PNAS
www.pnas.org

Supplementary Information for

A functional division of *Drosophila* sweet taste neurons that is value-based and task-specific

Hsueh-Ling Chen, Dorsa Motevalli, Ulrich Stern, Chung-Hui Yang*

* Corresponding author: yang@neuro.duke.edu

This PDF file includes:

Additional information on Methods

Fly strains. Flies were raised in standard cornmeal molasses food and maintained in the incubator with temperature set at 25°C and humidity set at 60%. The following stocks were used: w^{1118}, *Gr5a-GAL4* (1), $Gr5a^{LexA}$ (2, 3), $Gr43a^{GAL4}$ (4, 5), *Gr43a-LexA* (4, 5), *Gr61a-GAL4* (BL-57658) (6), $Gr64a^{GAL4}$ (2, 3), *Gr64a-GAL4* (BL-57662) (6), *Gr64f-GAL4* (BL-57668, BL-57669) (6), $Gr64f^{LexA}$ (2, 3), *GAD-LexA* (BL-60324), *TH-LexA* (7), *TDC2-LexA* (BL-52242), *TPN2-GAL4* (8), *Otd-nls::FLP* (9), *Tub-FRT-stop-FRT-GAL80* (BL-39213, 38878), *UAS-*$Kir_{2.1}$*::eGFP* (III) (10), *UAS-*$Kir_{2.1}$ (II) (BL-6596), *LexAop-*$Kir_{2.1}$ (a gift from Dr. Q. Yuan), *UAS-CsChrimson::mVenus* (BL-55136), *UAS-FRT-myr::TopHAT2-FRT-Syn21-Chrimson::tdTomato* (11), *UAS-FRT-mCherry-FRT-*$Kir_{2.1}$*::eGFP* (11), *UAS-FRT-*$Kir_{2.1}$*::eGFP-FRT-mCherry* (11), *LexAop-GAL80* (BL-32217), *LexAop-*P_2X_2 (12), GRASP reagents (BL-58755, BL-64314, BL-64315) (13, 14), *trans*-Tango reagents (BL-77124) (15).

Regular Egg-Laying Preference Assay. A detailed protocol including the design of our apparatus had been described previously (16). Briefly, flies were collected into food vials supplied with yeast paste made from active yeast and 0.5% propionic acid. Each vial typically contained about 30-40 females mixed with 10-15 males. After 3-4 days in the "yeasted" vials, individual females were then loaded into an apparatus with 48 individual two-choice arenas and allowed to lay eggs overnight (~14 hours). For preparing 10 ml of sucrose substrate, the protocol is as follows: we first added 750 μl of 2 M sucrose into a 50 ml conical tube and then poured pre-melted 1% agarose until the volume reached 10 ml, thus the final concentration of sucrose in the sucrose substrate is 150 mM. For preparing 10 ml of plain substrate, we replaced the 2 M sucrose stock solution with same volume of ddH_2O. The preference index for the sucrose substrate was calculated as follows: $(N_{Sucrose} - N_{Plain}) / (N_{Sucrose} + N_{Plain})$. $N_{Sucrose}$ and N_{Plain} represent the numbers of eggs laid on the sucrose-containing vs. the plain substrates, respectively. Note that we only calculated PI for a fly if it had laid at least 10 eggs.

Optogenetics-assisted Egg-laying and Positional Preference Assay. *SkinnerSys*: SkinnerSys is a new high-throughput closed-loop stimulation platform we developed for assaying how optogenetic activation of neurons of interest impacts egg-laying preferences. Briefly, it consists of the following custom components: 1) SkinnerTrax – software we previously developed for tracking the positions of multiple individual flies in real time and delivering light pulses to them depending on their behaviors (17, 18) (Note that the code is on GitHub at https://github.com/ulrichstern/SkinnerTrax). 2) A new custom-built egg-laying apparatus, each of which contains 40 individual two-choice arenas modeled after our regular high-throughput egg-laying apparatus, with the main difference that the arena sidewalls are less tall and slightly angled to make sure the sidewalls cannot hide flies from the cameras. 3) A custom-designed printed circuit board (PCB) with 80 individually controllable red (624 nm) 5mm 15° beam angle Cree LEDs, with two LEDs per arena. With both LEDs for a particular arena on, it is illuminated fully and roughly uniformly. The board uses TLC59711 LED drivers to offer fine-grain control over LED intensity, and hosts a Teensy 3.2 to allow low-latency (<0.1 ms) communication with SkinnerTrax over USB. 4) A custom-designed stand for the apparatus, allowing the apparatus to be quickly placed into the SkinnerSys setup. The stand holds also the cameras used for tracking (Microsoft LifeCam Cinemas) and the PCB in fixed positions. The stand stands on a lightpad (Artograph LightPad A920) that provides backlight for tracking (through two solder mask-free "windows" in the PCB) and is dimmed flicker-free to 2.5% of its native intensity with a 560Ω serial resistor. The resulting low backlight intensity is still sufficient for tracking

