

RESEARCH ARTICLE

A subset of brain neurons controls regurgitation in adult *Drosophila melanogaster*

Yu-Chieh David Chen¹, Sameera Ahmad², Kush Amin² and Anupama Dahanukar^{1,3,*}

ABSTRACT

Taste is essential for animals to evaluate food quality and make important decisions about food choice and intake. How complex brains process sensory information to produce behavior is an essential question in the field of sensory neurobiology. Currently, little is known about higher-order taste circuits in the brain as compared with those of other sensory systems. Here, we used the common vinegar fly, *Drosophila melanogaster*, to screen for candidate neurons labeled by different transgenic *GAL4* lines in controlling feeding behaviors. We found that activation of one line (*VT041723-GAL4*) produces ‘proboscis holding’ behavior (extrusion of the mouthpart without withdrawal). Further analysis showed that the proboscis holding phenotype indicates an aversive response, as flies pre-fed with either sucrose or water prior to neuronal activation exhibited regurgitation. Anatomical characterization of *VT041723-GAL4*-labeled neurons suggests that they receive sensory input from peripheral taste neurons. Overall, our study identifies a subset of brain neurons labeled by *VT041723-GAL4* that may be involved in a taste circuit that controls regurgitation.

KEY WORDS: Proboscis, Feeding behaviors, Neuronal circuits, Taste, *GAL4/UAS*

INTRODUCTION

One of the fundamental questions in the field of neuroscience is how the brain responds to different sensory inputs and mediates appropriate behaviors. To address this fundamental question, many have taken advantage of the vinegar fly, *Drosophila melanogaster*, as a neurogenetic model organism. With a numerically simpler nervous system compared with that in mammals, flies nevertheless exhibit complex behaviors. Importantly, fundamental principles of sensory coding and neuronal circuit function for processing sensory inputs and driving behaviors are often conserved across species. Therefore, *Drosophila* is a powerful model for functional dissection of neuronal circuits underlying behaviors.

The gustatory system, which influences selection of food, egg deposition sites and mates, among others, is an appealing sensory system to address such questions. The identification of chemosensory receptor genes (Clyne et al., 2000; Scott et al., 2001) and the development of methods to assess feeding behaviors (Ja et al., 2007; Deshpande et al., 2014; Itskov et al., 2014; Ro et al., 2014; Murphy

et al., 2017; Shell et al., 2018; Park et al., 2018; Moreira et al., 2019; Yapici et al., 2016; Shiraiwa and Carlson, 2007; Diegelmann et al., 2017) provided a foundation for dissecting the functions of peripheral taste neurons with precise molecular genetic tools. Much is now known about how peripheral taste neurons detect various chemicals (Ling et al., 2014; Weiss et al., 2011; Chen and Dahanukar, 2017; Ledue et al., 2015; He et al., 2019; Raad et al., 2016; Steck et al., 2018; Jaeger et al., 2018), but higher-order gustatory processing in the central brain remains poorly understood. A number of recent studies have utilized powerful genetic screens for higher-order neurons in the brain that process taste information and control feeding behaviors. For example, a number of interneurons and motor neurons have been found to selectively respond to sugars (Miyazaki et al., 2015; Kain and Dahanukar, 2015; Flood et al., 2013; Yapici et al., 2016; Gordon and Scott, 2009) or bitter compounds (Bohra et al., 2018; Kim et al., 2017) and mediate innate feeding responses such as proboscis extension and food ingestion as well as learned taste aversion. In addition, several neuromodulatory interneurons, which modulate taste responses to sugars and bitter compounds, have also been described (Ledue et al., 2016; Youn et al., 2018; Inagaki et al., 2014b; Inagaki et al., 2012). In this study, we aimed to identify candidate higher-order brain neurons involved in processing taste information and mediating feeding behaviors.

We used both *VT-GAL4* and *Janelia-GAL4* transgenic fly lines to access different subsets of neurons in the adult fly brain (Kvon et al., 2014; Jenett et al., 2012) and asked which if any can induce proboscis extension when activated. We expressed dTrpA1, a heat-activated ion channel (Kang et al., 2011), under the control of a *UAS* promoter in subsets of neurons labeled by the selected *VT-GAL4* and *Janelia-GAL4* lines and examined heat-activated proboscis extension responses (PERs) (Shiraiwa and Carlson, 2007). We identified one candidate line (*VT041723-GAL4*), which labels a neuronal population that mediates regurgitation. Activation of *VT041723-GAL4*-labeled neurons induces prolonged proboscis extension (proboscis holding) for as long as 7 min without retraction. Similar results were observed by optogenetic activation of these neurons. Pre-feeding of flies with sucrose or water prior to neuronal activation leads to regurgitation, suggesting an aversive response for this prolonged proboscis extension. Using the GFP reconstitution across synaptic partners (GRASP) technique, we found that the *VT041723-GAL4*-labeled neurons have synaptic connections with peripheral taste neurons in the pharynx. Altogether, our results identify a subset of brain neurons labeled by *VT041723-GAL4* that control regurgitation. Our behavioral data also suggest that proboscis extension, a commonly used acceptance feeding behavior readout, might not be a reliable indicator of appetitive feeding behavior.

MATERIALS AND METHODS

Fly strains

Flies were reared on standard cornmeal-dextrose-agar food at 25°C and 60–70% relative humidity under a 12 h:12 h dark:light cycle.

¹Interdepartmental Neuroscience Program, University of California, Riverside, CA 92521, USA. ²Department of Biology, University of California, Riverside, CA 92521, USA. ³Department of Molecular, Cell and Systems Biology, University of California, Riverside, CA 92521, USA.

*Author for correspondence (anupama.dahanukar@ucr.edu)

 Y.-C.D.C., 0000-0002-2597-7577; A.D., 0000-0003-0534-7700

The following fly strains were used in this study: *VT041723-GAL4* (Vienna *Drosophila* Resource Center) (Kvon et al., 2014), *Gr43a-LexA* (Miyamoto and Amrein, 2014), *Ir76b-LexA* (Ganguly et al., 2017), *Poxn^{AM22-B5}* (Boll and Noll, 2002), *Poxn⁷⁰* (Awasaki and Kimura, 1997), *UAS-mCD8-GFP* (Weiss et al., 2011), *UAS-Syt-GFP*, *UAS-DenMark* (BDSC 33064), *UAS-dTrpA1* (BDSC 26263), *UAS-CsChrimson* (BDSC 55135), *UAS-spGFP1-10::Nrx* (Fan et al., 2013), *LexAop-spGFP11::CD4* (Gordon and Scott, 2009) and *LexAop2-6XmCherry-HA* (BDSC 52271, 52272).

Immunohistochemistry

Flies aged 4–8 days were anesthetized on ice, and brain tissues were dissected in 1× PBST (PBS with 0.3% Triton X-100) followed by fixing with 4% paraformaldehyde in 1× PBST for 30 min at room temperature. After three washes with 1× PBST, samples were blocked with 5% normal goat serum (Sigma-Aldrich, G9023) in 1× PBST. Tissues were incubated in primary antibody solutions for 3 days at 4°C. Primary antibodies were: chicken anti-GFP (1:5000; Abcam, ab13970), rabbit anti-DsRed (1:200; Clontech, 632496) and mouse anti-nc82 (1:20; Developmental Studies Hybridoma Bank). Secondary antibodies (1:400; Invitrogen) were: goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa Fluor 546, goat anti-mouse Alexa Fluor 568 and goat anti-mouse Alexa Fluor 647. Samples were mounted in Vectashield antifade mounting medium (Vector Laboratories, H-1000) and stored at 4°C. Fluorescent images were acquired using a Leica SP5 confocal microscope with 400 Hz scan speed in 512×512 or 1024×1024 pixel format. Image stacks were acquired at 1 μm optical sections. All images are presented as maximum projections of the z-stack generated using Leica LAS AF software.

GRASP

Immunofluorescence staining procedures were similar to those described above with the following minor modifications for detecting GRASP signals between *Ir76b-LexA*-labeled peripheral taste neurons and *VT041723-GAL4*-labeled central neurons in the brain. To detect native reconstituted GFP signal, only the primary antibody of mouse anti-nc82 (1:20; Developmental Studies Hybridoma Bank) was used for staining neuropil. The two transgene controls were stained together with experimental genotypes at the same time and imaged with the same settings using a Leica SP5 confocal microscope. Image stacks were acquired at 1 μm optical sections. All images are presented as maximum projections of the z-stack generated using Leica LAS AF software.

Thermogenetically activated PER assay

Flies of both sexes, aged 4–8 days, were immobilized on glass coverslips with drops of clear, non-toxic nail polish and then allowed to acclimate for 30–60 min in a humidified chamber prepared by filling a pipette tip box with water and placing damp Kimwipes (Kimberly-Clark Kimtech) on top. One by one, each coverslip containing an individual fly was placed on a 31°C heat block and proboscis extensions were observed under a light microscope. In the initial screening of 194 *VT-GAL4* and *Janelia-GAL4* lines (Fig. 1A), we scored flies showing full proboscis extension as an indication of food acceptance. In subsequent experiments focusing on the *VT041723-GAL4* line, we recorded trial number, sex, proboscis extension and extension duration for each experimental trial. Proboscis holding was scored when flies fully extended their proboscis for more than 10 s without retraction. For the experiments

examining regurgitation phenotype, flies were starved for 24 h on either water-saturated tissues, and then pre-fed 0.5 μl of 100 mmol l⁻¹ sucrose (Sigma-Aldrich, S7903) (Fig. 4B,C), or dry tissues, and then pre-fed 0.5 μl of distilled water (Fig. 4D). Flies that did not consume the pre-feeding tastant solutions in their entirety were excluded from the analysis. Flies that consumed all of the pre-feeding tastant solutions were transferred to a 31°C heat block and the number of flies showing regurgitation was recorded. Regurgitation was defined by the presence of a liquid bubble at the tip of the proboscis (Fig. 4A). In all experiments, we tested both *GAL4* and *UAS* controls together with experimental flies in parallel, in random order, and experimenters were blind to genotype. Among all control flies, we did not observe any that showed proboscis holding or regurgitation behaviors.

Optogenetically activated PER assay

Two to four days after eclosion, flies were transferred to standard commeal-dextrose-agar food supplemented with 1 mmol l⁻¹ all-trans-retinal (ATR; Sigma-Aldrich, R2500), and placed in aluminium foil-wrapped vials at 25°C for 2–3 days. Sibling flies were transferred to the same food vials without ATR to serve as controls. Flies were prepared as for the thermogenetically activated PER assay described above, with the exception that they were prepared under low-light conditions, in which the intensity of room lights was too low to activate CsChrimson. Flies were then stimulated with 626 nm LED light (Super Bright LEDs Inc.), and the number of flies showing proboscis holding was recorded. In all experiments, we performed tests on both control and experimental flies on each day, in random order, and experimenters were blind to fly genotype and rearing conditions.

