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RESEARCH ARTICLE

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

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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.

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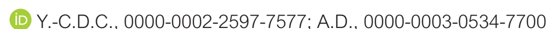
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The following fly strains were used in this study: *VT041723-GAL4* (Vienna *Drosophila* Resource Center) (Kvon et al., 2014), *Gr43a-LexA* (Miyamoto and Amrein, 2014), *Ir76b-LexA* (Ganguly et al., 2017), $Poxn^{\Delta M22\text{-}B5}$ (Boll and Noll, 2002), $Poxn^{70}$ (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 bind 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 cornmeal-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

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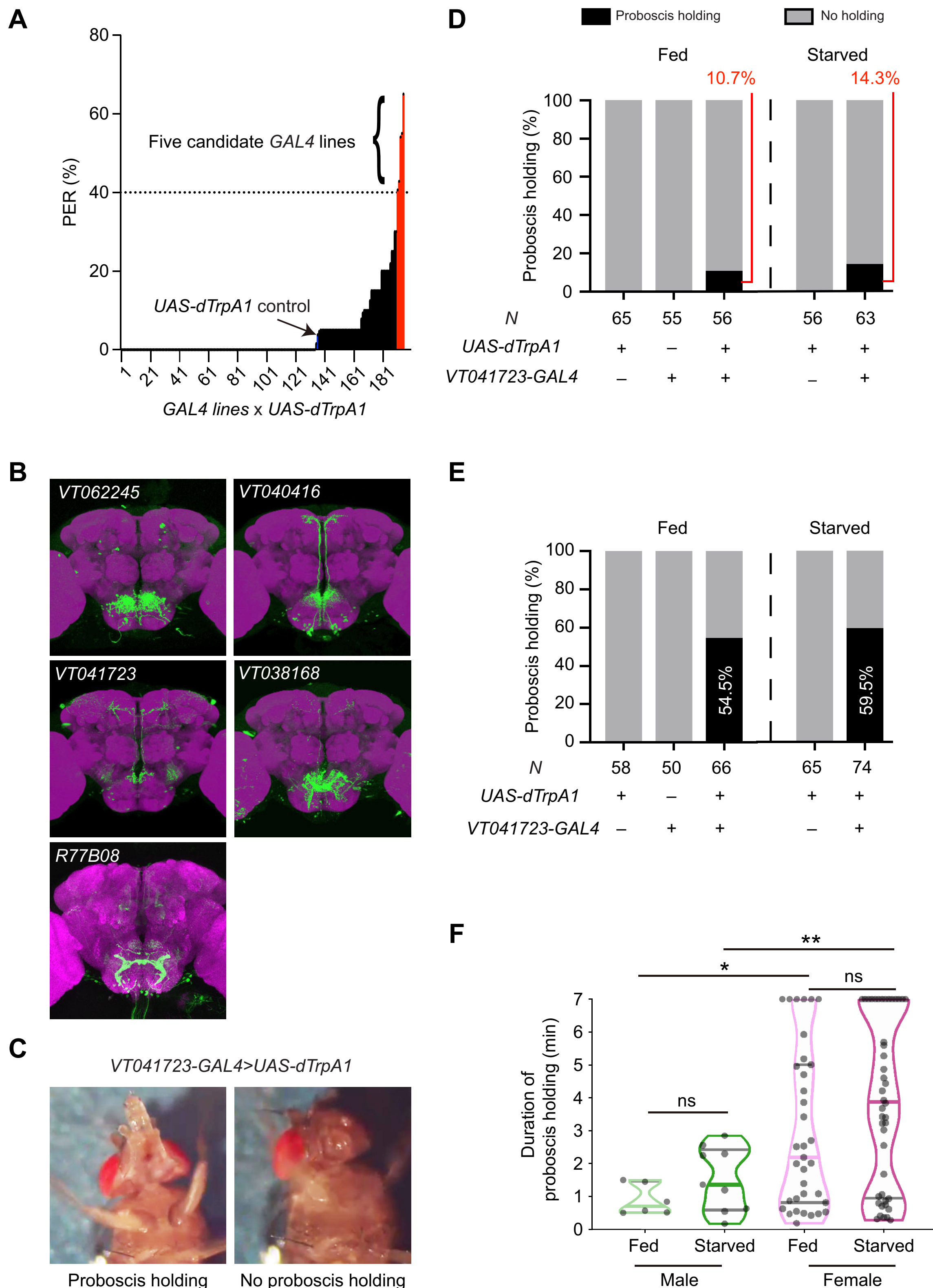