(at longer exposure and a frame rate of 7.5 fps) yet is too low to activate channelrhodopsins. At 7.5 fps, SkinnerTrax can quite easily handle two apparatuses on a single i7 machine. To avoid tracking problems when the red LEDs are on (the ones close to the camera axis shine essentially directly into the camera), we placed two layers of blue-pass filter (LEE Filters 172 Lagoon Blue) in front of the cameras. The Mastercam files for the egg-laying chamber and stand as well as the schematic and Gerber files for the PCB are available on GitHub at https://github.com/ulrichstern/SkinnerTrax. *Stimulation protocol*: After loading the flies into individual arenas (under dim light condition), we typically allowed them to lay eggs overnight (>14 hours) inside SkinnerSys. The stimulation protocol is as follows: if and only if SkinnerTrax determined that the fly in an arena was over the agarose for which we wanted stimulation, it pulsed both LEDs for the arena at 2 Hz (250 ms on/off), illuminating the arena with about 6.8 $\mu W/mm^2$ red light when the LEDs were on. *Preparation of flies to be assayed*: Flies were collected and prepared as described in the section of Egg-laying Preference Assay except vials were kept in the dark and that both the molasses food and the active yeast paste contained 200 mM of *all-trans*-retinal. Note that for these optogenetic studies, we added sorbitol (final concentration 100 mM), a tasteless substance for flies, into the plain agarose to ensure that flies had access to nutrition during egg-laying. We loaded the flies in dim light and kept them covered before tracking/stimulation.

Proboscis Extension Reflex (PER) Assay. Prior to testing, female flies were collected and incubated in dark with the retinal-containing molasses food and active yeast paste (400 mM all-trans-retinal) for 3 days. Individual flies were then introduced into P100 pipette tips with the pointed end part cut off to expose the head of the flies. After allowing the flies to recover for about 30 to 60 minutes, each fly was stimulated with red LED light at 5% intensity (2.29 $\mu W/mm^2$) for 5 times with a 5-minute rest in between each stimulation. PER response from each fly was scored by counting the % of times the fly responded and only full extension of the proboscis was scored as a response. For example, a fly would receive a score of 0% if it never responded to the 5 stimulations and 100% if it responded to all. The assay was performed under dim background illumination (0.08 $\mu W/mm^2$) provided by a lightpad (Artograph LightPad 920).

Fly Surgery for *trans*-Tango. Shortly after eclosion, flies were anesthetized on a CO_2 pad and had the first three tarsal segments from all six of their legs removed by a pair of sharp forceps. After the surgery, flies were aged to about 10-14 days old and their brains and VNCs were dissected and stained for imaging.

Ex vivo GCaMP imaging. Individual VNCs were immersed in imaging buffer (its ingredients were described in our previous work (19)) in a custom chamber and imaged using a 40X water lens and the Zen software. Response for each VNC was calculated as $\Delta F/F_0$, where F_0 represents the baseline of GCaMP signals and ΔF (i.e., $F_{peak} - F_0$) represents the largest fluorescence change evoked by stimulation. F_0 was calculated by averaging the acquired signal for ~2 min prior to stimulation and F_{peak} was the highest signal evoked by stimulation with either ATP (400 μM) or buffer. All included controls (that did not show any responses) responded to KCl.

Immunohistochemistry. Immunohistochemistry experiments were conducted following standard protocols with modifications on animals' age and fixation time. *Fly age:* for regular experiments, flies were dissected when they were about 7 days old, but for *trans*-Tango experiments, flies were generally aged for at least 14 days so as to increase the labeling strength of the traced postsynaptic targets. *Fixation time:* brains and VNCs were

fixed for about 40 minutes whereas proboscis and legs were fixed for about 1 hour. The primary antibodies used in this study were as follows: anti-NC82 mouse (1:50), anti-HA mouse (1:250), and anti-GFP rabbit (1:1000), and anti-GFP mouse (1:5000). The secondary antibodies used were as follows: donkey-anti mouse Cy3 (1:500), donkey-anti mouse Alexa Fluor 488 (1:500), and donkey-anti rabbit Alexa Fluor 488 (1:500). Samples were imaged using a Zeiss LSM700 with either the 20x air objective or the 40x oil objective and the acquired confocal images were then post-processed with Fiji.

Quantification and Statistical Analysis. GraphPad Prism 8 was used to perform statistical tests. We followed the guideline provided by GraphPad Statistics Guide in selecting tests and generally set the significance level at $\alpha = 0.05$. *When analyzing results that contained one or two groups*: we used Unpaired t-test with Welch's correction to compare two data groups and one-sample t-test from 0 to compare a sample mean against 0. *When analyzing results that contained more than two groups*: we used Welch's ANOVA test with Dunnett's multiple comparisons post-test to compare every group against one particular group. In general, we used ns for $p \geq 0.05$, * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$ in all unpaired t-tests and one-way ANOVA test and "ω" for significance from 0 in one-sample t-test.

