 12 hr light/dark cycle with food and water available ad libitum. Animals were group housed prior to surgery and single-housed following surgery. Transgenic mice were from the following strains: VGAT-IRES-Cre (1), DAT-IRES-Cre (2), Vglut2-IRES-Cre (1) and TH-IRES-Cre (3).

Surgical procedures

Mice were anesthetized using ketamine and xylazine (100mg/kg-10mg/kg), surgically injected with viral vectors and implanted with fiber optic cables. To photoactivate GABAergic LH projections, VGAT-IRES-Cre positive mice were bilaterally microinjected with 0.5 μ l of purified and concentrated adeno-associated virus ($\sim 10^{12}$ infections units per ml; UNC Vector Core) into the LH with the following stereotactic coordinates: A/P= -1.2; M/L= ± 1.0 and D/V=-5.2. Each LH microinjection contained an AAV5 viral construct coding cre-inducible ChR2-eYFP under control of the EF1 α promoter (AAV5-EF1 α -DIO-hChR2(H134R)-eYFP). Eight weeks after the virus injection, bilateral optical fibers were implanted above the VTA or other projection site (Fig. 2). To photoactivate and photoinhibit dopaminergic, GABAergic and glutamatergic neurons during in vivo behavioral experiments, DAT-IRES-Cre, VGAT-IRES-Cre and Vglut2-IRES-Cre mice were bilaterally microinjected with virus into VTA or

SNc (VTA A/P= -3.6 mm; M/L= \pm 1.6 mm; D/V=-4.9 mm and SNc A/P= -3.2mm; M/L= \pm 1.2 mm D/V=-4.3 mm. For lesion experiments we injected mice with DIO-ChR2 in the LH as described; additionally, we targeted to the VTA or peri-LC bilateral injections of 0.5 μ l virus encoding either cre-inducible caspase or YFP and implanted optical fibers above this site. For cell body stimulation/inhibition of GABAergic, glutamatergic, or noradrenergic neurons in the LC/peri-LC region we injected VGAT-IRES-cre, Vglut2-IRES-cre and TH-IRES-cre mice with 0,3 μ l of DIO-ChR2/ DIO-Arch 3.0 (A/P= -5.4 mm; M/L= \pm 0.75 mm; D/V= -3.75 mm). For stimulation of GABAergic neurons in the arcuate nucleus of the hypothalamus we injected VGAT-IRES-cre with 0.1 μ l of DIO-ChR2 (A/P= -1.2 mm; ML= \pm 0.2 mm; D/V= -5.8mm).

Behavioral testing

For all behavioral experiments, mice were moved from the vivarium to the behavioral facility and allowed to acclimate in a holding area for 30 minutes prior to the start of the test. Feeding tests were conducted at 10 AM (3 hours into the light cycle) in a 40 x 40 x 40 cm clear acrylic box. The light was uniformly distributed with an intensity of 74 ± 0.5 Lux. Approximately fifty 0.5-gram pellets of standard chow were available on the floor with a random distribution throughout in the box. Latency to approach the food under the effect of the optogenetic stimulation-inhibition was measured. Light was delivered under twelve total combinations of laser power (0.3-3-30mW) and frequency (5-10-20-40 Hz). Before the test, mice were left in the box without stimulation for 5 minutes. Animals received one-minute trials of each frequency and laser power, with one-minute breaks between trials. Eating was never seen during stimulation-off periods. Open field testing was conducted in the same chamber without food pellets. Mice remained in the chamber for 8 minutes and were recorded by video, with

distance traveled tracked by Ethovision software (Noldus). For optical self-stimulation, mice were first trained in three 60-min daily sessions to lever press for stimulation on a fixed ratio 1 schedule in a standard mouse operant chamber (MED-Associates). Each active lever press resulted in a single 500 ms (20 Hz) optical pulse train. Mice underwent daily sessions of 40 minutes, during which they performed 10 minutes of self-stimulation with each of four combinations of frequency (5 Hz, 10 Hz, 20 Hz and 40 Hz) in increasing order, for three consecutive days with one laser power tested each day. During those session mice were not exposed to a cue paired with the lever and they had the opportunity to choose between an active and an inactive lever.

Histology

After behavioral testing, all mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. For immunohistochemistry, fixed brains were sectioned on a vibratome at 40 μm . Sections were incubated overnight with mouse anti-tyrosine hydroxylase (catalog #T1299, 1:5,000, Sigma Aldrich). RNAscope (Advanced Cell Diagnostics) *in situ* hybridization was used to detect decrease in number of cells expressing Slc32a1 mRNA (encoding VGAT). Brains for RNAscope were fresh frozen on dry ice, and sectioned into 14 μm sections on a cryostat. RNAscope assays were performed using probe MmSLC32A1-C2-VGAT following the manufacturer's recommended protocol.

Electrophysiology

Mice were deeply anesthetized with isoflurane and killed by decapitation. Brains were quickly removed and placed in warmed (30°C) modified Krebs buffer containing (in mM): 126 NaCl,