Fig. 1. A proboscis extension screen of *GAL4* transgenic fly lines identifies *VT041723-GAL4* neurons as candidates for higher-order taste neurons controlling feeding behavior. (A) Heat-activated proboscis extension responses (PERs) of 195 *GAL4>dTrpA1* lines. The *UAS-dTrpA1* control is shown in blue (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.

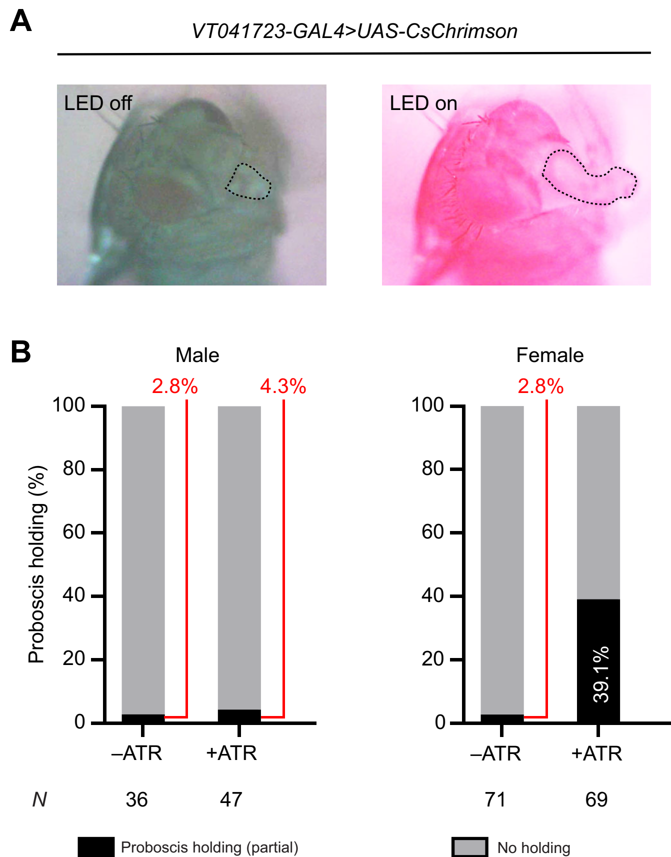

Fig. 2. Optogenetic activation of *VT041723-GAL4* neurons induces proboscis holding. (A) Sample images of the head before (left) and after (right) optogenetic activation of *VT041723-GAL4* neurons with 626 nm LED. The dotted line outlines the proboscis. Note that the proboscis is not fully extended as 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-GAL>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).