Figure S1

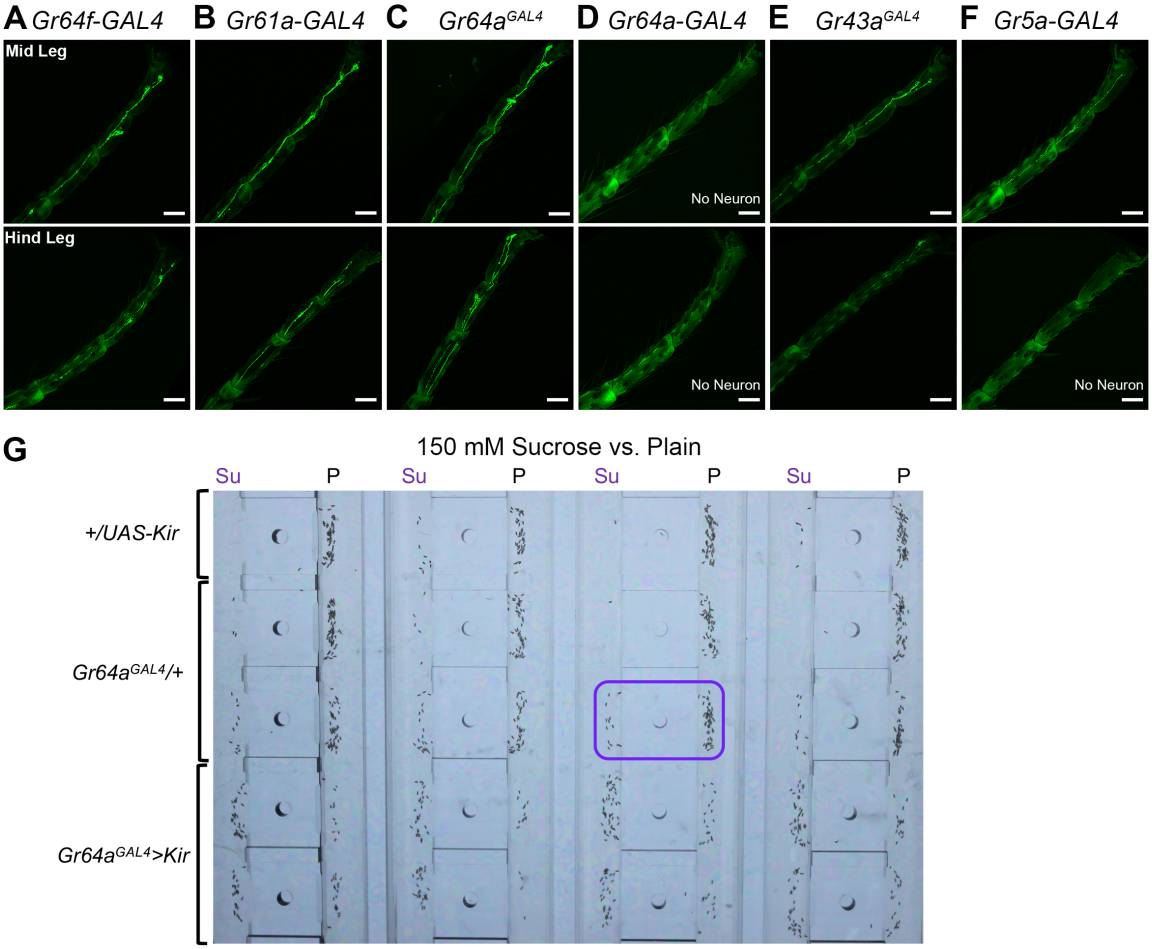

Figure S1. Leg sweet neurons labeled by different sweet *Gr-GAL4*s and the effect of silencing *Gr64a*^{GAL4}-expressing neurons on sucrose rejection

(A-F) Representative images showing the expression of different sweet *Gr-GAL4*s on the mid-legs (top row) and the hindlegs (bottom row). Scale bars: 60 μm.

(G) A representative image showing egg-laying preferences of 20 individual flies in the *sucrose vs. plain* task. Note that control flies (top three rows) preferred plain whereas experimental flies (lower two rows) preferred sucrose. The purple rectangle shows the approximate area for a single arena in our apparatus.