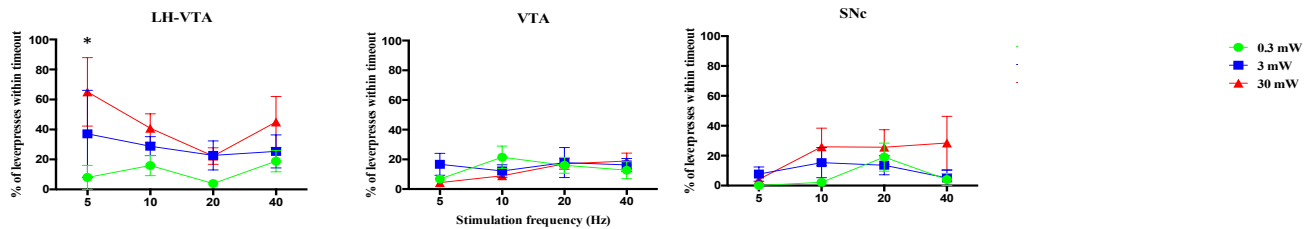
2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 11 D-glucose, and 21.5 NaHCO₃, bubbled with 95/5% O₂/CO₂ with 5 μM MK-801 to reduce excitotoxicity and increase slice viability. In the same solution, horizontal brain slices (200 μm) were obtained using a vibrating microtome (Leica Biosystems) and maintained at 32° C. Neurons in or surrounding the LC were identified by their location relative to the 4th ventricle. Dopamine neurons in the VTA were identified by their location relative to the MT and their electrophysiological properties. Whole-cell patch-clamp recordings were obtained from neurons at 35 °±1 ° C with a Multiclamp 700B amplifier (Molecular Devices) and Digidata 1440a digitizer (Molecular Devices) and Clampex software (Molecular Devices) in modified Krebs buffer (as above) with 3 μM NBQX to isolate GABAergic synaptic transmission and 100 μM 4-AP to increase neurotransmitter release. Pipette resistances were 2.5-4.6 MOhms when filled with internal solution containing (in mM) 104.56 K-methylsulfate, 5.30 NaCl, 4.06 MgCl₂, 4.06 CaCl₂, 7.07 HEPES(K), 3.25 BAPTA(K₄), 0.26 GTP (sodium salt), 4.87 ATP (sodium salt), 4.59 creatine phosphate (sodium salt), pH 7.3 with 0.1% neurobiotin. GABA receptor-mediated synaptic currents were evoked by optical stimulation using a single or train (5 pulses, 60Hz) 473nm LED laser pulses (1 ms, V_{hold} -65 mV or -88 mV. In the experiments shown in Fig.S4, voltage-clamp recordings were made using glass pipettes with resistance 3.8-5 MOhms, filled with internal solution containing (in mM): 117 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 Mg-ATP, and 0.25 Na-GTP, pH 7.2–7.3 and 280–285 mOsm. Light-evoked paired pulses were acquired at 0 mV by having a laser stimulation of 3 mW intensity. The second stimulus was evoked at 50 ms after the first.

Statistical analysis

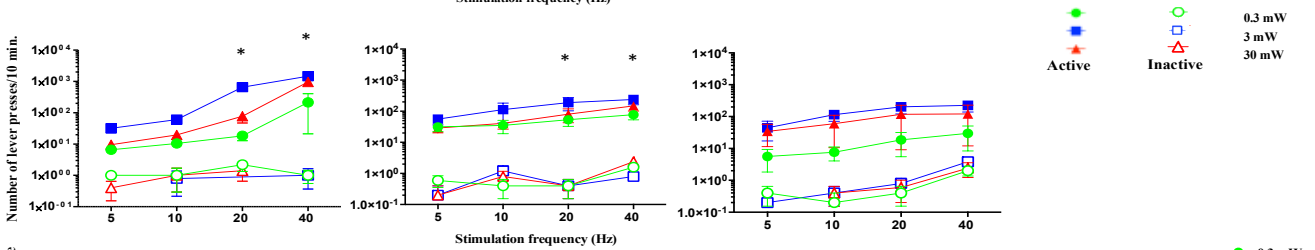
Effects of laser stimulation and experimental treatment were analyzed with a linear mixed effects model using R function lme from package “nlme”. Post-hoc tests, when warranted, were performed using two-tailed Student’s t-tests with Bonferroni-adjusted significance cutoff. One sample t-tests were used for eating experiments to compare against null hypothesis of time eating = 0 seconds. Paired t-tests were used for self-stimulation experiments to compare log-transformed active vs inactive lever-press counts for a specific set of stimulation parameters. Independent samples t-tests were used in caspase lesion experiments to compare time eating in control vs lesion groups at a given laser frequency. All graphical depictions of group data are presented as mean \pm SEM

Fig. S1

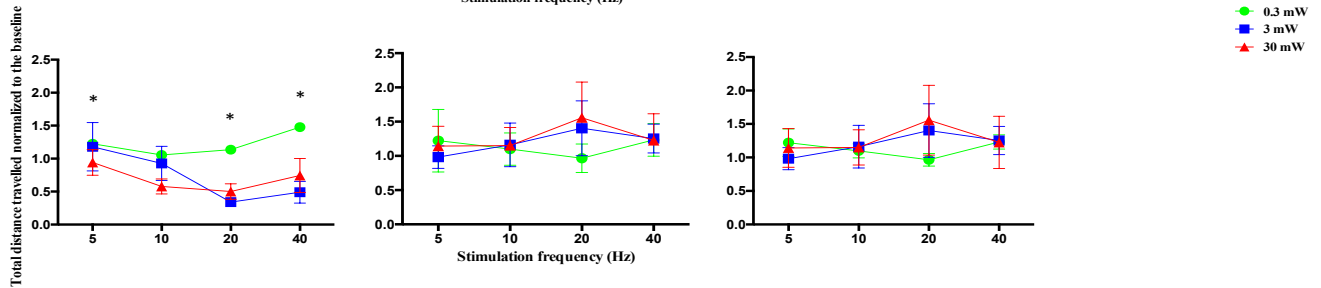
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B