Statistical analyses

All data are presented as median±interquartile range. Statistical tests were conducted using Prism 8 (GraphPad software). Differences between means of different groups were evaluated for statistical significance with unpaired *t*-tests. All control and experimental genotypes were always tested in parallel, and experimenters were blind to all genotypes and rearing conditions. All independent trials were performed over 2 days.

RESULTS

A thermogenetic activation screen of transgenic *GAL4* lines identifies *VT041723-GAL4*, which triggers a proboscis holding behavior

To identify higher-order brain neurons involved in feeding behaviors, we took advantage of available transgenic resources in the Vienna Tiles *GAL4* (*VT-GAL4*) Library at the Vienna *Drosophila* Resource Center (VDRC) and the *Janelia-GAL4* collection at the Janelia Farm Research Campus. Transgenic *GAL4* lines created with different promoter DNA sequences show different labeling patterns that can be visualized with different reporters, such as *UAS-GFP*. The expression patterns of *VT-GAL4* and *Janelia-GAL4* lines in the adult *Drosophila* brain have been well documented (Pfeiffer et al., 2008; Jenett et al., 2012; Kvon et al., 2014). Using the Virtual Fly Brain online database (www.virtualflybrain.org) (Milyaev et al., 2012), we first did a preliminary image-based screen for neurons that arborize in and around the subesophageal zone (SEZ), the primary taste center in the fly brain, and selected several candidate lines for further analysis. Among these, *GAL4* lines that showed sparse labeling in the adult brain were prioritized for subsequent behavioral screening. To determine whether any of the selected *GAL4* lines labeled neurons involved

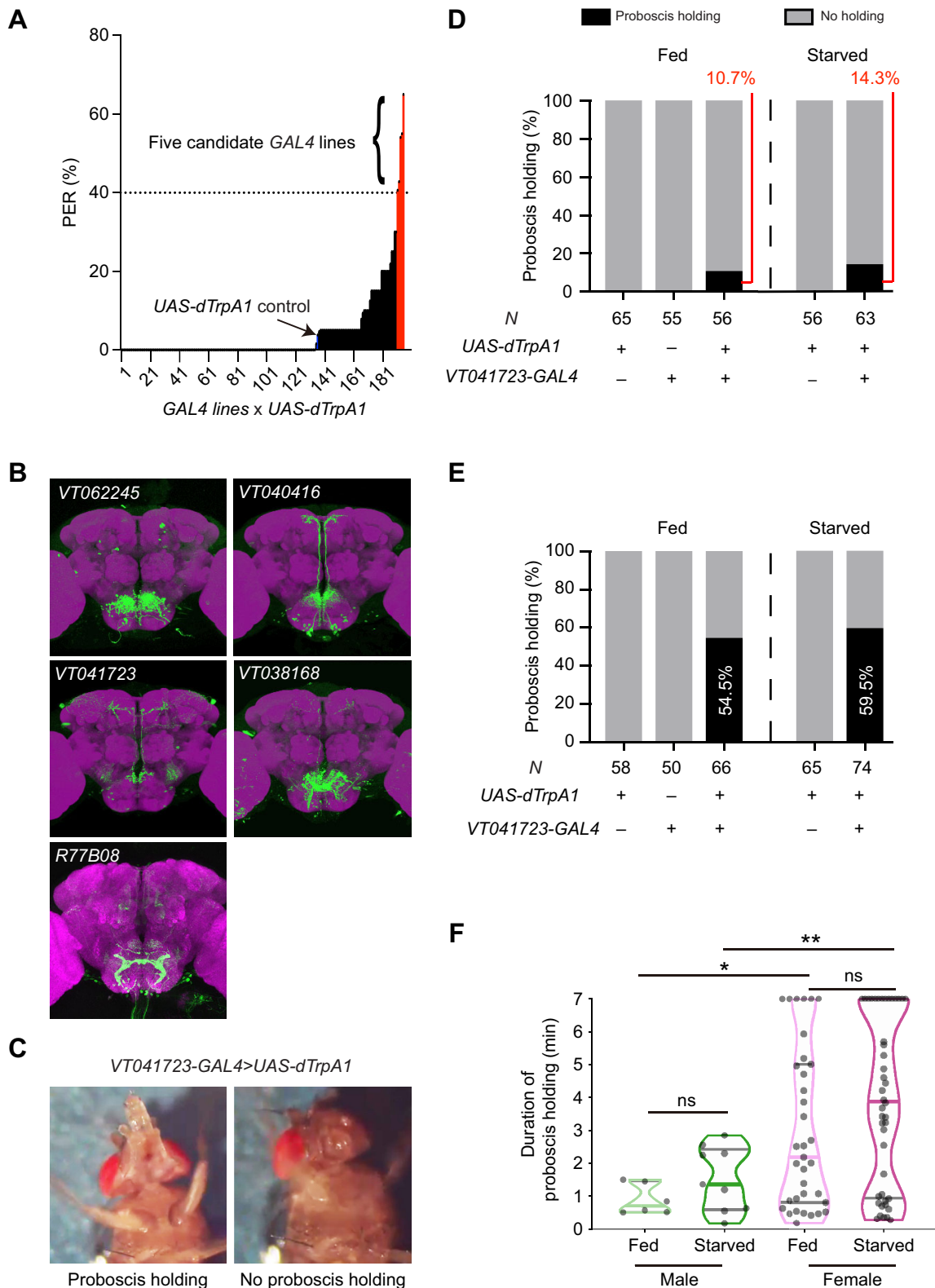


Fig. 1. A proboscis extension screen of *GAL4* transgenic fly lines identifies *VT041723-GAL4* neurons as candidates for higher-order taste neurons controlling feeding behavior. (A) Heat-activated proboscis extension responses (PERs) of 195 *GAL4>dTrpA1* lines. The *UAS-dTrpA1* control is shown in blue (arrow). Red bars indicate the five candidate *GAL4* lines with >40% PER. (B) Green fluorescent protein (GFP) expression patterns in the adult *Drosophila* brain driven by the five candidate *GAL4* lines. Dickson Lab VT line images (Tirian & Dickson, 2017 preprint) were taken from Virtual Fly Brain online database (www.virtualflybrain.org) (Milyaev et al., 2012). (C) Sample images of proboscis holding upon thermogenetic activation of *VT041723-GAL4* neurons (see also Movie 1). (D,E) Results of thermogenetic activation experiments with male (D) and female (E) flies of the indicated genotypes, tested without starvation (fed) or after 24 h starvation on wet tissues (starved). *UAS* and *GAL4* controls were tested in parallel with the experimental flies, and experimenters were blind to genotype. *N*=50–74. (F) Duration of proboscis holding in a 7 min thermogenetic activation assay. *N*=6–44. **P*<0.05, ***P*<0.01; ns, not significant; unpaired *t*-tests.

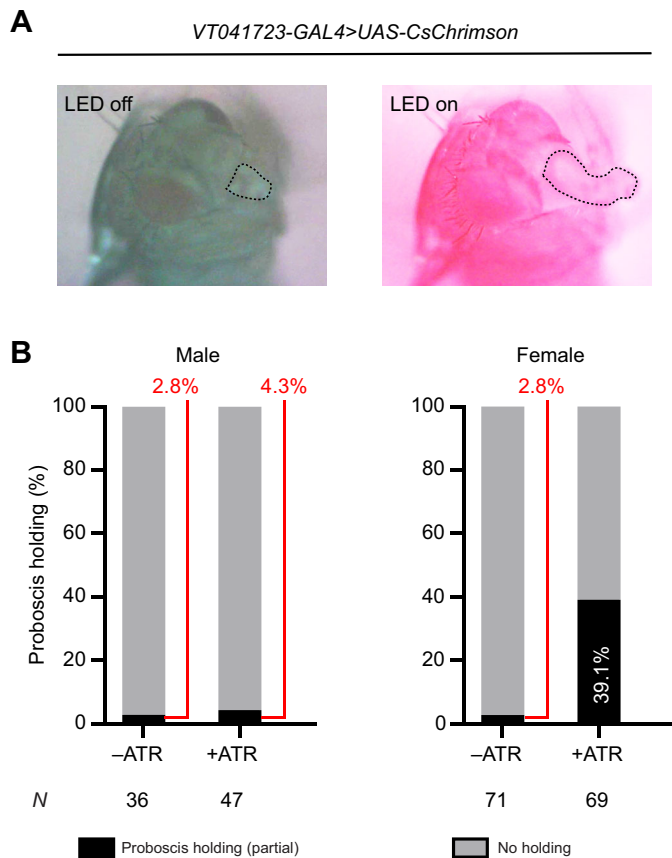


Fig. 2. Optogenetic activation of *VT041723-GAL4* neurons induces proboscis holding. (A) Sample images of the head before (left) and after (right) optogenetic activation of *VT041723-GAL4* neurons with 626 nm LED. The dotted line outlines the proboscis. Note that the proboscis is not fully extended as compared with Fig. 1C; however, flies held it in the partially extended position for the 7 min duration of the assay (see also Movie 2). (B) Percentage of *VT041723-GAL4>UAS-CsChrimson* flies fed with food with (+) or without (-) all-trans-retinal (ATR) showing proboscis holding upon red LED activation. The experimenters were blind to the fed state of the flies. $N=36-71$.

in feeding behaviors, we expressed the *Drosophila* transient receptor potential channel, subfamily A, member 1 (*dTrpA1*), a heat-activated cation channel (Kang et al., 2011), using the *GAL4/UAS* binary expression system (Brand and Perrimon, 1993). By elevating the ambient temperature to 31°C, we could thermogenetically activate these neurons and record the PER, in which the fly protrudes its mouthpart (proboscis), as a readout of feeding behavior (Shiraiwa and Carlson, 2007). From a preliminary screen of 194 *GAL4* lines (155 *VT-GAL4* lines and 39 *Janelia-GAL4* lines) (Table S1), we found five lines (*VT062245-GAL4*, *VT040416-GAL4*, *VT041723-GAL4*, *VT038168-GAL4* and *R77B08-GAL4*) that exhibited more than 40% PER (Fig. 1A). Closer examination of the expression patterns of the five lines excluded three (*VT062245-GAL4*, *VT038168-GAL4* and *R77B08-GAL4*) based on expression in peripheral taste neurons that project to the SEZ (Fig. 1B) (Kwon et al., 2014). Interestingly, PER activated by the *VT041723-GAL4* line was unique in that the flies did not retract the proboscis after extension, but rather maintained it in the extended position at length (Fig. 1C; Movie 1). We termed this unusual PER response ‘proboscis holding’ and selected the *VT041723-GAL4* line for further analysis.