Figure S2

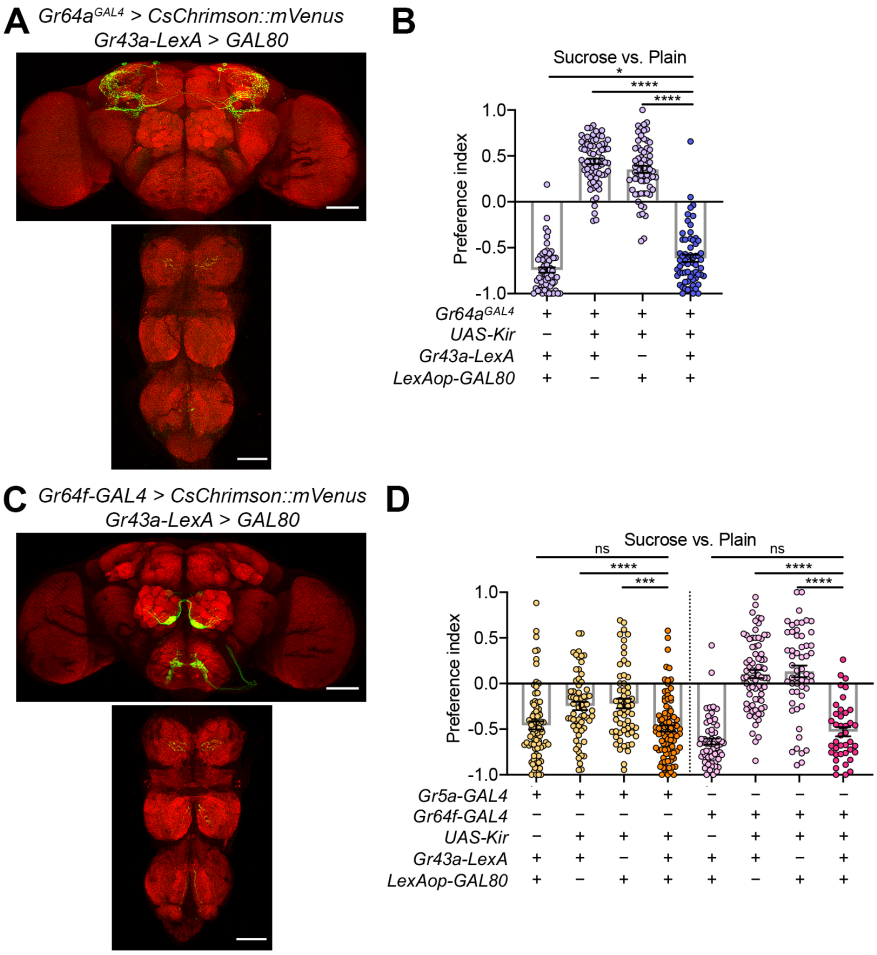

Figure S2. Additional evidence supporting the importance of the leg sweet neurons in sucrose rejection in a *sucrose vs. plain* task

(A) Representative images showing the expression of *Gr64a*^{GAL4} in both the brain and the VNC in the presence of *Gr43a-LexA>GAL80.* Scale bars: 60 μm.

(B) Egg-laying PI of females whose *Gr64a*^{GAL4}-expressing neurons were silenced in the presence and absence of *Gr43a-LexA>GAL80* in the *sucrose vs. plain* task. Welch's ANOVA test with Dunnett's posttest; n = 62-70.

(C) Representative images showing the expression of *Gr64f-GAL4* in both the brain and the VNC in the presence of *Gr43a-LexA>GAL80.* Scale bars: 60 μm.

(D) Egg-laying PI of females whose *Gr5a-GAL4*- and *Gr64f-GAL4*-expressing neurons were silenced in the presence and absence of *Gr43a-LexA>GAL80* in the *sucrose vs. plain* task. Welch's ANOVA test with Dunnett's posttest; n = 40-92.

For all comparisons performed in this figure, ns for $p \geq 0.05$, *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$.

Figure S3

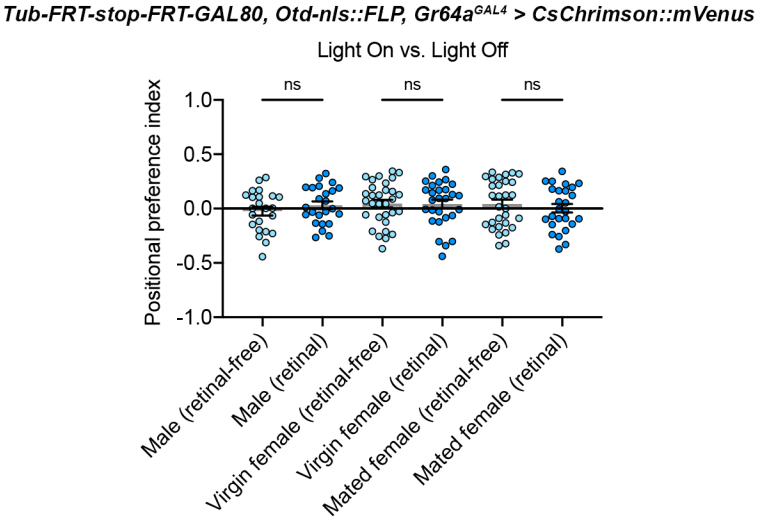

Figure S3. Males, virgins, and non-egg-laying mated females do not prefer to spend time on an option on which their leg sweet neurons are artificially activated

Positional PI of males, virgins, and mated – but non-egg-laying – females that expressed *CsChrimson* selectively in only their *Gr64a*^{GAL4}-labeled neurons on the legs in the *light-on vs. light-off* two-choice task. Unpaired t test with Welch's correction and 1-sample t test against 0, ns for $p \geq 0.05$; n = 23-31.