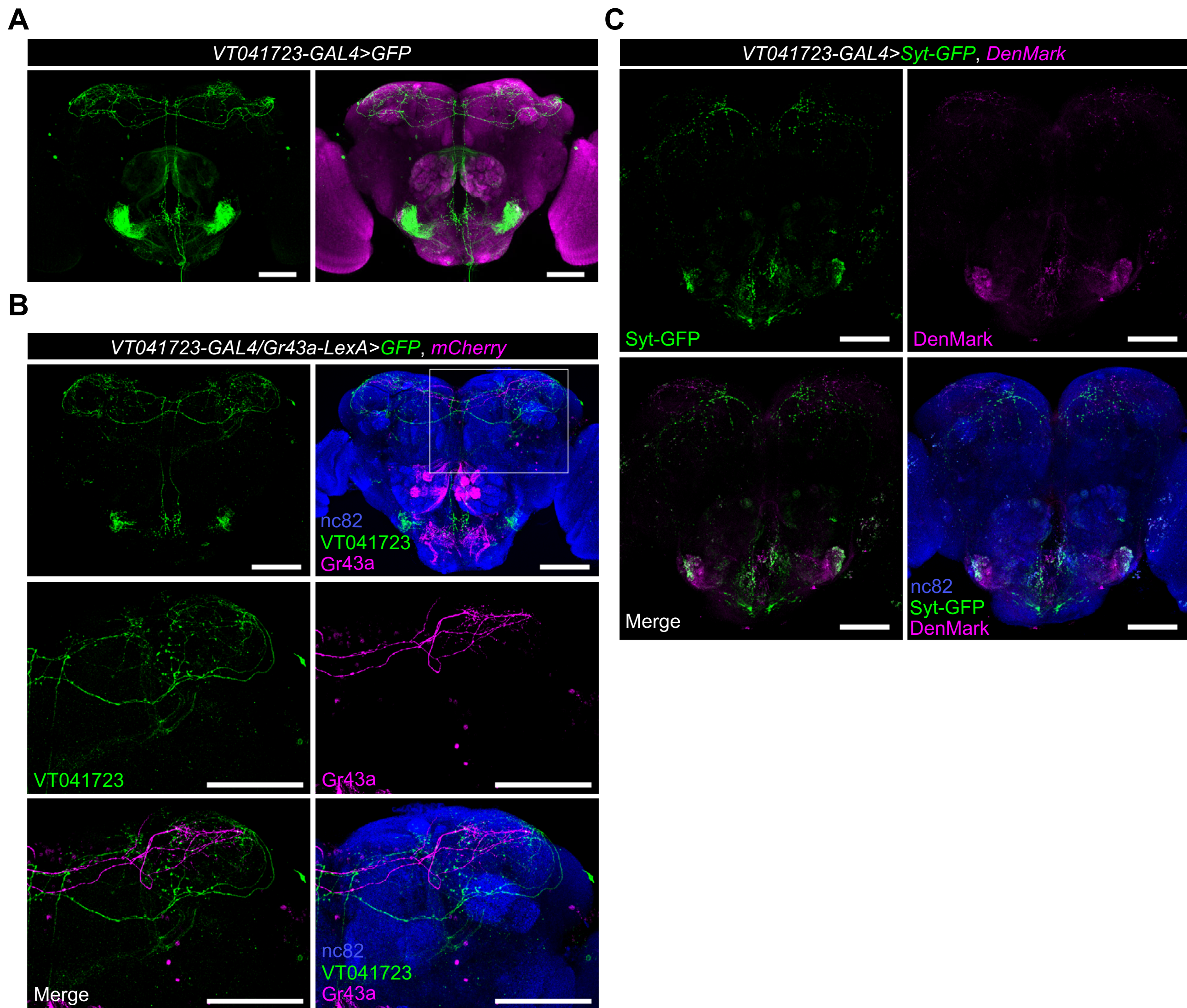

Fig. 3. Neuroanatomical analysis of *VT041723-GAL4* neurons. (A) Expression of a GFP reporter driven by *VT041723-GAL4* in the adult *Drosophila* brain. Neuropil is stained with anti-nc82 (magenta). Scale bars: 100 µm. (B) GFP and mCherry reporter expression driven by *VT041723-GAL4* (green) and *Gr43a-LexA* (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 µm. (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 µm.