Thermogenetic activation of *VT041723-GAL4* neurons induces a sexually dimorphic proboscis holding that is independent of starvation

To determine whether both males and females exhibited proboscis holding upon activation of *VT041723-GAL4* neurons, we performed the heat-activated PER assay with mated male and female flies of both experimental and control genotypes (Fig. 1D,E). The proboscis holding phenotype was recorded on an all-or-nothing basis. If a fly extended its proboscis for 10 s or longer upon heat activation, it was considered to have proboscis holding. If the fly did not extend its proboscis, or if the duration of proboscis extension was less than 10 s, it was considered to have no proboscis holding. As expected, both male and female control flies with either *VT041723-GAL4* or *UAS-dTrpA1* transgenes did not show any proboscis holding under any test conditions. The experimental *VT041723-GAL4>UAS-dTrpA1* flies demonstrated varying levels of proboscis holding between sexes. We found that 10.7% of male flies ($N=56$) and 54.5% of mated female flies ($N=66$) showed the proboscis holding response (Fig. 1D,E). As starvation increases the PER response in flies (Dethier, 1976), we next assessed whether *VT041723-GAL4* neuron-activated proboscis holding behavior is modulated by starvation. We tested flies that were starved for 24 h ($N=63$ for males and $N=74$ for females) and found that similar fractions of fed and starved flies exhibited proboscis holding (Fig. 1D,E).

To further investigate the nature of proboscis holding in *VT041723>dTrpA1* flies, we recorded the duration of proboscis holding in fed and starved flies that showed this behavior. For feasibility, we capped measurement of proboscis holding time at 7 min. Our results showed that the average proboscis holding duration was not significantly different between fed and starved flies of the same sex (unpaired *t*-test for males and Mann–Whitney test for females, $P>0.05$). However, mated female flies showed significantly longer times of proboscis holding compared with males in both fed and starved conditions (unpaired *t*-tests, $P<0.05$) (Fig. 1F). In fact, many female flies held the proboscis in the extended position for the maximum recording time (7 min) (Movie 1). Together, our results show that activation of *VT041723-GAL4* neurons induces proboscis holding in a sexually dimorphic manner, with females exhibiting proboscis holding at a higher frequency and for a longer duration.

Optogenetic activation of *VT041723-GAL4* neurons induces a sexually dimorphic partial proboscis holding response

We next verified the role of *VT041723-GAL4* neurons in proboscis holding in an independent optogenetic activation paradigm using a red-shifted channelrhodopsin, *CsChrimson* (Klapoetke et al., 2014). Experimental flies were transferred to food supplemented with ATR for 2–3 days in the dark and tested for behavioral responses with 626 nm red LED stimulation. Consistent with the results of thermogenetic activation experiments (Fig. 1), optogenetic activation of *VT041723-GAL4* neurons resulted in proboscis holding (Fig. 2A; Movie 2). We noted, however, that in most cases the proboscis was not fully extended (partial proboscis holding) by optogenetic activation. Nonetheless, these flies also maintained the partial proboscis holding for up to 7 min under continuous red LED exposure, at which point the trial was completed (see Movie 2). Further, the partial proboscis holding responses were sexually dimorphic; 4.3% of male flies ($N=47$) and 39.1% of mated female flies ($N=69$) exhibited the phenotype (Fig. 2B). Control flies that were not given ATR food (-ATR) showed little if any proboscis holding upon light stimulation ($N=36$ for males and $N=71$ for females).

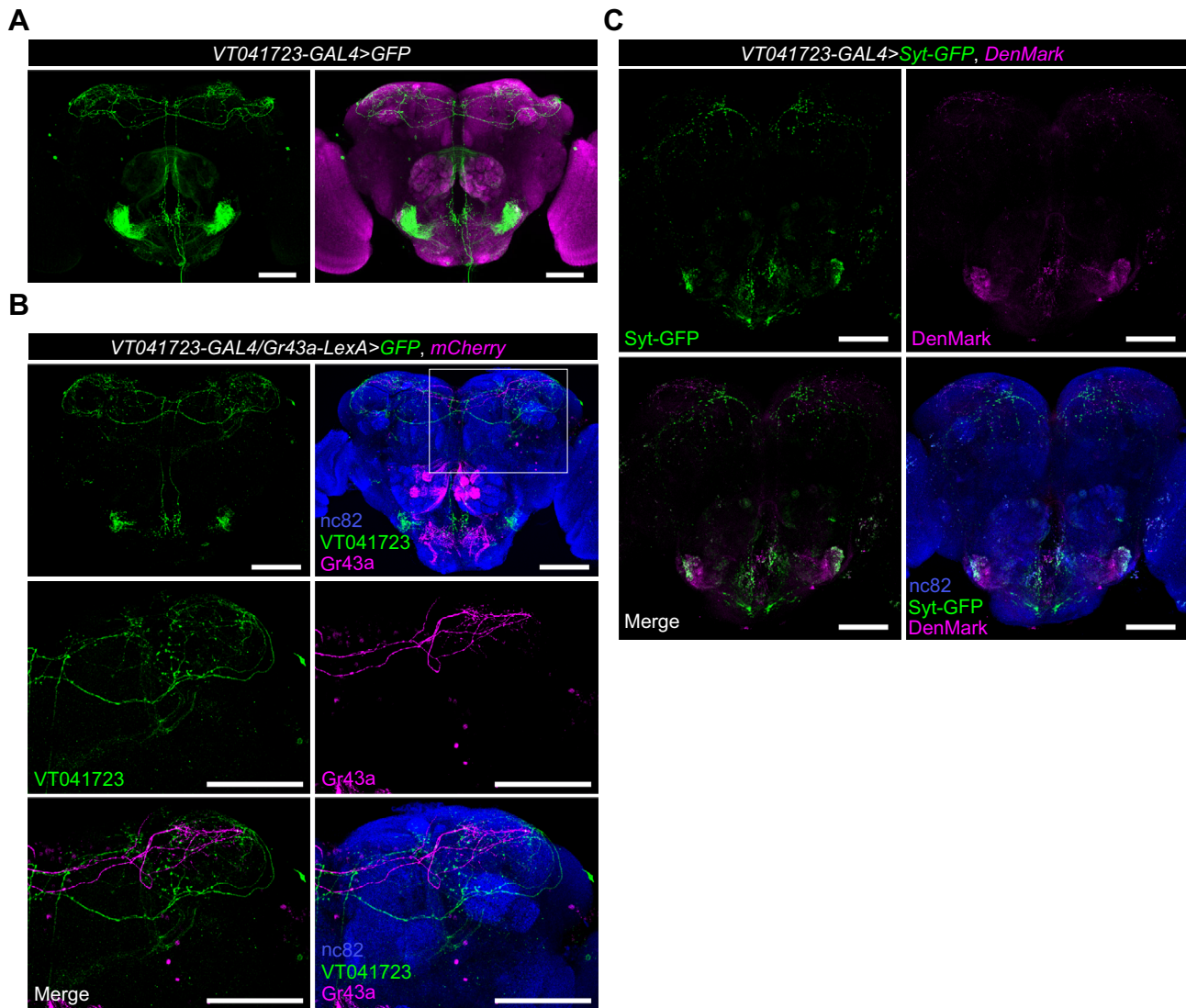


Fig. 3. Neuroanatomical analysis of *VT041723-GAL4* neurons. (A) Expression of a GFP reporter driven by *VT041723-GAL4* in the adult *Drosophila* brain. Neuropil is stained with anti-nc82 (magenta). Scale bars: 100 μ m. (B) GFP and mCherry reporter expression driven by *VT041723-GAL4* and *Gr43a-LexA* (magenta) in the adult *Drosophila* brain. Neuropil is stained with anti-nc82 (blue). The boxed region is enlarged in the images below. Scale bars: 100 μ m. (C) Expression of the pre-synaptic marker Syt-GFP (green) and dendritic marker DenMark (magenta) in *VT041723-GAL4* neurons in the adult *Drosophila* brain. Neuropil is stained with anti-nc82 (blue). Scale bars: 100 μ m.

***VT041723-GAL4* neurons in the dorsolateral protocerebrum and anterior SEZ**

We next examined the expression pattern of *VT041723-GAL4* in the brain using *UAS-GFP*. Similar to the expression pattern described previously (Kvon et al., 2014), we found labeling in neurons that showed dense innervation in the antennal mechanosensory and motor center (AMMC), and some labeled neurites traveling across the midline between the SEZ and the pars intercerebralis regions (Fig. 3A). Some weakly labeled cell bodies were observed within the SEZ. Notably, one pair of neurons in the dorsolateral protocerebrum was strongly labeled, and their anatomical characteristics were reminiscent of previously reported *Gr43a*⁺ fructose-sensing neurons in the brain (Miyamoto et al., 2012). To confirm whether *VT041723-GAL4* labeled *Gr43a*⁺ neurons, we performed double-labeling experiments with two fluorescent reporters driven by *VT041723-GAL4* and *Gr43a-LexA*, respectively (Fig. 3B). We found no overlap between expression of the two reporters, indicating that *VT041723-GAL4* labels a different set of neurons in the brain.

To characterize the neuroanatomy of *VT041723-GAL4* neurons in more detail, we expressed the presynaptic marker Syt-GFP (Zhang et al., 2002) and the postsynaptic marker DenMark (Nicolai et al., 2010) and examined their distribution in the brain (Fig. 3C). We found the Syt-GFP signal was located medially relative to DenMark in the protocerebrum region. Both Syt-GFP and DenMark signals were observed in the AMMC and the SEZ. In the AMMC, DenMark was distributed across the whole neuropil whereas Syt-GFP was confined to the lateral AMMC region. In summary, the *VT041723-GAL4* line labels neurons in the anterior SEZ as well as the dorsolateral protocerebrum of the fly brain, consistent with a role in controlling proboscis extension and holding.