Figure S4

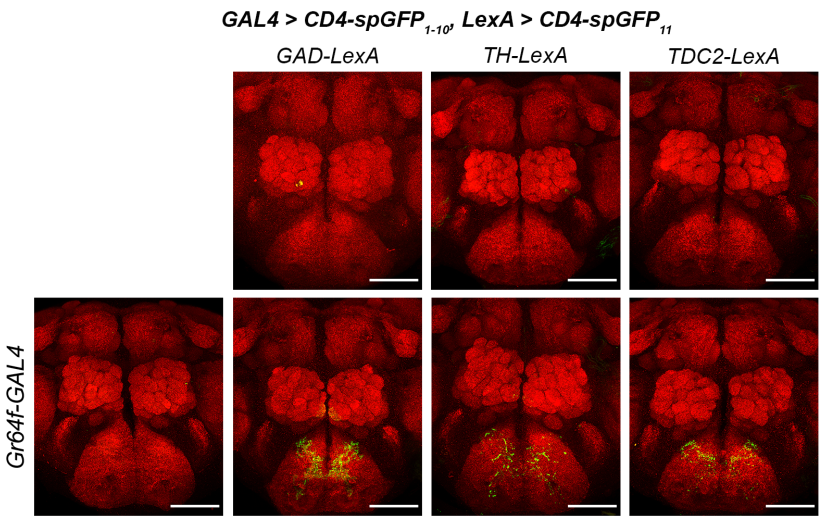

Figure S4. *Gr64f*-expressing sweet neurons form direct contacts with multiple classes of neuromodulatory neurons

Representative images showing sweet neurons (labeled by *Gr64f-GAL4*) have direct contacts with GABAergic (GABA), dopaminergic (DA), and octopaminergic (OA) neurons in the SEZ. Such contacts are detected by expressing one half of the GFP (*CD4::splitGFP*$_{1\text{-}10}$) in the *Gr64f-GAL4*-expressing neurons and the other half of the GFP (*CD4::splitGFP*$_{11}$) in the *GAD-LexA*-, *TH-LexA*- or *TDC2-LexA*-expressing neurons. Scale bars: 60 μm. We used *GAD-LexA* for labeling the GABAergic neurons, *TH-LexA* for labeling the DA neurons, and *TDC2-LexA* for labeling the OA neurons. The three panels on the top row and the first panel on the second row are control images.

Figure S5

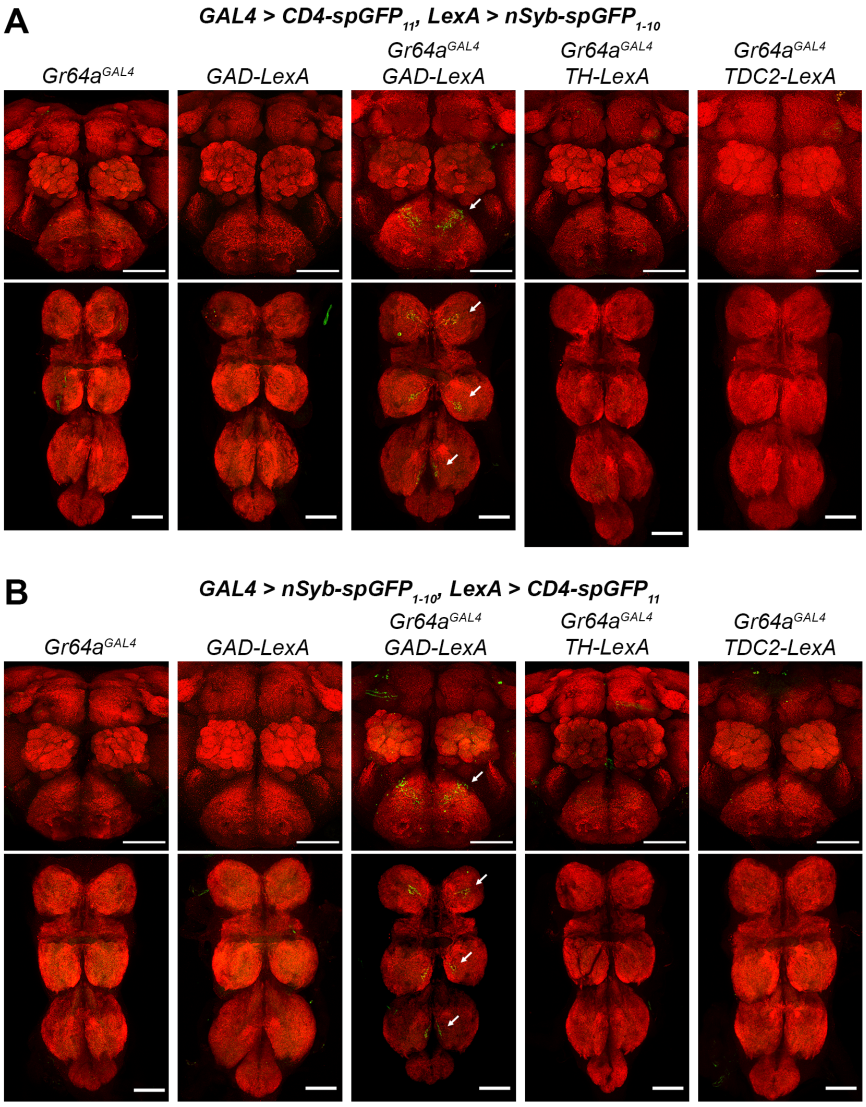

Figure S5. Sweet neurons on the legs have bidirectional direct contacts with GABAergic neurons but not with OA or DA neurons