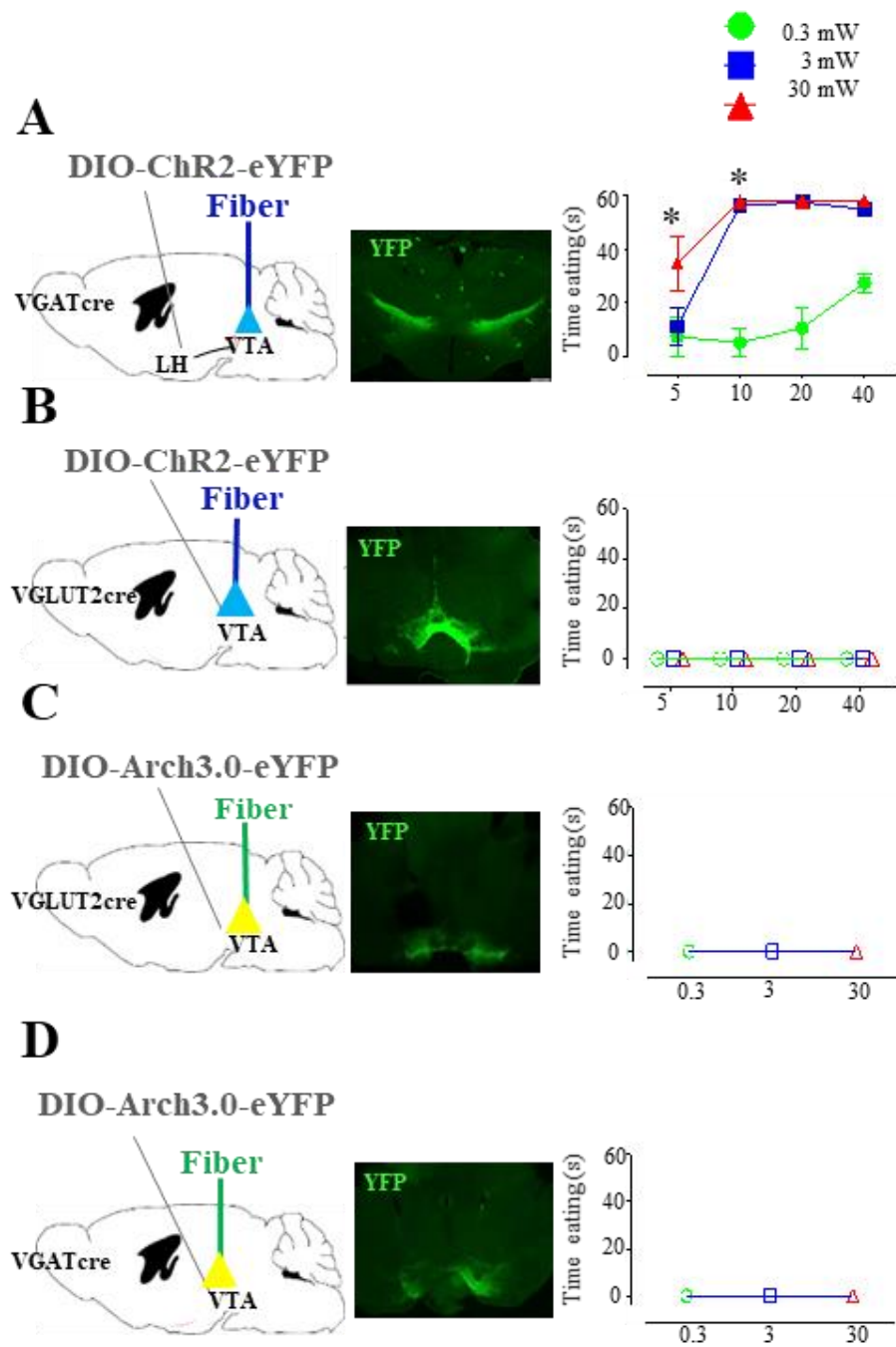
C



(A) VGAT LH-VTA-ChR2 mice perform a higher number of unrewarded lever presses within the timeout when compared with DAT VTA-ChR2 and DAT SNc-ChR2. Two-way ANOVA showed a significant main effect of cell type ($F_{2,12}=32.12$, $p=0.0106$). Bonferroni post-hoc analysis showed a significant difference in the % of lever presses during the timeout performed by VGAT LH-VTA mice at 30 mW and 5 Hz when compared to DAT VTA and DAT SNc mice. **(B) Number of active and inactive lever presses in 10 minutes.** Two-way ANOVA showed significant main effect of laser power ($F_{5,24} = 48.12$, $P < 0.0001$), frequency ($F_{3,72} = 53.3$, $p < 0.0001$) and their interaction ($F_{15,$

$F_{72} = 18.7$, $p < 0.0001$) in VGAT LH-VTA group. Bonferroni post-hoc analysis showed significant differences between active and inactive lever presses at 20 Hz 30 mW and at 40 Hz 3 and 30 mW. In DAT VTA group, two-way ANOVA showed significant main effect of laser power ($F_{5, 24} = 4.415$, $p=0.0054$), frequency ($F_{3, 72} = 14.37$, $p < 0.0001$) and their interaction ($F_{15, 72} = 4,243$, $p < 0.0001$). Bonferroni post-hoc analysis showed significant differences between active and inactive lever presses at 20 Hz 30 mW and at 40 Hz 3 and 30 mW. **(C) Total distance traveled performed during the stimulation normalized to the baseline.** In VGAT LH-VTA group two-way ANOVA showed significant main effect of laser power ($F_{2,12} = 48.07$, $p < 0.0001$), frequency ($F_{3, 36} = 18,82$, $p < 0.0001$) and their interaction ($F_{6,36} = 10.37$, $p < 0.0001$). Bonferroni post-hoc analysis showed significant differences in the total distance traveled at 5 Hz 30 mW, 10 Hz 3 and 30 mW and 40 Hz 3 and 30 mW. * $p < 0.05$.