Post-consumption activation of *VT041723-GAL4* neurons induces regurgitation

We next aimed to determine whether the *VT041723-GAL4*-activated proboscis holding phenotype is modulated by prior feeding experience. To test this possibility, we starved the

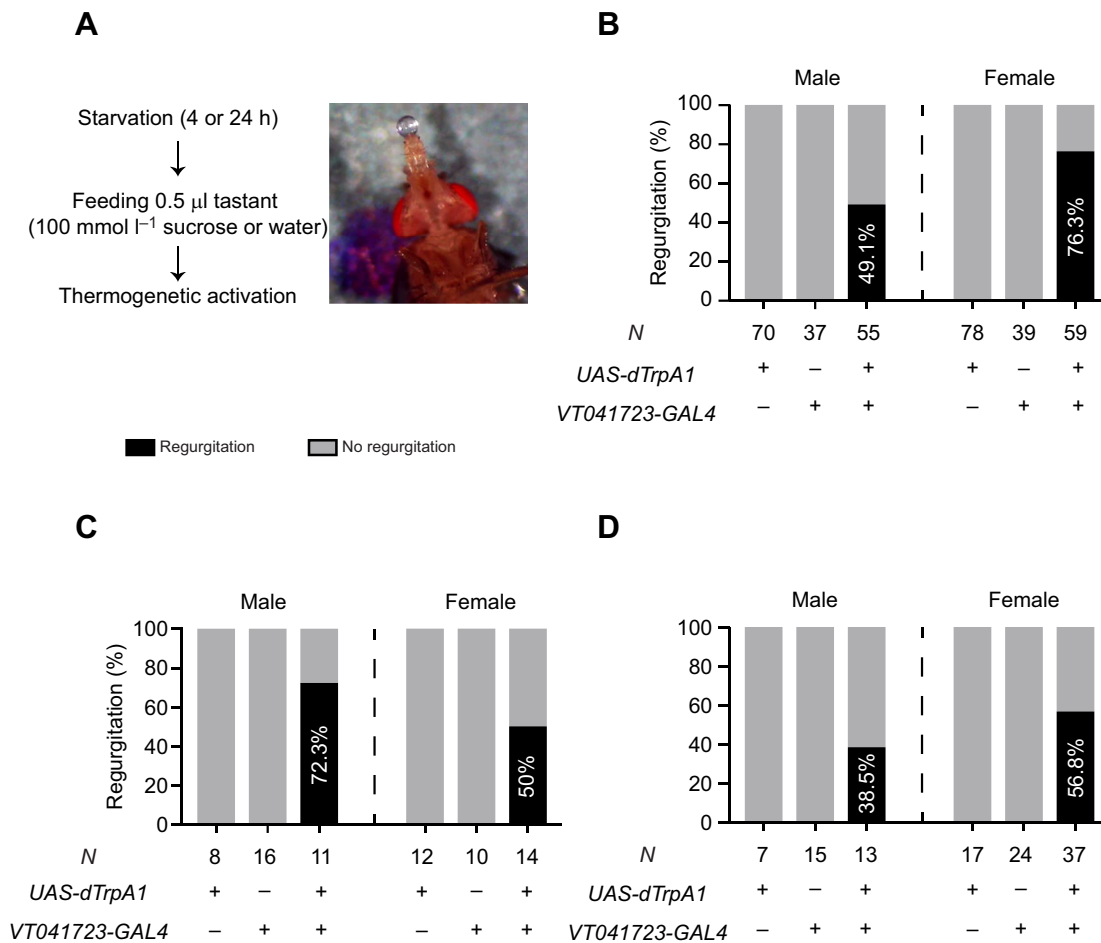


Fig. 4. Thermogenetic activation of VT041723-GAL4 neurons induces regurgitation after ingestion. (A) Summary of the experimental procedure for the regurgitation assays (left), and an image of a fly showing regurgitation (right; see Movie 3). (B,C) Distribution of phenotypes upon heat activation after 100 mmol l⁻¹ sucrose feeding. No significant difference was observed between flies with 24 h (B) and 4 h starvation (C) prior to sucrose feeding. *N*=8–78. (D) Distribution of phenotypes upon heat activation after water ingestion following 24 h starvation on dry tissues. *N*=7–37. In all experiments, UAS and GAL4 controls were tested in parallel with experimental flies, and experimenters were blind to genotype. No regurgitation was seen in any of the transgenic control flies.

VT041723-GAL4>UAS-dTrpA1 flies for 24 h and then pre-fed the flies with a fixed amount of 100 mmol l⁻¹ sucrose (0.5 μl) immediately before transferring them to the 31°C heat block for thermogenetic activation (Fig. 4A; Movie 3). Surprisingly, we found that half of male (49.1%) and more than half of mated female (76.3%) flies exhibited regurgitation (Fig. 4B), which was apparent by the formation of a liquid bubble at the tip of the proboscis (Fig. 4A). In addition, about 10% of the flies showed proboscis holding without regurgitation. These results suggest that activation of VT041723-GAL4 neurons conveys an aversive signal that causes regurgitation of an ingested meal.

Regurgitation mediated by VT041723-GAL4 neurons is independent of starvation state and meal quality

We next asked whether starvation time affects the regurgitation phenotype. For this purpose, we performed mild starvation (4 h) before pre-feeding flies with 0.5 μl of 100 mmol l⁻¹ sucrose. Similar to the results obtained with 24 h starvation, we found more than half of the male (72.3%) and half of mated female (50%) flies exhibited regurgitation upon activation of VT041723-GAL4 neurons (Fig. 4C). In addition, regurgitation behavior was also observed when flies were pre-fed with 0.5 μl of water after starvation on dry tissue paper, suggesting that the observed behavioral response is independent of tastants in the pre-fed meal (Fig. 4D). Thus,

VT041723-GAL4-induced regurgitation of a meal appears to be independent of starvation state and meal quality.

VT041723-GAL4 neurons have synaptic proximity with pharyngeal gustatory receptor neurons

We next investigated the possibility that VT041723-GAL4 neurons may be part of taste circuits by performing GRASP experiments (Fan et al., 2013). We first examined the expression of both VT041723-GAL4 and *Ir76b-LexA* in the fly brain. *Ir76b-LexA* labels some olfactory neuronal projections in the antennal lobes as well as projections in the SEZ from many gustatory receptor neurons (GRNs) from different taste organs, including those in labellar taste hairs, labellar taste pegs, pharynx and legs (Ganguly et al., 2017; Zhang et al., 2013; Hussain et al., 2016; Chen and Dahanukar, 2017; Steck et al., 2018; Jaeger et al., 2018; Chen and Amrein, 2017; Ahn et al., 2017). We found that neurites of VT041723-GAL4 neurons and *Ir76b*⁺ pharyngeal GRNs appeared to be in close proximity to each other in the SEZ (Fig. 5A). We then performed a GRASP experiment by expressing split GFP1-10 fused with a transmembrane protein involved in synapse formation (Knight et al., 2011), neuroligin, in VT041723-GAL4 neurons, and split GFP11 fused with CD4 in *Ir76b*⁺ neurons. We stained the neuropil using anti-nc82 and visualized direct GFP fluorescence. Controls lacking either VT041723-GAL4 or *Ir76b-LexA* did not show any GFP signal.

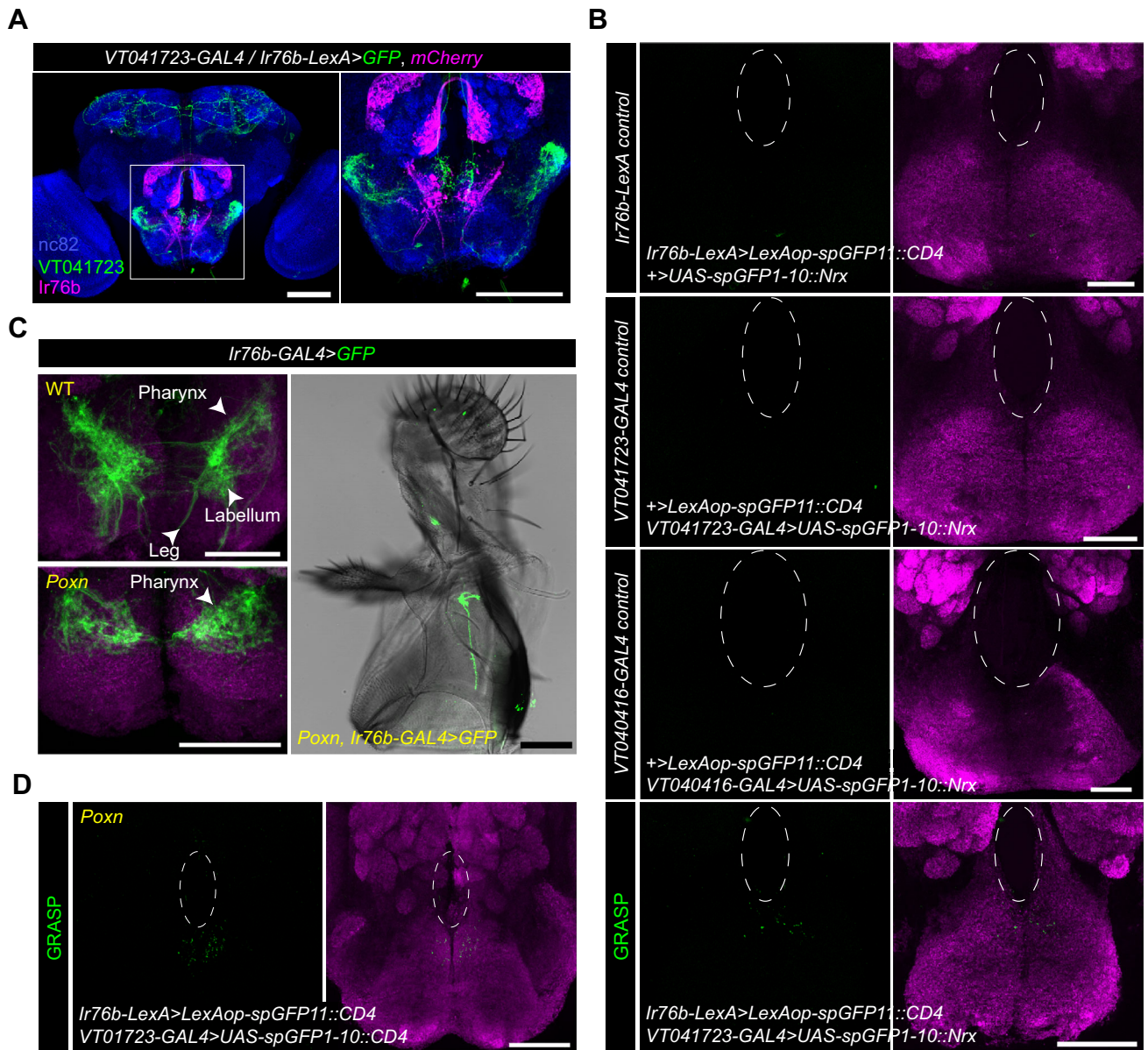


Fig. 5. *VT041723-GAL4* neurons show a GFP reconstitution across synaptic partners (GRASP) signal with *Ir76b*⁺ pharyngeal gustatory receptor neurons (GRNs). (A) GFP and mCherry reporter expression driven by *VT041723-GAL4* (green) and *Ir76b-LexA* (magenta) in the adult *Drosophila* brain. The boxed region in the left image is enlarged and shown on the right. Neuropil is stained with anti-nc82 (blue). Scale bars: 100 μm. (B) GRASP signal (green) in the brains of flies with the indicated transgenes. Neuropil is stained with anti-nc82 (blue). Dashed line outlines the region of the esophagus. Scale bars: 50 μm. (C) Left: images of the subesophageal zone (SEZ) showing axonal termini (green) labeled by *Ir76b-GAL4>GFP* in wild-type (WT, *w¹¹¹⁸*) and *Poxn* (*Poxn^{ΔM22-B5}/Poxn⁷⁰*) flies. Scale bars: 50 μm. Right: brightfield images of the proboscis showing GRNs (green) labeled by *Ir76b-GAL4>GFP* in the pharynx and a few taste pegs in a *Poxn* mutant background. Scale bar: 100 μm. (D) GRASP signal (green) in the brain of a *Poxn* mutant fly with indicated transgenes. Scale bar: 50 μm.

