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RESEARCH ARTICLE

Pharyngeal stimulation with sugar triggers local searching behavior in *Drosophila*

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ABSTRACT

Foraging behavior is essential for all organisms to find food containing nutritional chemicals. A hungry *Drosophila melanogaster* fly performs local searching behavior after drinking a small amount of sugar solution. Using video tracking, we examined how the searching behavior is regulated in *D. melanogaster*. We found that a small amount of highly concentrated sugar solution induced a long-lasting searching behavior. After the intake of sugar solution, a fly moved around in circles and repeatedly returned to the position where the sugar droplet had been placed. The non-nutritious sugar D-arabinose, but not the non-sweet nutritious sugar D-sorbitol, was effective in inducing the behavior, indicating that sweet sensation is essential. Furthermore, *pox-neuro* mutant flies, which have no external taste bristles, showed local searching behavior, suggesting the involvement of the pharyngeal taste organ. Experimental activation of pharyngeal sugar-sensitive gustatory receptor neurons by capsaicin using the GAL4/UAS system induced local searching behavior. In contrast, inhibition of pharyngeal sugar-responsive gustatory receptor neurons abolished the searching behavior. Together, our results indicate that, in *Drosophila*, the pharyngeal taste-receptor neurons trigger searching behavior immediately after ingestion.

KEY WORDS: Fly, Gustation, Feeding, Dance

INTRODUCTION

Innate behavior is triggered by a specific key stimulus in animals (Tinbergen, 1951). Behavioral responses are elicited depending on the quality and strength of the key stimulus but also on the internal state of animals. Identification of the neuronal and molecular network underlying innate behavioral responses is a fundamental problem in neurobiology. Foraging behavior in insects is one of the most sophisticated behaviors. Honey bees are capable of communicating the place of a foraging spot, and ants use pathfinding to successfully return to their nest site (von Frisch, 1967; Müller and Wehner, 1988). Dethier had previously demonstrated that a hungry blowfly, *Phormia regina*, performs a sugar-elicited local search behavior (Dethier, 1957) and suggested that this behavior might be a behavioral module co-opted in the bee's dance communication, but there is no clear supporting evidence for this idea (A.B. and T.T., unpublished). In general, two kinds of food search behaviors are distinguished: a hunger-induced large-scale roaming with heightened attention towards food cues such as odors, and a food-intake-elicited local search for more food (Bell, 1985, 1990a,b). Local searches for more food are characterized by an increase in turning behavior, which results in circular trajectories around the location of the original food item (Dethier, 1957; Bell, 1985; McGuire and Tully, 1986; Nagle and Bell, 1986). The logic is that the probability of finding another food item is higher in the vicinity of the food item found than further away. Many studies on flies, including *Drosophila*, have demonstrated that this behavior is dependent on the hunger state (Dethier, 1957; McGuire and Tully, 1986), genetic background (Nagle and Bell, 1986) and distribution of food items in the environment (Bell, 1985, 1990a,b).

Here, we present a study on sugar-elicited searching behavior in *Drosophila*. We intended to identify the sensory input pathway that triggers the searching behavior. In *Drosophila*, multiple taste organs are found on the legs, the mouthparts (labellum and labellar palps) and the internal pharynx (Stocker, 1994; Singh, 1997). Each taste organ is thought to have a specific role in regulating feeding behavior. Flies detect the presence of sugar using tarsal taste sensilla. Then, if a sugar concentration is high enough, they extend the proboscis. Stimulation of labellar taste sensilla induces the opening of labellar lobes and, if interpseudotracheal papillae taste sensilla are stimulated, they initiate drinking by the action of the cibarial pump. The solution sucked in is finally monitored by pharyngeal taste neurons deciding whether to continue ingesting (LeDue et al., 2015). Finally, the solution passes from the esophagus to the proventriculus and, if the flies are hungry, the solution will pass into the crop. Crop expansion is monitored by a recurrent nerve (Gelperin, 1971) and it is possible that gut-innervating neurons may also be involved in the control of searching behavior.

Thus, we investigated at which sensory step the searching behavior is initiated and regulated, and what kind of stimulation can trigger the behavior. First, we tested which chemical property of the sugar solution triggers the behavior. A previous study indicated that osmolarity is a key satiety signal (Gruber et al., 2013), but our results showed that sweetness is the essential key stimulus to induce searching behavior after ingesting a small amount of sugar. Second, we used *pox-neuro* (*poxn*) mutants, in which all external chemosensilla are transformed into mechanosensilla, and also the GAL4/UAS system to artificially activate specific gustatory receptor neurons (GRNs). Together, these two approaches demonstrate that pharyngeal sugar-responsive GRNs perceive and mediate the key stimulus triggering the sugar-elicited search behavior.

MATERIALS AND METHODS

Fly strains

Drosophila melanogaster were raised on standard glucose–cornmeal–yeast–wheatgerm medium under a 12 h:12 h light:dark cycle at 25°C. Canton-Special (CS), obtained from B. Gerber's

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laboratory (Leibniz Institute of Neurobiology, Germany) in 2014 and reared *en masse* in our laboratory, was used as a wild-type strain. *poxn*$^{70\text{-}23}$*/CyO* was crossed to *Df(2R)42WMG/CyO*, and *poxn*$^{70\text{-}23}$*/Df(2R)WMG* flies were used as the *poxn* mutant strain (Awasaki and Kimura, 1997). *Gr43a*^{GAL4} and *Gr64a*^{GAL4} flies were provided by H. Amrein (Texas A&M University, USA). *Gr5a-GAL4* and *Gr64e-GAL4* flies were provided by J. C. Carlson (Yale University, USA). *UAS-VR1E600K* flies were provided by K. Scott (UC Berkeley, USA). *UAS-TNT* and *UAS-IMPTNT* flies were obtained from Bloomington Stock Center. Female flies change their feeding behavior after mating (Carvalho et al., 2006) and we used male flies for most of our experiments, but female flies showed similar local searching behavior.