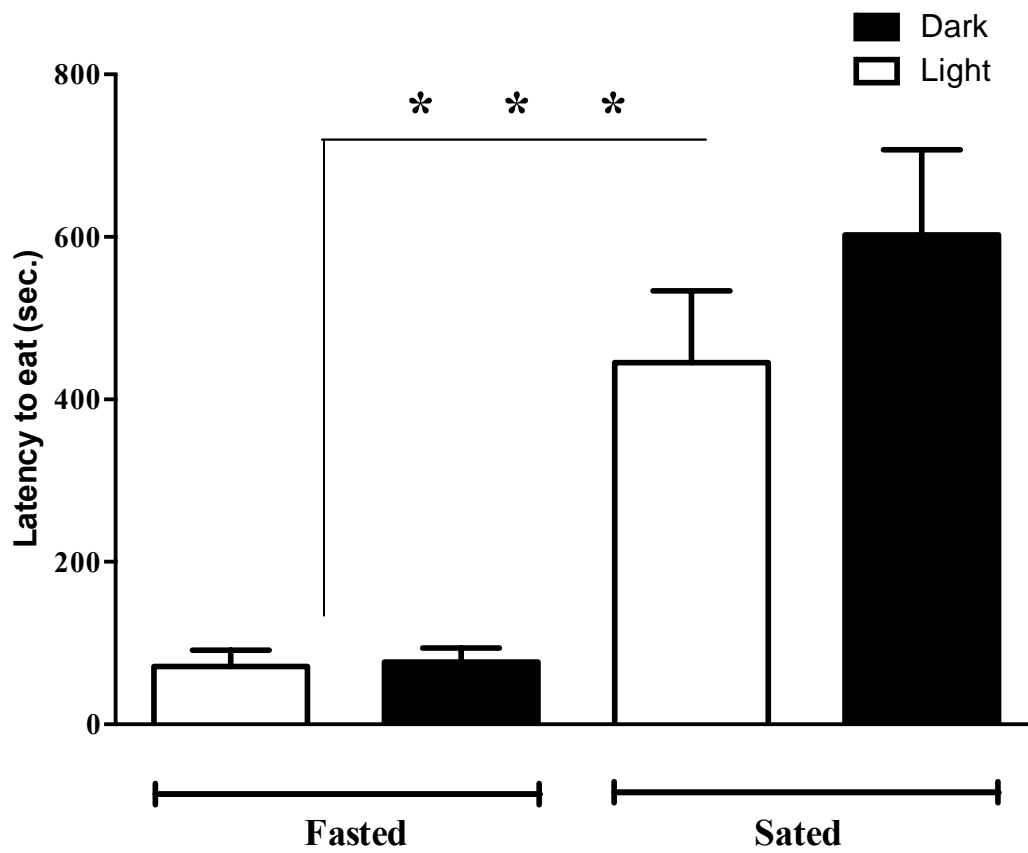
Fig. S2



Optogenetic manipulations of non-dopaminergic VTA populations do not elicit eating.

(A) VGAT LH-VTA circuit targeting. 10x image of the VGAT LH-VTA-ChR2-eYFP (green) circuit. Photostimulation of VGAT LH-VTA projection significantly produces robust eating in sated animals (B) 10x image of the Vglut2 VTA-ChR2-eYFP (green). Photostimulation of glutamatergic VTA cell bodies does not produce eating. (C) 10x image of the Vglut2 VTA-Arch 3.0-eYFP (green). Photoinhibition of glutamatergic VTA cell bodies does not produce eating. (D) 10x image of the VGAT VTA-Arch 3.0-eYFP (green). Photoinhibition of GABAergic VTA cell bodies does not produce eating in sated animals.* $p < 0.05$, post-hoc comparison vs null value.