Representative images showing that the leg sweet neurons (labeled by *Gr64a*^{GAL4}) have direct contacts with GABA neurons but not with DA and OA neurons. We used different GRASP reagents to assess the directionality of such contacts. **(A)** *Gr64a*^{GAL4} was used to direct expression of one-half of the GFP targeted to the cell membrane of sweet neurons (*CD4::splitGFP*$_{11}$) while the *LexA* drivers for GABA, DA, and OA neurons were used to direct the other half of the GFP targeted specifically to their presynaptic termini (*nSyb::splitGFP*$_{1\text{-}10}$). **(B)** *Gr64a*^{GAL4} was used to direct one-half of the GFP targeted to the presynaptic termini (*nSyb::splitGFP*$_{1\text{-}10}$) of the sweet neurons whereas *LexA* drivers for GABA, DA, and OA were used to direct the other half of the GFP (*CD4::splitGFP*$_{11}$) targeted to their cell membrane. Scale bars: 60 μm. The arrows indicate GRASP signals.

Figure S6

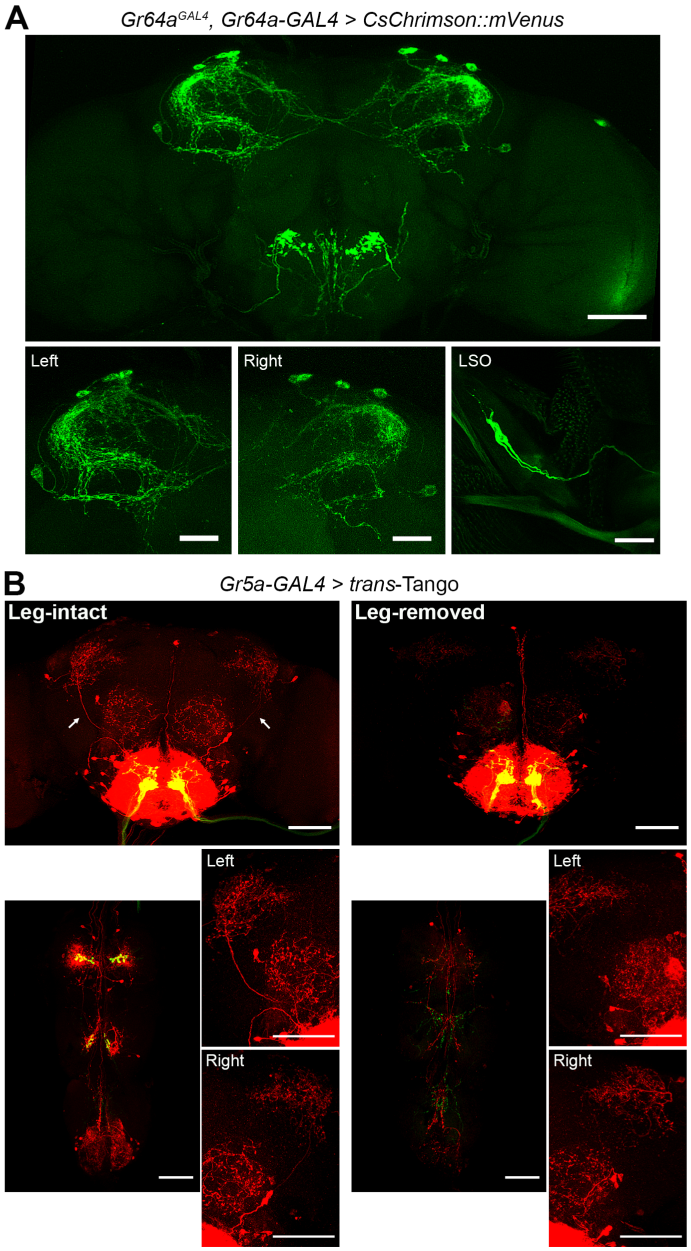

Figure S6. Additional evidence supporting that the sweet neurons on the legs have distinct postsynaptic target(s) from the rest of the sweet neurons

(A) Representative images showing the neurons co-labeled by *Gr64a*^{GAL4} and *Gr64a-GAL4* in the brain and on the esophagus. Because the number of neurons co-labeled by the two *GAL4s* did not differ from the numbers labeled by each *GAL4* individually, this result indicates that *Gr64a*^{GAL4} and *Gr64a-GAL4* labeled the same neurons in the brain and on the esophagus. Scale bars: 60 μm (top) and 30 μm (bottom).