A different candidate line from our screen (Fig. 1A,B), *VT040416-GAL4*, that labels extensive neurite arborization in the SEZ (Fig. S1), also did not show any positive GRASP signal with *Ir76b*⁺ GRNs. Notably, we observed reconstitution of GFP fluorescence in the SEZ when *VT041723-GAL4* and *Ir76b-LexA* were used to express the two split GFP components (Fig. 5B), suggesting that termini of *VT041723-GAL4* neurons are in close proximity with those of *Ir76b-LexA* GRNs, and may receive taste input from *Ir76b*⁺ neurons.

One previous study showed that thermogenetic activation of *Gr66a*-expressing taste neurons in the mouthpart caused regurgitation (Kang et al., 2011), which raised the possibility that *VT041723-GAL4* neurons receive input from pharyngeal *Gr66a*⁺

GRNs. To test this possibility, we used *Poxn-neuro* (*Poxn*) mutants in which all external taste hairs are transformed into mechanosensory hairs, leaving pharyngeal taste neurons intact (Chen et al., 2018; Chen and Dahanukar, 2017; Ledue et al., 2015). Consistent with our previous report (Chen and Dahanukar, 2017), *Poxn* mutants retained *Ir76b*⁺ projections from the pharynx and a few taste pegs, while lacking projections from all external taste organs. GRASP experiments in a *Poxn* mutant background revealed a positive GRASP signal between *VT041723-GAL4* and *Ir76b-LexA* GRNs in the SEZ (Fig. 5D). The results support the idea that *VT041723-GAL4* neurons receive taste input from pharyngeal GRNs and regulate regurgitation.

DISCUSSION

Knowledge about how neural circuits are wired in the brain is crucial for understanding how sensory information is translated into behavior. In *Drosophila*, higher-order brain regions that process olfactory information, such as the lateral horn and mushroom body, have been described in detail (Dolan et al., 2019; Jefferis et al., 2007; Marin et al., 2002; Wong et al., 2002), but much less is known about processing of gustatory information after the first relay in the SEZ, with reports of only a few central neurons that have been anatomically or functionally characterized (Bohra et al., 2018; Kim et al., 2017; Yapici et al., 2016; Miyazaki et al., 2015; Kain and Dahanukar, 2015; Flood et al., 2013). In this study, we identified that activation of *VT041723-GAL4*-labeled neurons causes proboscis holding and regurgitation behavior in adult *Drosophila*.

Proboscis extension has been characterized as an appetitive behavioral response and is widely used as a read-out of food acceptance (Shiraiwa and Carlson, 2007). Several previous reports have shown that activation of external sweet taste neurons via *Gr5a-GAL4* causes proboscis extension (Inagaki et al., 2012; Inagaki et al., 2014a; Dawydow et al., 2014; Du et al., 2016; Kain and Dahanukar, 2015; Yapici et al., 2016; Keene and Masek, 2012). Under these conditions, flies usually exhibit proboscis extensions followed by quick retractions. As activation of *VT041723-GAL4* neurons resulted in a single extension without retraction for the duration of the assay, we considered that it may not be indicative of an appetitive response but rather that it represented an aversive response. Consistent with this idea, post-consumption activation of *VT041723-GAL4* neurons induced regurgitation, similar to that observed in flies with stimulation of deterrent taste neurons (Kang et al., 2011) or with overconsumption (Pool et al., 2014). However, *VT041723-GAL4* neurons induced regurgitation that was often accompanied by proboscis holding, and sustained proboscis extension is typically observed only when the fly is actively ingesting. We cannot, therefore, exclude the possibility that proboscis holding and regurgitation are controlled by different subsets of *VT041723-GAL4* neurons. Alternatively, proboscis holding may be a common feature of feeding and regurgitation behaviors.

In this study, we found that the frequency of proboscis holding behavior is strikingly higher in females than in males. In *Drosophila*, *doublesex (dsx)* and *fruitless (fru)* are known as sex-determining transcription factors that specify sexually dimorphic neuronal circuits and behaviors (Erdman and Burtis, 1993; Ito et al., 1996; Ryner et al., 1996; Auer and Benton, 2016; Asahina, 2018). Although we found no sexual dimorphism in the pattern of *VT041723-GAL4* expression in the brain (data not shown), a closer look at the expression of sex-specific *fru* and *dsx* in *VT041723-GAL4* neurons would provide further insight into possible mechanisms underlying sexual dimorphism. In addition, sex-specific differences in feeding responses to salt (Walker et al., 2015), yeast (Ribeiro and Dickson, 2010), amino acids (Ganguly et al., 2017) and sugars (Chandegra et al., 2017) have been reported. Given the possibility of functional connectivity between *VT041723-GAL4* neurons and peripheral taste neurons, it will be of interest to determine whether specific gustatory input is involved in sex-dependent variation in the proboscis holding phenotype. Moreover, as the sexual difference is lost when flies are pre-fed with either water or sucrose and tested in thermogenetic activation experiments, it appears that prior feeding experience differentially influences the proboscis holding phenotype in males and females.

VT041723-GAL4 labels multiple neurons that can be largely separated into two anatomical groups, one near the dorsolateral protocerebrum and a second around the SEZ with extensive neurite

arborization in the AMMC. Although our study did not identify which of the two populations is involved in regurgitation behavior, GRASP experiments implicate the latter, which are poised to receive input from pharyngeal *Ir76b*⁺ GRNs, which encompass *Gr66a*⁺ GRNs in the number 8 and 9 sensilla of the labral sense organ (LSO) (Chen and Dahanukar, 2017) that induce regurgitation (Kang et al., 2011). *Gr66a* is broadly expressed in many bitter taste neurons and mediates feeding avoidance of various aversive compounds (Weiss et al., 2011; Moon et al., 2006; Marella et al., 2006; Wang et al., 2004; Thorne et al., 2004). It is plausible, therefore, that pharyngeal *Gr66a*⁺ GRNs act as a final checkpoint for food consumption and sense cues that induce regurgitation of unsavory meals via activation of *VT041723-GAL4* neurons.

PER requires precise coordination of various motor programs, including rostrum lifting, haustellum extension, labella extension and labella spreading. Recently, motoneurons controlling the individual motor sequence of the PER have been described at the single-cell level (Schwarz et al., 2017). However, motor circuits controlling regurgitation have not been explored and, consequently, little is known about whether PER and regurgitation share common motor programs. Based on our observations, we posit that *VT041723-GAL4* neurons provide a good starting point to address such questions. Future experiments using genetic intersectional strategies may identify the minimum subset of *VT041723-GAL4* neurons that are required for regurgitation behavior. Overall, our results lay the groundwork to analyze a simple behavior and the neuronal circuits and conditions that control it.

Acknowledgements

We would like to thank members of the Dahanukar laboratory for useful comments on the manuscript, and Sen Miao for her help with the initial thermogenetic screen.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.-C.D.C., A.D.; Methodology: Y.-C.D.C., A.D.; Validation: Y.-C.D.C., S.A., K.A.; Formal analysis: Y.-C.D.C.; Investigation: Y.-C.D.C., S.A., K.A.; Writing - original draft: Y.-C.D.C.; Writing - review & editing: Y.-C.D.C., S.A., K.A., A.D.; Visualization: Y.-C.D.C.; Supervision: A.D.; Funding acquisition: A.D.

Funding

This work was funded by grants from the National Institutes of Health (NIH R01DC013587) and National Science Foundation (IOS1149667), and funds from the University of California AES Mission Funding program to A.D. Y.-C.D.C. is a Howard Hughes Medical Institute International Student Research Fellow. Stocks were also obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537). Deposited in PMC for release after 12 months.

Data availability

Data are available from the Mendeley data repository: <https://dx.doi.org/10.17632/yvy34k52t9.1>

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.210724.supplemental>

References

- Ahn, J.-E., Chen, Y. and Amrein, H. (2017). Molecular basis of fatty acid taste in *Drosophila*. *eLife* **6**, e30115. doi:10.7554/eLife.30115
- Asahina, K. (2018). Sex differences in *Drosophila* behavior: qualitative and quantitative dimorphism. *Curr. Opin. Physiol.* **6**, 35-45. doi:10.1016/j.cophys.2018.04.004
- Auer, T. O. and Benton, R. (2016). Sexual circuitry in *Drosophila*. *Curr. Opin. Neurobiol.* **38**, 18-26. doi:10.1016/j.conb.2016.01.004
- Awasaki, T. and Kimura, K. (1997). *pox-neuro* is required for development of chemosensory bristles in *Drosophila*. *J. Neurobiol.* **32**, 707-721. doi:10.1002/(SICI)1097-4695(19970620)32:7<707::AID-NEU6>3.0.CO;2-8