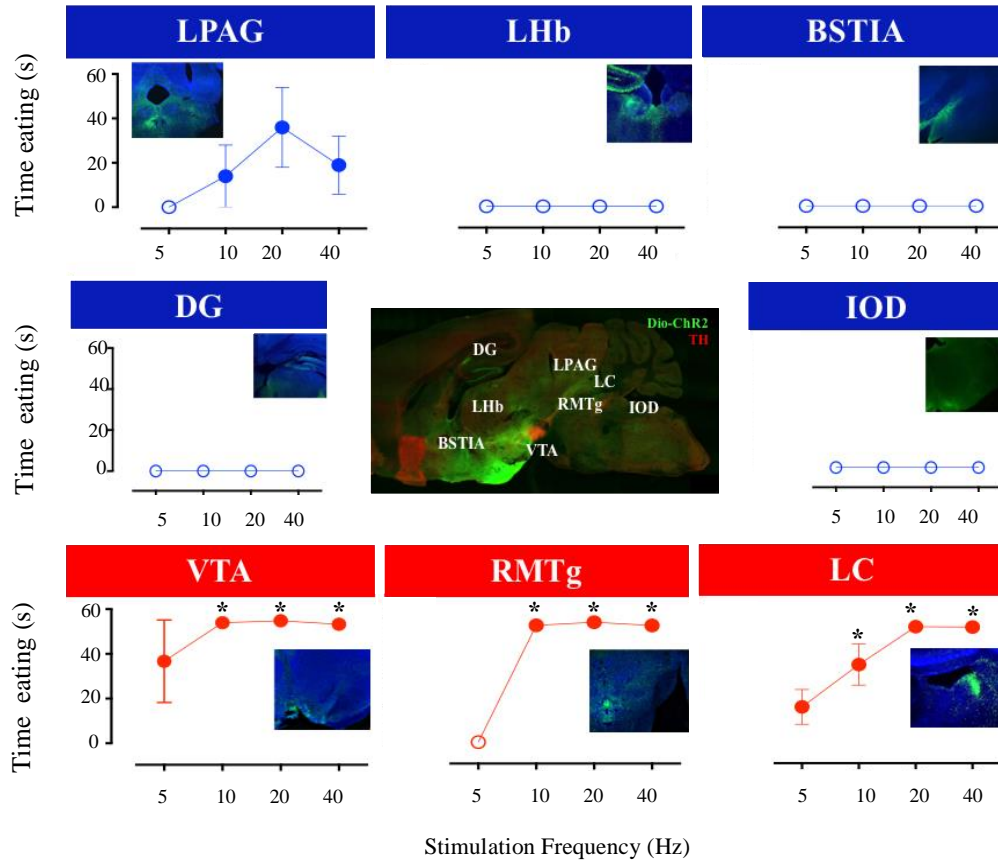
Fig. S3



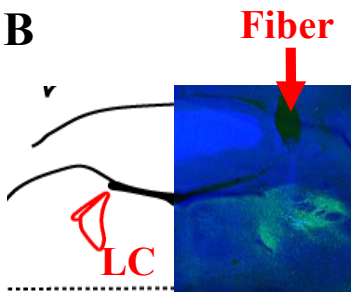
Latency to feed in food-restricted and sated mice. Two-way ANOVA showed a significant main effect of satiety status, ($F_{1, 18} = 46.66$), $p < 0.0001$. No significant difference between the light and dark groups was observed.

Fig. S4

A



B



LH GABA neurons project caudally to the locus coeruleus, where terminal stimulation

elicits eating. (A) Center image, Sagittal representative image of VGAT LH ChR2-eYFP

(green) and TH staining (red). The image shows strong rostral projections anterior to the bed

nucleus of the stria terminalis (BSTIA), dentate gyrus (DG), and lateral habenula (LHb). Caudal

projections are seen to ventral tegmental area (VTA), rostromedial tegmental nucleus (RMTg),

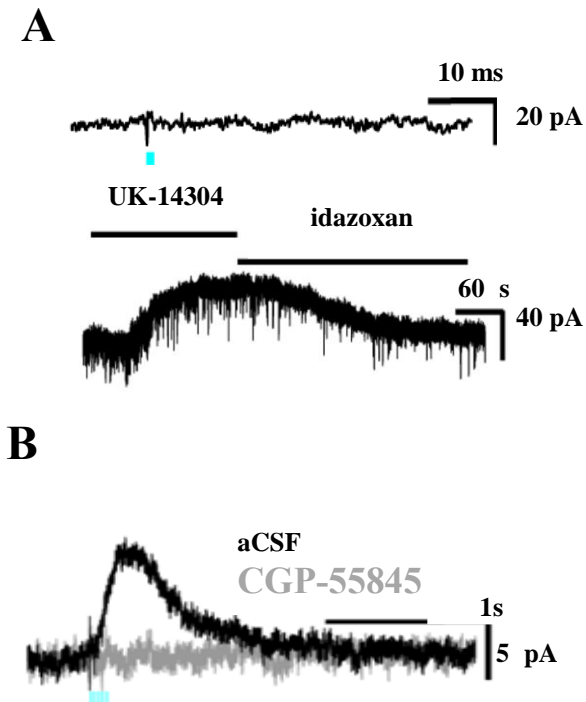
lateral periaqueductal gray (LPAG), locus coeruleus (LC), and dorsal inferior olive (IOD).

Photostimulations of VGAT LH projections to VTA, RMTg, and peri-LC produce eating. * $p <$

0.05 post-hoc vs null value. (B) Representative coronal section showing fluorescence of LH

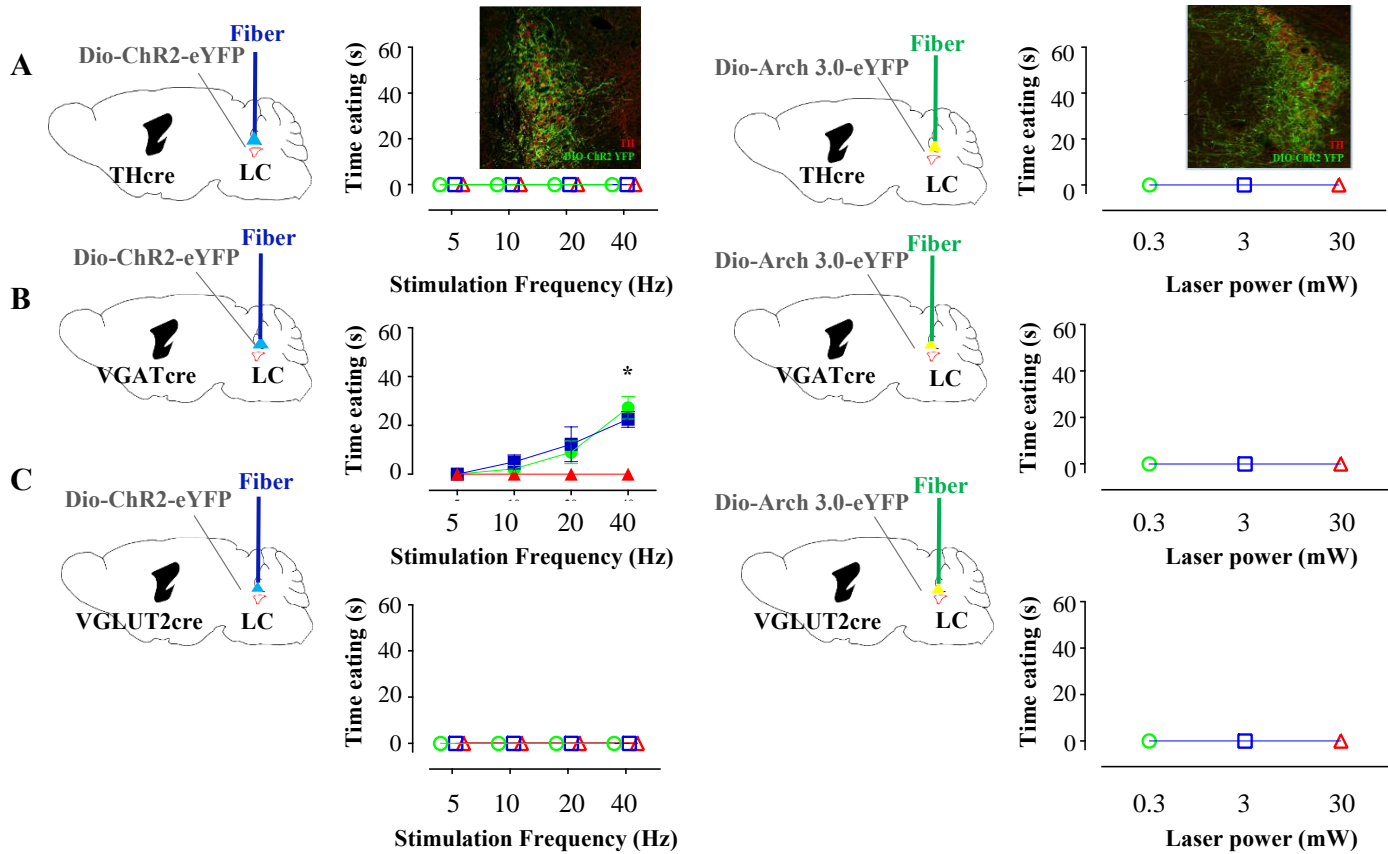
terminals in peri-LC and fiber placement.