(B) Representative images showing *trans*-Tango tracing of *Gr5a-GAL4*-expressing neurons in intact flies (left) and in flies with their legs amputated shortly after eclosion (right). Scale bars: 60 μm.

Figure S7

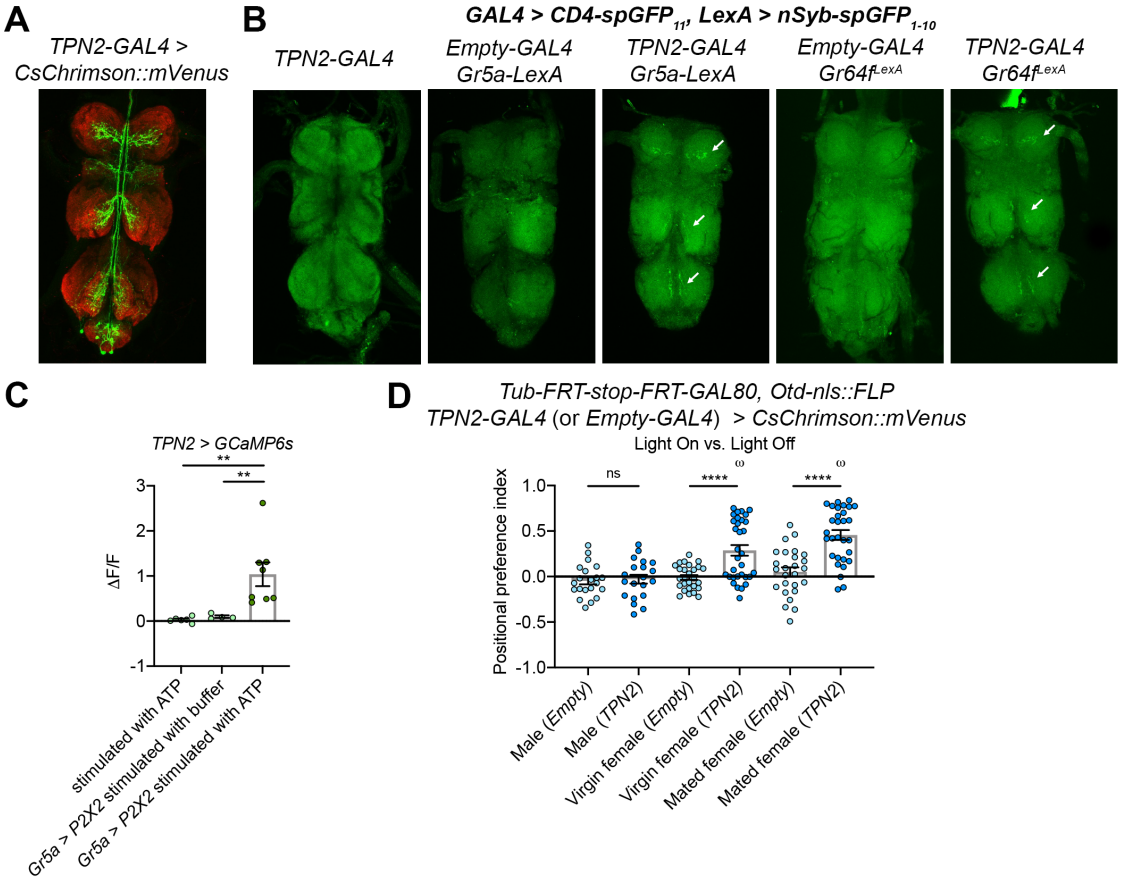

Figure S7. Additional characterization of TPN2 neurons and their behavioral impact on non-egg-laying animals

(A) Processes of *TPN2-GAL4*-expressing neurons in the VNC.

(B) Representative images showing that *TPN2-GAL4*-expressing neurons have direct contacts with the leg sweet neurons. *Gr5a-LexA* and *Gr64f*^{LexA} were used to direct *nSyb::splitGFP*$_{1-10}$ expression in the leg sweet neurons while *TPN2-GAL4* was used to direct the expression of *CD4::splitGFP*$_{11}$. The arrows indicate GRASP signals.

(C) Ca^{2+}/GCaMP responses of *TPN2-GAL4*-expressing neurons when *Gr5a-LexA*-expressing sweet neurons on the legs were artificially stimulated by the *P*$_2$*X*$_2$-ATP system. Unpaired t test with Welch's correction; n = 4-8. See also **Movie S3**.

(D) Positional PI of males, virgins, and mated – but non-egg-laying – females that expressed *CsChrimson* selectively in only their *TPN2-GAL4*-expressing neurons in the *light-on vs. light-off* task. Unpaired t test with Welch's correction and 1-sample t test against 0; n = 20-32.

For all comparisons performed in this figure, ns for $p \geq 0.05$, ** $p < 0.01$, and **** $p < 0.0001$; ω, statistical significance for 1-sample t test against 0.