- Bohra, A. A., Kallman, B. R., Reichert, H. and Vijayraghavan, K.** (2018). Identification of a single pair of interneurons for bitter taste processing in the *Drosophila* brain. *Curr. Biol.* **28**, 847-858.e3. doi:10.1016/j.cub.2018.01.084
- Boll, W. and Noll, M.** (2002). The *Drosophila Pox neuro* gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. *Development* **129**, 5667-5681. doi:10.1242/dev.00157
- Brand, A. H. and Perrimon, N.** (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
- Chandegra, B., Tang, J. L. Y., Chi, H. and Alic, N.** (2017). Sexually dimorphic effects of dietary sugar on lifespan, feeding and starvation resistance in *Drosophila*. *Aging (Albany NY)* **9**, 2521-2528. doi:10.18632/aging.101335
- Chen, Y. and Amrein, H.** (2017). Ionotropic receptors mediate *Drosophila* oviposition preference through sour gustatory receptor neurons. *Curr. Biol.* **27**, 2741-2750.e4. doi:10.1016/j.cub.2017.08.003
- Chen, Y.-C. D. and Dahanukar, A.** (2017). Molecular and cellular organization of taste neurons in adult *Drosophila* pharynx. *Cell Rep.* **21**, 2978-2991. doi:10.1016/j.celrep.2017.11.041
- Chen, Y.-C. D., Park, S. J., Ja, W. W. and Dahanukar, A.** (2018). Using *Pox-neuro* (*Poxn*) mutants in *Drosophila* gustation research: a double-edged sword. *Front. Cell Neurosci.* **12**, 382. doi:10.3389/fncel.2018.00382
- Clyne, P. J., Warr, C. G. and Carlson, J. R.** (2000). Candidate taste receptors in *Drosophila*. *Science* **287**, 1830-1834. doi:10.1126/science.287.5459.1830
- Dawydow, A., Gueta, R., Ljaschenko, D., Ullrich, S., Hermann, M., Ehmann, N., Gao, S., Fiala, A., Langenhan, T., Nagel, G. et al.** (2014). Channelrhodopsin-2-XXL, a powerful optogenetic tool for low-light applications. *Proc. Natl. Acad. Sci. USA* **111**, 13972-13977. doi:10.1073/pnas.1408269111
- Deshpande, S. A., Carvalho, G. B., Amador, A., Phillips, A. M., Hoxha, S., Lizotte, K. J. and Ja, W. W.** (2014). Quantifying *Drosophila* food intake: comparative analysis of current methodology. *Nat. Methods* **11**, 535-540. doi:10.1038/nmeth.2899
- Dethier, V. G.** (1976). *The Hungry Fly: A Physiological Study of the Behavior Associated with Feeding*. Cambridge, MA: Harvard University Press.
- Diegelmann, S., Jansen, A., Jois, S., Kastenholz, K., Velo Escarcena, L., Strudthoff, N. and Scholz, H.** (2017). The capillary feeder assay measures food intake in *Drosophila melanogaster*. *J. Vis. Exp.* **121**, e55024. doi:10.3791/55024
- Dolan, M.-J., Frechter, S., Bates, A. S., Dan, C., Huovalia, P., Roberts, R. J. V., Schlegel, P., Dhawan, S., Tabano, R., Dionne, H. et al.** (2019). Neurogenetic dissection of the *Drosophila* lateral horn reveals major outputs, diverse behavioural functions, and interactions with the mushroom body. *eLife* **8**, e43079. doi:10.7554/eLife.43079
- Du, E. J., Ahn, T. J., Wen, X., Seo, D.-W., Na, D. L., Kwon, J. Y., Choi, M., Kim, H.-W., Cho, H. and Kang, K.** (2016). Nucleophile sensitivity of *Drosophila* TRPA1 underlies light-induced feeding deterrence. *eLife* **5**, e18425. doi:10.7554/eLife.18425
- Erdman, S. E. and Burtis, K. C.** (1993). The *Drosophila* doublesex proteins share a novel zinc finger related DNA binding domain. *EMBO J.* **12**, 527-535. doi:10.1002/j.1460-2075.1993.tb05684.x
- Fan, P., Manoli, D. S., Ahmed, O. M., Chen, Y., Agarwal, N., Kwong, S., Cai, A. G., Neitz, J., Renslo, A., Baker, B. S. et al.** (2013). Genetic and neural mechanisms that inhibit *Drosophila* from mating with other species. *Cell* **154**, 89-102. doi:10.1016/j.cell.2013.06.008
- Flood, T. F., Iguchi, S., Gorczyca, M., White, B., Ito, K. and Yoshihara, M.** (2013). A single pair of interneurons commands the *Drosophila* feeding motor program. *Nature* **499**, 83-87. doi:10.1038/nature12208
- Ganguly, A., Pang, L., Duong, V.-K., Lee, A., Schoniger, H., Varady, E. and Dahanukar, A.** (2017). A molecular and cellular context-dependent role for Ir76b in detection of amino acid taste. *Cell Rep.* **18**, 737-750. doi:10.1016/j.celrep.2016.12.071
- Gordon, M. D. and Scott, K.** (2009). Motor control in a *Drosophila* taste circuit. *Neuron* **61**, 373-384. doi:10.1016/j.neuron.2008.12.033
- He, Z., Luo, Y., Shang, X., Sun, J. S. and Carlson, J. R.** (2019). Chemosensory sensilla of the *Drosophila* wing express a candidate ionotropic pheromone receptor. *PLoS Biol.* **17**, e2006619. doi:10.1371/journal.pbio.2006619
- Hussain, A., Zhang, M., Üçpunar, H. K., Svensson, T., Quillery, E., Gompel, N., Ignell, R. and Grunwald Kadov, I. C.** (2016). Ionotropic chemosensory receptors mediate the taste and smell of polyamines. *PLoS Biol.* **14**, e1002454. doi:10.1371/journal.pbio.1002454
- Inagaki, H. K., Ben-Tabou De-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R. and Anderson, D. J.** (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* **148**, 583-595. doi:10.1016/j.cell.2011.12.022
- Inagaki, H. K., Jung, Y., Hoopfer, E. D., Wong, A. M., Mishra, N., Lin, J. Y., Tsien, R. Y. and Anderson, D. J.** (2014a). Optogenetic control of *Drosophila* using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. *Nat. Methods* **11**, 325-332. doi:10.1038/nmeth.2765
- Inagaki, H. K., Panse, K. M. and Anderson, D. J.** (2014b). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in *Drosophila*. *Neuron* **84**, 806-820. doi:10.1016/j.neuron.2014.09.032
- Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S. and Yamamoto, D.** (1996). Sexual orientation in *Drosophila* is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. *Proc. Natl. Acad. Sci. USA* **93**, 9687-9692. doi:10.1073/pnas.93.18.9687
- Itskov, P. M., Moreira, J.-M., Vinnik, E., Lopes, G., Safarik, S., Dickinson, M. H. and Ribeiro, C.** (2014). Automated monitoring and quantitative analysis of feeding behaviour in *Drosophila*. *Nat. Commun.* **5**, 4560. doi:10.1038/ncomms5560
- Ja, W. W., Carvalho, G. B., Mak, E. M., De La Rosa, N. N., Fang, A. Y., Liong, J. C., Brummel, T. and Benzer, S.** (2007). Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. USA* **104**, 8253-8256. doi:10.1073/pnas.0702726104
- Jaeger, A. H., Stanley, M., Weiss, Z. F., Musso, P.-Y., Chan, R. C. W., Zhang, H., Feldman-Kiss, D. and Gordon, M. D.** (2018). A complex peripheral code for salt taste in *Drosophila*. *eLife* **7**, e37167. doi:10.7554/eLife.37167
- Jefferis, G. S. X. E., Potter, C. J., Chan, A. M., Marin, E. C., Rohlfing, T., Maurer, C. R., Jr and Luo, L.** (2007). Comprehensive maps of *Drosophila* higher olfactory centers: spatially segregated fruit and pheromone representation. *Cell* **128**, 1187-1203. doi:10.1016/j.cell.2007.01.040
- Jenett, A., Rubin, G. M., Ngo, T.-T. B., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B. D., Cavallaro, A., Hall, D., Jeter, J. et al.** (2012). A GAL4-driver line resource for *Drosophila* neurobiology. *Cell Rep.* **2**, 991-1001. doi:10.1016/j.celrep.2012.09.011
- Kain, P. and Dahanukar, A.** (2015). Secondary taste neurons that convey sweet taste and starvation in the *Drosophila* brain. *Neuron* **85**, 819-832. doi:10.1016/j.neuron.2015.01.005
- Kang, K., Panzano, V. C., Chang, E. C., Ni, L., Dainis, A. M., Jenkins, A. M., Regna, K., Muskavitch, M. A. T. and Garrity, P. A.** (2011). Modulation of TRPA1 thermal sensitivity enables sensory discrimination in *Drosophila*. *Nature* **481**, 76-80. doi:10.1038/nature10715
- Keene, A. C. and Masek, P.** (2012). Optogenetic induction of aversive taste memory. *Neuroscience* **222**, 173-180. doi:10.1016/j.neuroscience.2012.07.028
- Kim, H., Kirkhart, C. and Scott, K.** (2017). Long-range projection neurons in the taste circuit of *Drosophila*. *Elife* **6**, e23386. doi:10.7554/eLife.23386
- Klapoetke, N. C., Murata, Y., Kim, S. S., Pulver, S. R., Birdsey-Benson, A., Cho, Y. K., Morimoto, T. K., Chuong, A. S., Carpenter, E. J., Tian, Z. et al.** (2014). Independent optical excitation of distinct neural populations. *Nat. Methods* **11**, 338-346. doi:10.1038/nmeth.2836
- Knight, D., Xie, W. and Boulianne, G. L.** (2011). Neurexins and neuroligins: recent insights from invertebrates. *Mol. Neurobiol.* **44**, 426-440. doi:10.1007/s12035-011-8213-1
- Kvon, E. Z., Kazmar, T., Stampfel, G., Yáñez-Cuna, J. O., Pagani, M., Schernhuber, K., Dickson, B. J. and Stark, A.** (2014). Genome-scale functional characterization of *Drosophila* developmental enhancers in vivo. *Nature* **512**, 91-95. doi:10.1038/nature13395
- Kwon, J. Y., Dahanukar, A., Weiss, L. A. and Carlson, J. R.** (2014). A map of taste neuron projections in the *Drosophila* CNS. *J. Biosci.* **39**, 565-574. doi:10.1007/s12038-014-9448-6
- Ledue, E. E., Chen, Y.-C., Jung, A. Y., Dahanukar, A. and Gordon, M. D.** (2015). Pharyngeal sense organs drive robust sugar consumption in *Drosophila*. *Nat. Commun.* **6**, 6667. doi:10.1038/ncomms7667
- Ledue, E. E., Mann, K., Koch, E., Chu, B., Dakin, R. and Gordon, M. D.** (2016). Starvation-induced depotentiation of bitter taste in *Drosophila*. *Curr. Biol.* **26**, 2854-2861. doi:10.1016/j.cub.2016.08.028
- Ling, F., Dahanukar, A., Weiss, L. A., Kwon, J. Y. and Carlson, J. R.** (2014). The molecular and cellular basis of taste coding in the legs of *Drosophila*. *J. Neurosci.* **34**, 7148-7164. doi:10.1523/JNEUROSCI.0649-14.2014
- Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E. and Scott, K.** (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. *Neuron* **49**, 285-295. doi:10.1016/j.neuron.2005.11.037
- Marin, E. C., Jefferis, G. S. X. E., Komiyama, T., Zhu, H. and Luo, L.** (2002). Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell* **109**, 243-255. doi:10.1016/S0092-8674(02)00700-6
- Milyaev, N., Osumi-Sutherland, D., Reeve, S., Burton, N., Baldock, R. A. and Armstrong, J. D.** (2012). The Virtual Fly Brain browser and query interface. *Bioinformatics* **28**, 411-415. doi:10.1093/bioinformatics/btr677
- Miyamoto, T. and Amrein, H.** (2014). Diverse roles for the *Drosophila* fructose sensor Gr43a. *Fly (Austin)* **8**, 19-25. doi:10.4161/fly.27241
- Miyamoto, T., Slone, J., Song, X. and Amrein, H.** (2012). A fructose receptor functions as a nutrient sensor in the *Drosophila* brain. *Cell* **151**, 1113-1125. doi:10.1016/j.cell.2012.10.024
- Miyazaki, T., Lin, T.-Y., Ito, K., Lee, C.-H. and Stopfer, M.** (2015). A gustatory second-order neuron that connects sucrose-sensitive primary neurons and a distinct region of the gnathal ganglion in the *Drosophila* brain. *J. Neurogenet.* **29**, 144-155. doi:10.3109/01677063.2015.1054993
- Moon, S. J., Köttgen, M., Jiao, Y., Xu, H. and Montell, C.** (2006). A taste receptor required for the caffeine response in vivo. *Curr. Biol.* **16**, 1812-1817. doi:10.1016/j.cub.2006.07.024
- Moreira, J.-M., Itskov, P. M., Goldschmidt, D., Baltazar, C., Steck, K., Tastekin, I., Walker, S. J. and Ribeiro, C.** (2019). optoPAD, a closed-loop optogenetics