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

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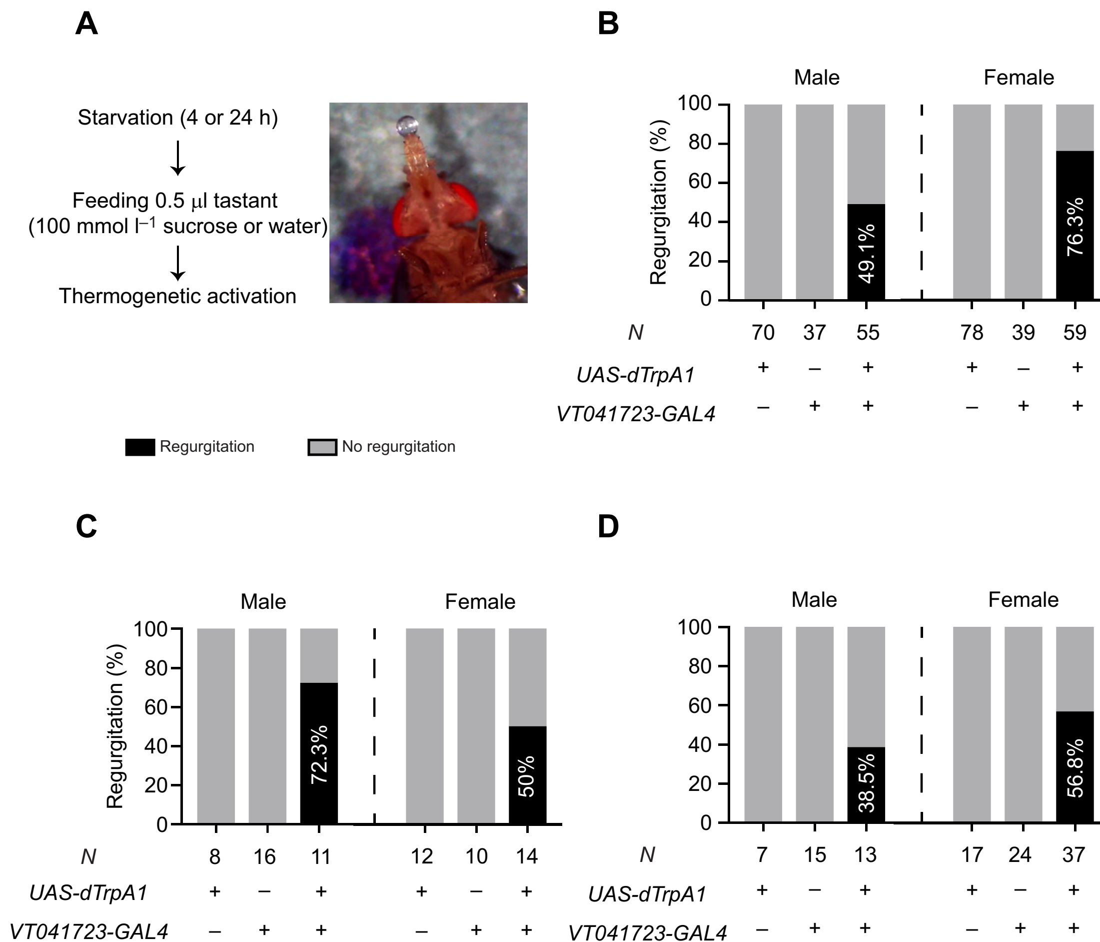

Fig. 4. Thermogenetic activation of *VT041723-GAL4* neurons induces regurgitation after ingestion. (A) Summary of the experimental procedure for the regurgitation assays (left), and an image of a fly showing regurgitation (right; see Movie 3). (B,C) Distribution of phenotypes upon heat activation after 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 thermogenic 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), neurexin, 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.