Movie S1. A virtual "sweet and plain" decision task

In this video clip, we showed the design of our virtual *sweet vs. plain* task administered by SkinenrSys. Three flies that expressed *CsChrimson* in their sweet neurons can be seen exploring two plain agarose options – one on the top and another at the bottom – in the arenas. The top agarose (outlined in rectangle) in the arena was "baited with light" such that each time the flies entered this area, light would be switched on and thus their sweet neurons would become activated. The bottom agarose was not baited with light and would stay unilluminated throughout the task.

Movie S2. SkinnerSys in action

In this video clip, we showed how SkinnerSys handled closed-loop stimulation of 20 individual flies in parallel. On the left, SkinnerSys displayed 20 flies being tracked and stimulated according to the rule we defined; on the right, SkinnerSys displayed the real-time positional heatmaps of these flies.

Movie S3. Response of TPN2 neurons to stimulation of the leg sweet neurons

In this video clip, we showed that the processes of TPN2 neurons at the T1 region of VNC (where they synapsed with axons of the leg sweet neurons) exhibited a clear GCaMP increase when we stimulated the leg sweet neurons (with the P_2X_2/ATP system) in an *ex vivo* preparation. Note that the A-P axis of the VNC in this video was rotated about 45 degree clockwise.

References for SI

1. S. Chyb, A. Dahanukar, A. Wickens, J. R. Carlson, Drosophila Gr5a encodes a taste receptor tuned to trehalose. *Proc Natl Acad Sci U S A* **100 Suppl 2**, 14526-14530 (2003).
2. S. Fujii *et al.*, Drosophila sugar receptors in sweet taste perception, olfaction, and internal nutrient sensing. *Curr Biol* **25**, 621-627 (2015).
3. A. Yavuz, C. Jagge, J. Slone, H. Amrein, A genetic tool kit for cellular and behavioral analyses of insect sugar receptors. *Fly (Austin)* **8**, 189-196 (2014).
4. T. Miyamoto, H. Amrein, Diverse roles for the Drosophila fructose sensor Gr43a. *Fly (Austin)* **8**, 19-25 (2014).
5. T. Miyamoto, J. Slone, X. Song, H. Amrein, A fructose receptor functions as a nutrient sensor in the Drosophila brain. *Cell* **151**, 1113-1125 (2012).
6. J. Y. Kwon, A. Dahanukar, L. A. Weiss, J. R. Carlson, Molecular and cellular organization of the taste system in the Drosophila larva. *J Neurosci* **31**, 15300-15309 (2011).
7. J. A. Berry, I. Cervantes-Sandoval, M. Chakraborty, R. L. Davis, Sleep Facilitates Memory by Blocking Dopamine Neuron-Mediated Forgetting. *Cell* **161**, 1656-1667 (2015).
8. H. Kim, C. Kirkhart, K. Scott, Long-range projection neurons in the taste circuit of Drosophila. *Elife* **6** (2017).
9. K. Asahina *et al.*, Tachykinin-expressing neurons control male-specific aggressive arousal in Drosophila. *Cell* **156**, 221-235 (2014).
10. R. A. Baines, J. P. Uhler, A. Thompson, S. T. Sweeney, M. Bate, Altered electrical properties in Drosophila neurons developing without synaptic transmission. *J Neurosci* **21**, 1523-1531 (2001).

11. K. Watanabe *et al.*, A Circuit Node that Integrates Convergent Input from Neuromodulatory and Social Behavior-Promoting Neurons to Control Aggression in Drosophila. *Neuron* **95**, 1112-1128 e1117 (2017).
12. B. Gou, Y. Liu, A. R. Guntur, U. Stern, C. H. Yang, Mechanosensitive neurons on the internal reproductive tract contribute to egg-laying-induced acetic acid attraction in Drosophila. *Cell Rep* **9**, 522-530 (2014).
13. E. H. Feinberg *et al.*, GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. *Neuron* **57**, 353-363 (2008).
14. L. J. Macpherson *et al.*, Dynamic labelling of neural connections in multiple colours by trans-synaptic fluorescence complementation. *Nat Commun* **6**, 10024 (2015).
15. M. Talay *et al.*, Transsynaptic Mapping of Second-Order Taste Neurons in Flies by trans-Tango. *Neuron* **96**, 783-795 e784 (2017).
16. B. Gou, E. Zhu, R. He, U. Stern, C. H. Yang, High Throughput Assay to Examine Egg-Laying Preferences of Individual Drosophila melanogaster. *J Vis Exp* 10.3791/53716, e53716 (2016).
17. U. Stern *et al.*, Learning a Spatial Task by Trial and Error in Drosophila. *Curr Biol* **29**, 2517-2525 e2515 (2019).
18. U. Stern, C. H. Yang, SkinnerTrax: high-throughput behavior-dependent optogenetic stimulation of Drosophila. *bioRxiv* https://doi.org/10.1101/080614 (2017).
19. H. L. Chen, U. Stern, C. H. Yang, Molecular control limiting sensitivity of sweet taste neurons in Drosophila. *Proc Natl Acad Sci U S A* **116**, 20158-20168 (2019).