- system to study the circuit basis of feeding behaviors. *eLife* **8**, e43924. doi:10.7554/eLife.43924
- Murphy, K. R., Park, J. H., Huber, R. and Ja, W. W.** (2017). Simultaneous measurement of sleep and feeding in individual *Drosophila*. *Nat. Protoc.* **12**, 2355-2366. doi:10.1038/nprot.2017.096
- Nicolai, L. J. J., Ramaekers, A., Raemaekers, T., Drozdzecki, A., Mauss, A. S., Yan, J., Landgraf, M., Annaert, W. and Hassan, B. A.** (2010). Genetically encoded dendritic marker sheds light on neuronal connectivity in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **107**, 20553-20558. doi:10.1073/pnas.1010198107
- Park, A., Tran, T. and Atkinson, N. S.** (2018). Monitoring food preference in *Drosophila* by oligonucleotide tagging. *Proc. Natl. Acad. Sci. USA* **115**, 9020-9025. doi:10.1073/pnas.1716880115
- Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T.-T. B., Misra, S., Murphy, C., Scully, A., Carlson, J. W., Wan, K. H., Lavery, T. R. et al.** (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 9715-9720. doi:10.1073/pnas.0803697105
- Pool, A.-H., Kvello, P., Mann, K., Cheung, S. K., Gordon, M. D., Wang, L. and Scott, K.** (2014). Four GABAergic interneurons impose feeding restraint in *Drosophila*. *Neuron* **83**, 164-177. doi:10.1016/j.neuron.2014.05.006
- Raad, H., Ferveur, J.-F., Ledger, N., Capovilla, M. and Robichon, A.** (2016). Functional gustatory role of chemoreceptors in *Drosophila* wings. *Cell Rep.* **15**, 1442-1454. doi:10.1016/j.celrep.2016.04.040
- Ribeiro, C. and Dickson, B. J.** (2010). Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in *Drosophila*. *Curr. Biol.* **20**, 1000-1005. doi:10.1016/j.cub.2010.03.061
- Ro, J., Harvanek, Z. M. and Pletcher, S. D.** (2014). FLIC: high-throughput, continuous analysis of feeding behaviors in *Drosophila*. *PLoS One* **9**, e101107. doi:10.1371/journal.pone.0101107
- Ryner, L. C., Goodwin, S. F., Castrillon, D. H., Anand, A., Vilella, A., Baker, B. S., Hall, J. C., Taylor, B. J. and Wasserman, S. A.** (1996). Control of male sexual behavior and sexual orientation in *Drosophila* by the fruitless gene. *Cell* **87**, 1079-1089. doi:10.1016/S0092-8674(00)81802-4
- Schwarz, O., Bohra, A. A., Liu, X., Reichert, H., Vijayraghavan, K. and Pielage, J.** (2017). Motor control of *Drosophila* feeding behavior. *Elife* **6**, e19892. doi:10.7554/eLife.19892
- Scott, K., Brady, R., Jr, Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C. and Axel, R.** (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* **104**, 661-673. doi:10.1016/S0092-8674(01)00263-X
- Shell, B. C., Schmitt, R. E., Lee, K. M., Johnson, J. C., Chung, B. Y., Pletcher, S. D. and Grotewiel, M.** (2018). Measurement of solid food intake in *Drosophila* via consumption-excretion of a dye tracer. *Sci. Rep.* **8**, 11536. doi:10.1038/s41598-018-29813-9
- Shiraiwa, T. and Carlson, J. R.** (2007). Proboscis extension response (PER) assay in *Drosophila*. *J. Vis. Exp.* **3**, e193. doi:10.3791/193
- Steck, K., Walker, S. J., Itskov, P. M., Baltazar, C., Moreira, J.-M. and Ribeiro, C.** (2018). Internal amino acid state modulates yeast taste neurons to support protein homeostasis in *Drosophila*. *eLife* **7**, e31625. doi:10.7554/eLife.31625
- Thorne, N., Chromey, C., Bray, S. and Amrein, H.** (2004). Taste perception and coding in *Drosophila*. *Curr. Biol.* **14**, 1065-1079. doi:10.1016/j.cub.2004.05.019
- Tirian, L. and Dickson, B. J.** (2017). The VT GAL4, LexA, and split-GAL4 driver line collections for targeted expression in the *Drosophila* nervous system. bioRxiv. doi:https://doi.org/10.1101/198648
- Walker, S. J., Corrales-Carvajal, V. M. and Ribeiro, C.** (2015). Postmating circuitry modulates salt taste processing to increase reproductive output in *Drosophila*. *Curr. Biol.* **25**, 2621-2630. doi:10.1016/j.cub.2015.08.043
- Wang, Z., Singhvi, A., Kong, P. and Scott, K.** (2004). Taste representations in the *Drosophila* brain. *Cell* **117**, 981-991. doi:10.1016/j.cell.2004.06.011
- Weiss, L. A., Dahanukar, A., Kwon, J. Y., Banerjee, D. and Carlson, J. R.** (2011). The molecular and cellular basis of bitter taste in *Drosophila*. *Neuron* **69**, 258-272. doi:10.1016/j.neuron.2011.01.001
- Wong, A. M., Wang, J. W. and Axel, R.** (2002). Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* **109**, 229-241. doi:10.1016/S0092-8674(02)00707-9
- Yapici, N., Cohn, R., Schusterreiter, C., Ruta, V. and Vosshall, L. B.** (2016). A taste circuit that regulates ingestion by integrating food and hunger signals. *Cell* **165**, 715-729. doi:10.1016/j.cell.2016.02.061
- Youn, H., Kirkhart, C., Chia, J. and Scott, K.** (2018). A subset of octopaminergic neurons that promotes feeding initiation in *Drosophila melanogaster*. *PLoS ONE* **13**, e0198362. doi:10.1371/journal.pone.0198362
- Zhang, Y. Q., Rodesch, C. K. and Broadie, K.** (2002). Living synaptic vesicle marker: synaptotagmin-GFP. *Genesis* **34**, 142-145. doi:10.1002/gene.10144
- Zhang, Y. V., Ni, J. and Montell, C.** (2013). The molecular basis for attractive salt-taste coding in *Drosophila*. *Science* **340**, 1334-1338. doi:10.1126/science.1234133

SUPPLEMENTARY FIGURES

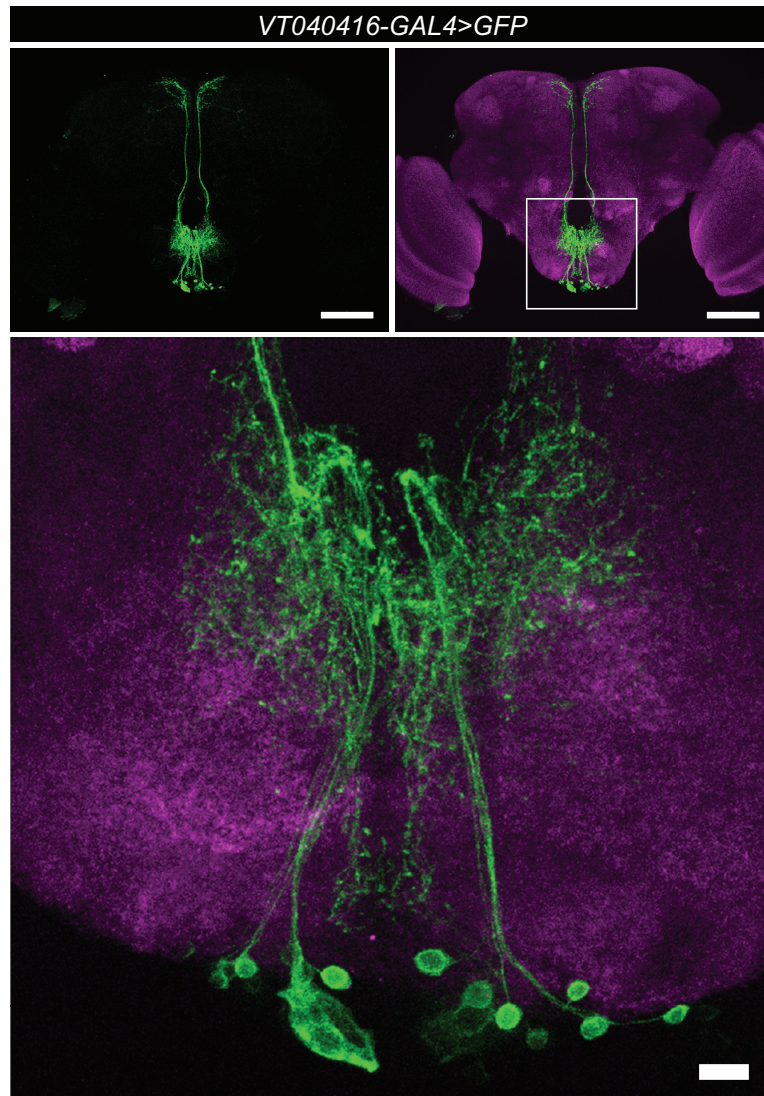


Fig. S1. Expression of *VT040416-GAL4* in the brain. (A) GFP expression in the adult *Drosophila* brain of *VT040416-GAL4>GFP* flies. Neuropil is stained with anti-nc82 (magenta). Scale bar: 100 μm (top), 10 μm (bottom).