Fig. S5



Stimulation of LH GABA terminals in peri-LC tissue evokes GABA receptor-mediated currents in non-noradrenergic neurons. (A) Photostimulation of LH-LC GABA inputs failed to produce a synaptic response in LC noradrenergic neurons, whose identity was confirmed by an outward current in response to application of the alpha 2-adrenergic receptor agonist UK-14304 (3 microM), reversed by idazoxan (1 microM). (B) Photostimulation of LH-peri-LC GABA inputs produces a slow inhibitory GABA-B postsynaptic current blocked CGP55845 (300 nM). Inward currents were measured from $V_{\text{hold}} = -88$ mV; outward currents were measured from $V_{\text{hold}} = -65$ mV).

Fig. S6

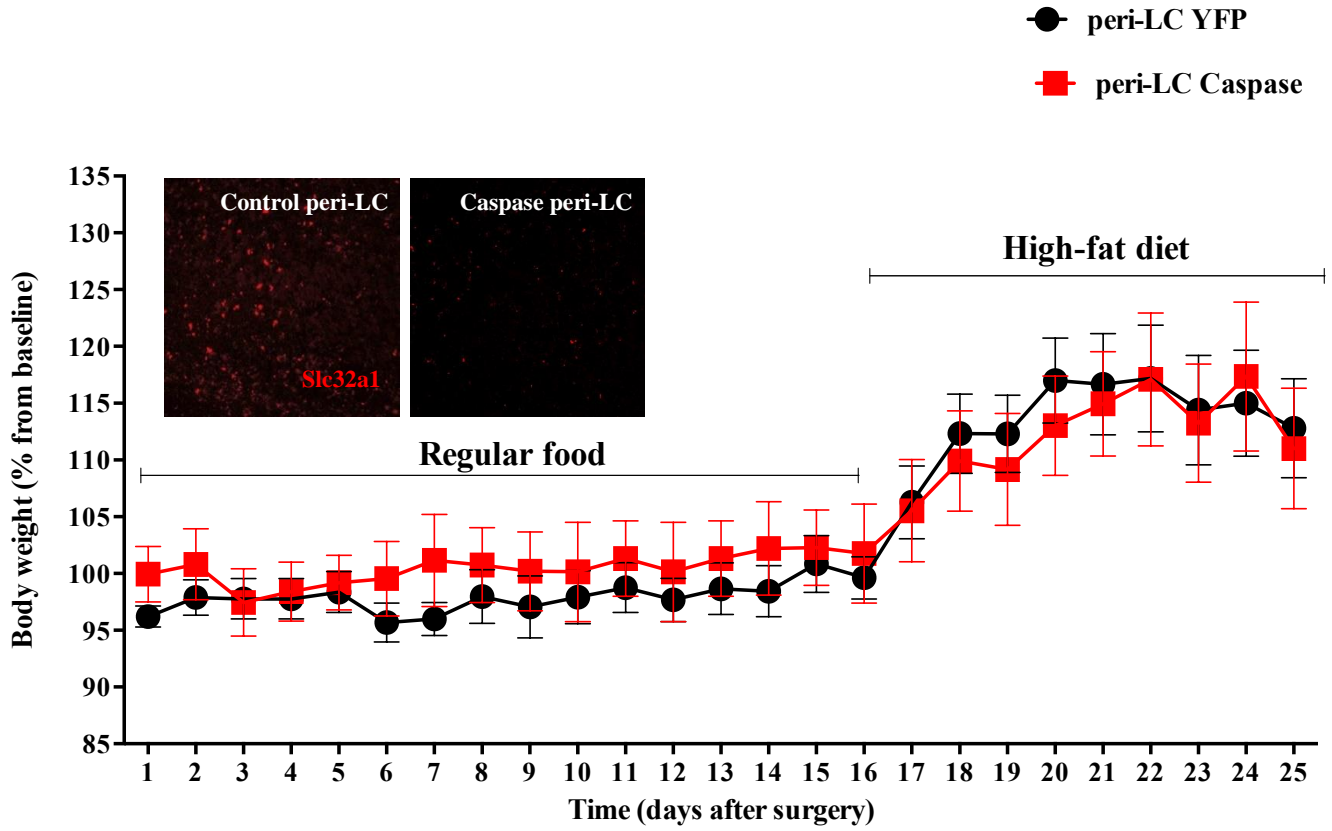


Stimulation of peri-LC GABA cell bodies elicits feeding. (A) 10x image of the TH LC-ChR2-eYFP/TH LC-Arch 3.0-eYFP (green) and TH staining (red). Photostimulation or photoinhibition of noradrenergic