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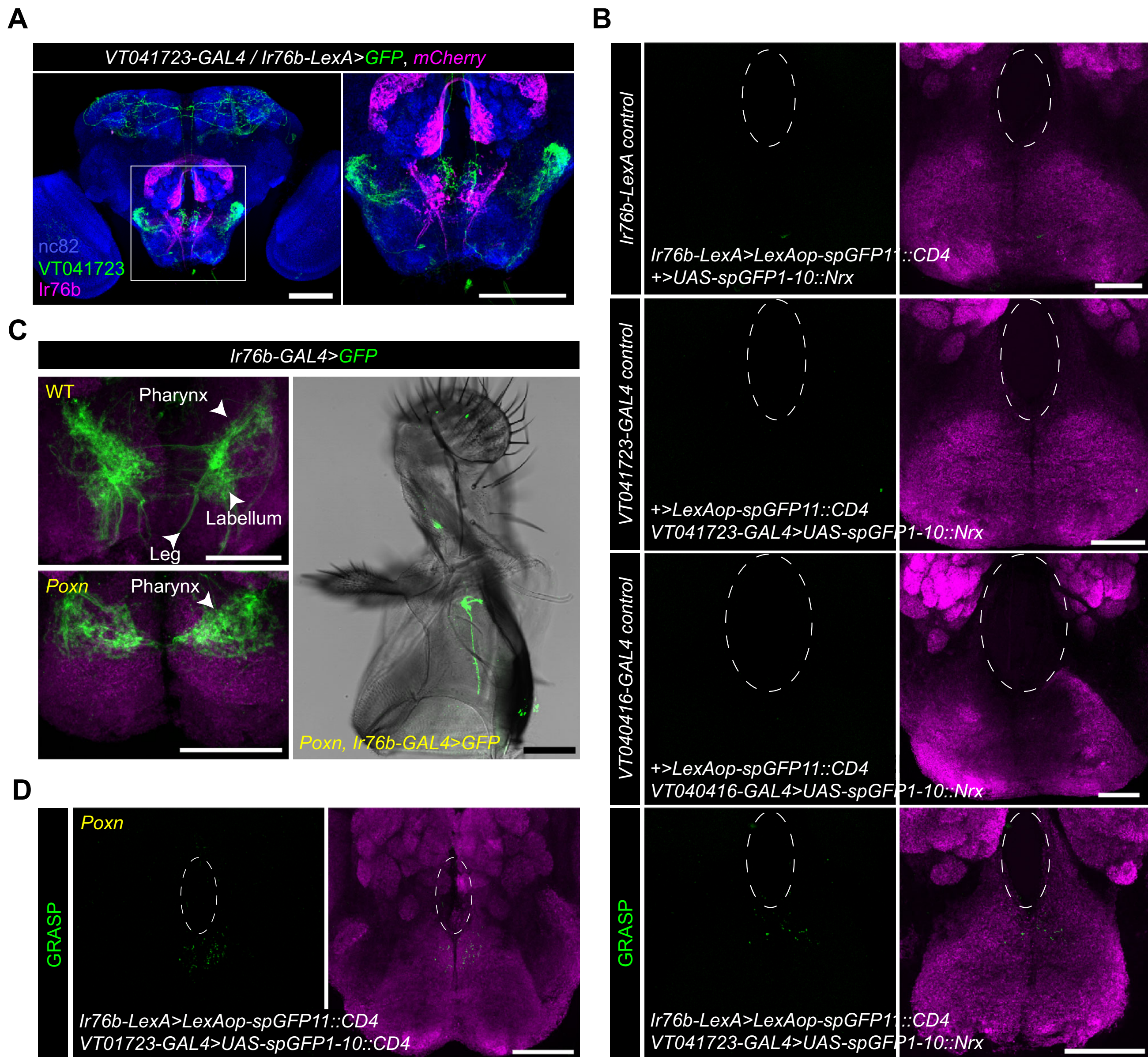

Fig. 5. *VT041723-GAL4* neurons show a GFP reconstitution across synaptic partners (GRASP) signal with *Ir76b*$^+$ pharyngeal gustatory receptor neurons (GRNs). (A) GFP and mCherry reporter expression driven by *VT041723-GAL4* (green) and *Ir76b-LexA* (magenta) in the adult *Drosophila* brain. The boxed 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 (magenta). 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, w^{1118}) and *Poxn* (*Poxn*$^{\Delta M22\text{-}B5}$/*Poxn*^{70}) 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 *Pox-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.

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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.

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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
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