SUPPLEMENTARY TABLE**Table S1. Summary of PER results in the thermogenetic activation screen with 194 selected *VT-GAL4* and *Janelia-GAL4* lines.**

Line number	Stock number	Inserted construct	PER (%)
1	BDSC 48723	GMR16D02-GAL4	0
2	BDSC 39332	GMR65A10-GAL4	0
3	BDSC 48522	GMR12G03-GAL4	0
4	BDSC 49238	GMR10G07-GAL4	0
5	BDSC 48891	GMR20D04-GAL4	0
6	BDSC 48755	GMR17B03-GAL4	0
7	BDSC 50020	GMR38G08-GAL4	0
8	BDSC 39898	GMR75F02-GAL4	0
9	BDSC 50326	GMR47G06-GAL4	0
10	BDSC 39288	GMR64A07-GAL4	0
11	BDSC 48527	GMR12G12-GAL4	0
12	BDSC 46435	GMR60F03-GAL4	0
13	BDSC 39316	GMR64G05-GAL4	0
14	BDSC 47335	GMR23F01-GAL4	0
15	BDSC 50039	GMR39C07-GAL4	0
16	BDSC 49700	GMR32B04-GAL4	0
17	BDSC 49004	GMR22H09-GAL4	0
18	BDSC 47839	GMR10A11-GAL4	0
19	BDSC 39885	GMR75B11-GAL4	0
20	BDSC 50296	GMR47B12-GAL4	0
21	BDSC 50034	GMR39A11-GAL4	0
22	BDSC 48367	GMR81E10-GAL4	0
23	BDSC 50291	GMR47A12-GAL4	0
24	BDSC 48515	GMR12F05-GAL4	0
25	BDSC 50297	GMR47C03-GAL4	0
26	BDSC 47253	GMR94C10-GAL4	0
27	BDSC 50207	GMR44D07-GAL4	0
28	VDRC 207671	VT057287	0
29	VDRC 206433	VT057286	0
30	VDRC 200916	VT057280	0
31	VDRC 200828	VT057294	0
32	VDRC 206712	VT043663	0
33	VDRC 205899	VT043688	0
34	VDRC 204490	VT043670	0
35	VDRC 202967	VT043685	0
36	VDRC 202712	VT043658	0
37	VDRC 201610	VT043686	0
38	VDRC 201134	VT043678	0
39	VDRC 200755	VT043699	0
40	VDRC 201193	VT043701	0
41	VDRC 200285	VT043698	0
42	VDRC 207582	VT046252	0

43	VDRC 205020	VT056866	0
44	VDRC 203856	VT023747	0
45	VDRC 206542	VT029749	0
46	VDRC 204934	VT043645	0
47	VDRC 206065	VT039485	0
48	VDRC 207679	VT063218	0
49	VDRC 207662	VT042573	0
50	VDRC 204287	VT043697	0
51	VDRC 201032	VT017247	0
52	VDRC 203341	VT031562	0
53	VDRC 203183	VT010262	0
54	VDRC 207777	VT021853	0
55	VDRC 207746	VT062774	0
56	VDRC 204334	VT037601	0
57	VDRC 207914	VT017745	0
58	VDRC 204710	VT048649	0
59	VDRC 202764	VT058427	0
60	VDRC 204652	VT023783	0
61	VDRC 204475	VT022100	0
62	VDRC 205316	VT041269	0
63	VDRC 201067	VT034804	0
64	VDRC 204569	VT026174	0
65	VDRC 201918	VT050226	0
66	VDRC 205643	VT063191	0
67	VDRC 200494	VT002857	0
68	VDRC 201761	VT014604	0
69	VDRC 203479	VT026020	0
70	VDRC 208182	VT038884	0
71	VDRC 202173	VT008279	0
72	VDRC 204924	VT032280	0
73	VDRC 203610	VT048140	0
74	VDRC 201756	VT002210	0
75	VDRC 201439	VT040540	0
76	VDRC 201230	VT015784	0
77	VDRC 205866	VT007768	0
78	VDRC 204711	VT048651	0
79	VDRC 204949	VT048656	0
80	VDRC 205136	VT039361	0
81	VDRC 207835	VT016057	0
82	VDRC 200076	VT012300	0
83	VDRC 205765	VT009667	0
84	VDRC 202860	VT027938	0
85	VDRC 200626	VT037726	0
86	VDRC 202148	VT049374	0
87	VDRC 201299	VT058699	0

88	VDRC 207526	VT022224	0
89	VDRC 206883	VT015971	0
90	VDRC 203133	VT007359	0
91	VDRC 206930	VT026759	0
92	VDRC 202180	VT008480	0
93	VDRC 202959	VT028872	0
94	VDRC 206202	VT026782	0
95	VDRC 204216	VT043928	0
96	VDRC 200348	VT038814	0
97	VDRC 205738	VT056808	0
98	VDRC 206660	VT013039	0
99	VDRC 202186	VT008489	0
100	VDRC 206852	VT041658	0
101	VDRC 208087	VT030526	0
102	VDRC 201715	VT017415	0
103	VDRC 206982	VT026753	0
104	VDRC 203106	VT016969	0
105	VDRC 203875	VT050233	0
106	VDRC 201792	VT025779	0
107	VDRC 203086	VT007709	0
108	VDRC 203039	VT014724	0
109	VDRC 202909	VT007767	0
110	VDRC 201324	VT049500	0
111	VDRC 201212	VT006395	0
112	VDRC 203757	VT007747	0
113	VDRC 200786	VT039997	0
114	VDRC 200755	VT043699	0
115	VDRC 204493	VT044843	0
116	VDRC 202191	VT003238	0
117	VDRC 203180	VT009835	0
118	VDRC 204287	VT043697	0
119	VDRC 201907	VT040033	0
120	VDRC 203008	VT010054	0
121	VDRC 206753	VT029822	0
122	VDRC 207897	VT015256	0
123	VDRC 207668	VT054815	0
124	VDRC 204630	VT044175	0
125	VDRC 202663	VT046307	0
126	VDRC 207554	VT058854	0
127	VDRC 206455	VT045791	0
128	VDRC 203935	VT016848	0
129	VDRC 202538	VT032907	0
130	VDRC 203984	VT062245	0
131	VDRC 205034	VT060196	0
132	VDRC 207789	VT041298	0

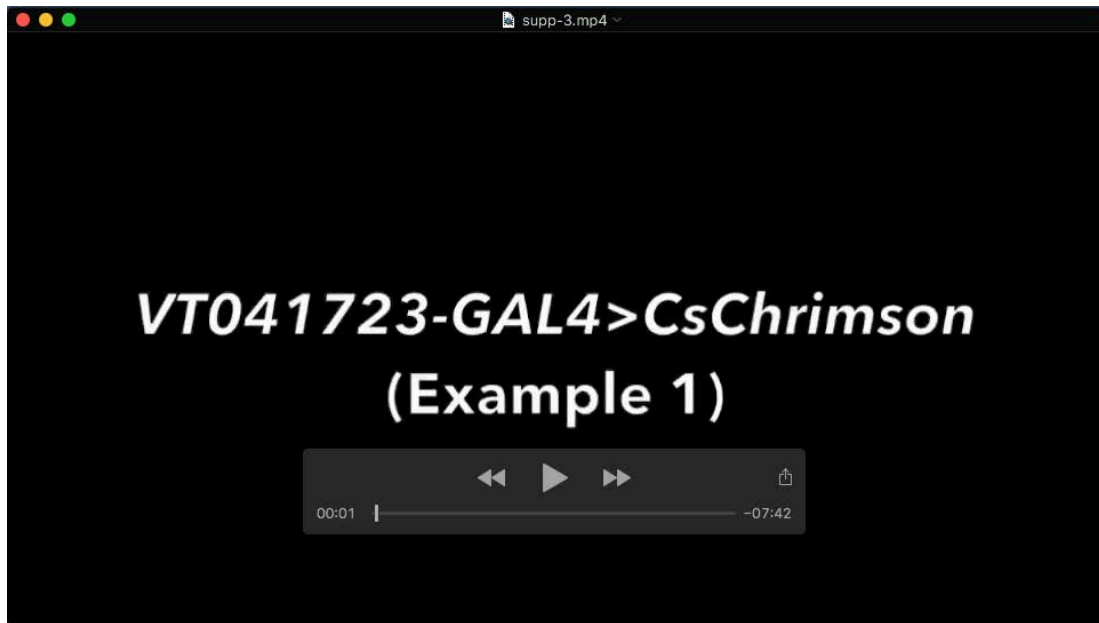
133	VDRC 206418	VT049371	0
134	VDRC 203267	VT029203	0
135	VDRC 203267	VT029203	1.5625
136	BDSC 26263	UAS-dTrpA1	3.84615
137	VDRC 206660	VT013039	4.6875
138	BDSC 49961	GMR37F05-GAL4	5
139	BDSC 50146	GMR42B10-GAL4	5
140	BDSC 38810	GMR52A06-GAL4	5
141	BDSC 39973	GMR77F07-GAL4	5
142	BDSC 40458	GMR86D08-GAL4	5
143	VDRC 201830	VT043635	5
144	VDRC 207088	VT050546	5
145	VDRC 207914	VT017745	5
146	VDRC 202842	VT059229	5
147	VDRC 206712	VT043663	5
148	VDRC 205377	VT049112	5
149	VDRC 203901	VT063556	5
150	VDRC 201617	VT049245	5
151	VDRC 206420	VT049570	5
152	VDRC 204187	VT062763	5
153	VDRC 204778	VT028452	5
154	VDRC 200252	VT034795	5
155	VDRC 201744	VT056509	5
156	VDRC 208860	VT030527	5
157	VDRC 204797	VT030528	5
158	VDRC 204931	VT038156	5
159	VDRC 202537	VT032906	5
160	VDRC 204703	VT046912	5
161	VDRC 200084	VT019428	5
162	VDRC 206523	VT020016	5
163	VDRC 203128	VT021366	5
164	VDRC 201412	VT045153	5
165	VDRC 207611	VT037802	5
166	VDRC 208087	VT030526	7.8125
167	VDRC 204949	VT048656	9.375
168	BDSC 47826	GMR88C04-GAL4	10
169	VDRC 202269	VT025803	10
170	VDRC 203052	VT017205	10
171	VDRC 200911	VT041424	10
172	VDRC 201230	VT015784	12.5
173	BDSC 49396	GMR53D04-GAL4	15
174	VDRC 205453	VT036779	15
175	VDRC 203966	VT040981	15
176	VDRC 205806	VT000456	15
177	VDRC 200309	VT050245	15

178	VDRC 206939	VT037470	15
179	VDRC 204317	VT030091	15
180	BDSC 50149	GMR42C05-GAL4	20
181	BDSC 50386	GMR48G07-GAL4	20
182	BDSC 48967	GMR22B01-GAL4	20
183	VDRC 204407	VT059796	20
184	VDRC 203800	VT038168	20
185	VDRC 206142	VT058927	20
186	VDRC 204139	VT017376	21.875
187	VDRC 204934	VT043645	25
188	VDRC 203186	VT013509	25
189	BDSC 48016	GMR9A03-GAL4	30
190	VDRC 20055	VT064520	30
191	VDRC 203984	VT062245	40.625
192	VDRC 204969	VT040416	42.85
193	VDRC 202699	VT041723	54.117
194	VDRC 203800	VT038168	55
195	BDSC 39951	GMR77B08-GAL4	65



Movie 1. Thermogenetic activation of *VT041723-GAL4* neurons induces proboscis holding.

Proboscis holding observed upon placing a *VT041723-GAL4>dTrpA1* fed female fly on a 31°C heated block. Note that the fly fully extended its proboscis and held it in that position for the whole 7-minute recording time.



Movie 2. Optogenetic activation of *VT041723-GAL4* neurons induces proboscis holding.

Proboscis holding observed upon activation red LED exposure (at time 0.15 seconds) on a *VT041723-GAL4>CsChrimson* fed female fly. Note that the fly partially extended its proboscis and held it in that position for the whole 7-minute recording time.



Movie 3. Thermogenetic activation of *VT041723-GAL4* neurons induces regurgitation after ingestion. Regurgitation observed upon placing a *VT041723-GAL4>dTrpA1* female fly pre-fed with 0.5 μ L of 100 mM sucrose on a 31°C heated block. Note that the fly held the proboscis with a bubble at the tip for the whole 7-minute recording time.