neurons does not produce feeding. (B) Photostimulation of VGAT LC-ChR2-eYFP displays laser-induced feeding while the photoinhibition does not. Analysis of stimulation groups showed significant main effect of cell type ($F_{2,10}=32.12$, $p<0.0001$) and cell type x power x frequency interaction ($F_{2,133}=10.00$, $p<0.0001$). Post-hoc analysis of mice with stimulation of GABAergic cell bodies showed significant feeding responses at 40 Hz stimulation with 3 and 30

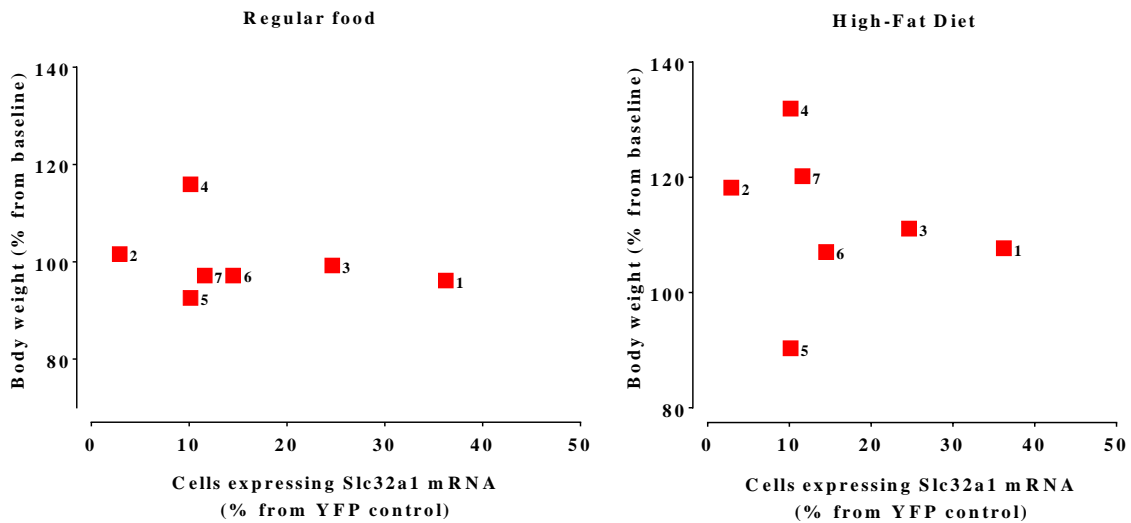
mW laser power. (C) Photostimulation of Vglut2 LC-ChR2-eYFP and photoinhibition of Vglut2 LC-Arch 3.0-eYFP do not show eating behavior under any condition tested.

Fig. S7

A



B



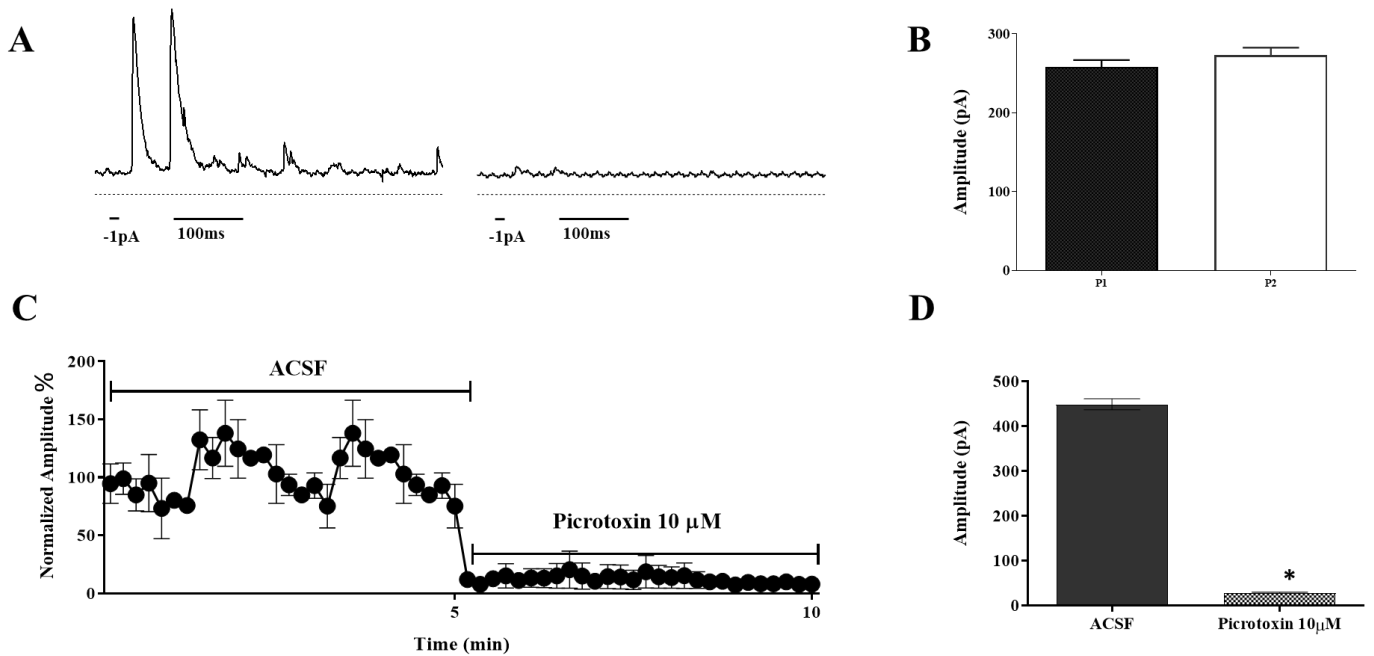
Lesion of GABA neurons in peri-LC does not promote weight loss. (A) We performed cre-dependent lesion using DIO-caspase virus injection of the peri-LC region in VGAT-cre mice (n=7,

the decrease in number of neurons expressing Slc32a1 mRNA was $84.26\% \pm 11.14$ with significant reduction in number of neurons between YFP and caspase-injected mice ($t=6.157$, $df=9$, $p=0.002$).

We exposed the animals to a regular and high-fat diet. Two-way ANOVA showed a significant effect of the time due to the high-fat diet ($F_{24,32}=23,37$; $p < 0.0001$), but no effect of the lesion.

(B) Correlation between % of body weight from the baseline and % of neurons expressing Slc32a1 mRNA compared to the YFP controls (Regular food $r^2= 0.07494$, $p= 0.55$; High-fat diet $r^2= 0.04976$, $p= 0.6307$).

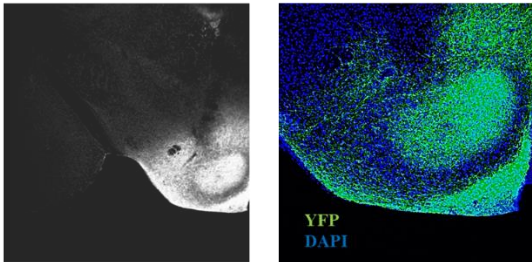
Fig. S8



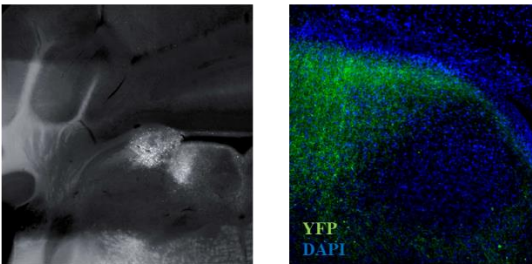
Optogenetic stimulation of LH inputs to peri-LC evokes outward currents mediated by the activation of GABA-A receptor. (A) Representative trace of light-evoked paired-pulse in peri-LC. The voltage was held at 0 mV and light was delivered with an intensity of 3 mW. (B) No significant difference between the amplitude average of the first stimulus when compared with the second. (C) Bath application of picrotoxin 10 μ M reduces significantly the current evoked by the light delivery. (D) Significant reduction of the amplitude of the current after the bath application of GABA-A antagonist. * $p < 0.05$.

Fig. S9

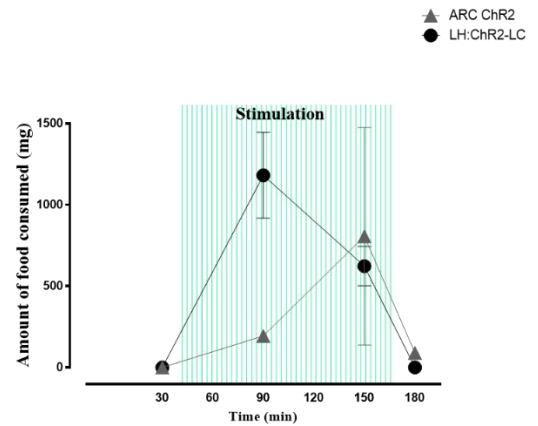
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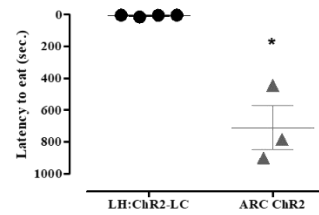
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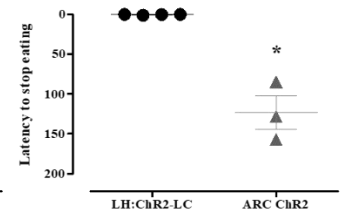
C



D



E



Stimulation of the LH-LC pathway produces feeding that begins right after the ignition of the laser and stops at the end of the stimulation. Comparison between feeding behavior evoked by the activation of GABAergic inputs from LH to LC (n=4) and the activation of GABAergic cell bodies in the hypothalamic arcuate nucleus (ARC) (n=3). (A) Representative image of DIO ChR2 injection in VGAT-cre mouse at 4 and 10x magnification. (B) Representative image of DIO ChR2 fluorescence in a mouse VGAT-cre projecting from LH to peri-LC at 4 and 10 x magnification.

(C) Amount of food consumed before (30 min), during (120 min) and after (30 min) the stimulation. Two-way ANOVA showed significant effect of the time ($F_{3,15} = 6.02$, $p = 0.0067$). Bonferroni post-hoc showed significant differences in the amount of food consumed at 90 min (1 hour after the onset of the stimulation). (D) Latency to approach the food under the effect of 3 mW and 20 Hz stimulation. T-test showed significant differences in the LH-LC group when compared with ARC. $p < 0.0001$. (E) Latency to stop eating after the end of the stimulation. T-test showed significant differences between the two groups, $p < 0.0001$. * $p < 0.05$.

Movie S1

Video showing a representative response of a mouse to 40 Hz optogenetic stimulation of LH-peri-LC circuitry.

References

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- 3) J.Lindeberg, et al., Transgenic expression of Cre recombinase from the tyrosine hydroxylase locus. *Genesis* 40(2):67-73 (2004).