Chemicals

The tastants D-glucose and D-arabinose were obtained from Sigma-Aldrich (St Louis, MO, USA); D-fructose, D-sorbitol and capsaicin from Wako Pure Chemical Industries (Tokyo, Japan); sucrose from NACALAI TESQUE (Kyoto, Japan); and trehalose from Nagase & Co., Ltd (Tokyo, Japan). Food Blue No. 1 was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Silicone oil was obtained from NACALAI TESQUE.

Food-starvation tolerance

We determined the food-starvation tolerance for each strain to standardize the hunger state among strains and experiments. Flies eclosed within 24 h were kept on standard medium for 24 h and then deprived of food with access to water (Evian™). Cut-out pieces of Kimwipe™ paper were plugged on the bottom of vials and were wetted with a sufficient amount of water. The number of surviving flies was counted at 1 h intervals. Ten flies of each strain were placed in a vial (N=3).

Sugar-elicited search assay

Shortly before the experiment, single starved flies were transferred into 0.5 ml microcentrifuge tubes and left for about 3 min on the LED light panel (On-Lap1303H; GeChic, China) to allow them to adapt to the experiment arena. A Petri dish was placed on the LED light panel and the center of it was coated with a very small amount of silicone oil with a cotton swab to maintain the spheroidal shape of the droplet; thus, the flies drink the whole droplet. The droplet was colored with a blue food dye to reveal the presence of any leftover. We confirmed that the presence of food dye does not affect the searching behavior. Then we put the fly container over the droplet and waited until the fly found the droplet. Immediately after the fly had started to ingest the droplet we removed the fly container, surrounded the arena with a white polyvinyl chloride pipe (67 mm inner diameter×100 mm height) to ensure a uniform visual environment and started video recording at 30 frames s^{-1} (Logicool HD Webcam C615; Logicool, Japan). Recordings were terminated when the fly escaped from the arena or after 3 min. In this arena flies can make a free decision to stop searching behavior and to fly away, as they would do under natural conditions. Videos were analyzed by Ctrax (K. M. Branson, California Institute of Technology, USA) to convert fly position into *xy* coordinates (Branson et al., 2009; see also http://ctrax.sourceforge.net and https://groups.google.com/forum/#!forum/ctrax). For each fly we determined the duration of food search, total path length, distance traveled from the starting point, activity rate and average speed. Activity rate was defined as the percentage of the walking periods above 2 mm s^{-1} in food-searching time. Average speed was calculated as path length divided by food-searching time. All experiments were performed between 2 and 6 h after lights on, when flies show constantly high activity.

To measure the crop volume, the flies were quickly anesthetized on ice just after ingestion of 0.1 µl of water or 200 mmol l^{-1} sucrose solution. The flies were submerged in ethanol and rapidly dissected in *Drosophila* Ringer's solution. The crops were carefully dissected out and photographed under the stereomicroscope.

All procedures comply with applicable law.

Genetics

To genetically activate specific taste-receptor neurons by capsaicin, *Gr43a*^{GAL4}, *Gr64a*^{GAL4}, *Gr64e-GAL4* and *Gr5a-GAL4* flies were crossed to *UAS-VR1E600K* flies. A two-choice preference test was performed to estimate the intensity of capsaicin stimulation and determine the concentration of capsaicin mixed with sugar solution for sugar-seeking behavior (Toshima and Tanimura, 2012). A total of 100 mmol l^{-1} capsaicin in 99.5% EtOH was diluted in sucrose solution to a final concentration of 1 mmol l^{-1}. EtOH was added to the sucrose solutions at a concentration equal to the capsaicin mixture (1%). Because capsaicin itself induced a weak searching behavior at 1 mmol l^{-1} and we did not want to use higher concentrations of capsaicin because of its possible toxic effect, we mixed capsaicin with sucrose.

Statistics

All data are presented as means±s.e.m. For the statistical analysis, we used either a Student's *t*-test or an ANOVA with Tukey's *post hoc* test.

RESULTS

Quantification of searching behavior by video tracking

The duration of food starvation affects the feeding responses and percentage of time spent moving during local searching behavior (Bell et al., 1985). Therefore, the starvation tolerance of each strain was determined before the test to standardize the hunger state among strains. A total of 90% of CS flies survived at 34 h starvation, and 24 h starvation was enough to induce consumption of a 0.1 µl droplet and local searching behavior in females and males. We determined the 90% survival time for all the strains, and the experimental starvation time was based on the data for CS (Table S1).

A hungry fly engages in local searching behavior after ingesting a small amount of sugar solution (Dethier, 1957; Bell et al., 1985). In order to study how the sugar-seeking behavior is regulated in adult *Drosophila*, we used a video-tracking setup that enabled us to record the position of a single fly in the arena (see Materials and methods). A small droplet of sugar solution was served as a trigger for local searching behavior. To compare the strength of searching behavior elicited by different stimuli, we determined the food-searching time, activity, average speed, path length and distance from the starting point.

It is known that sugar concentration affects local searching behavior in *P. regina* (Dethier, 1957). In *Drosophila*, we found that the duration of food search and the path length correlated positively with glucose concentration (Fig. 1). However, there were no significant differences in average speed and activity. Thus, we used the path length to compare the strength of the searching behavior.

Chemicals that induce local searching behavior

To ascertain the nature of the chemicals that trigger local searching behavior, we presented hungry flies with a 0.1 µl droplet of distilled water and six different sugar solutions (200 mmol l^{-1} sucrose,

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 3231-3237 doi:10.1242/jeb.161646

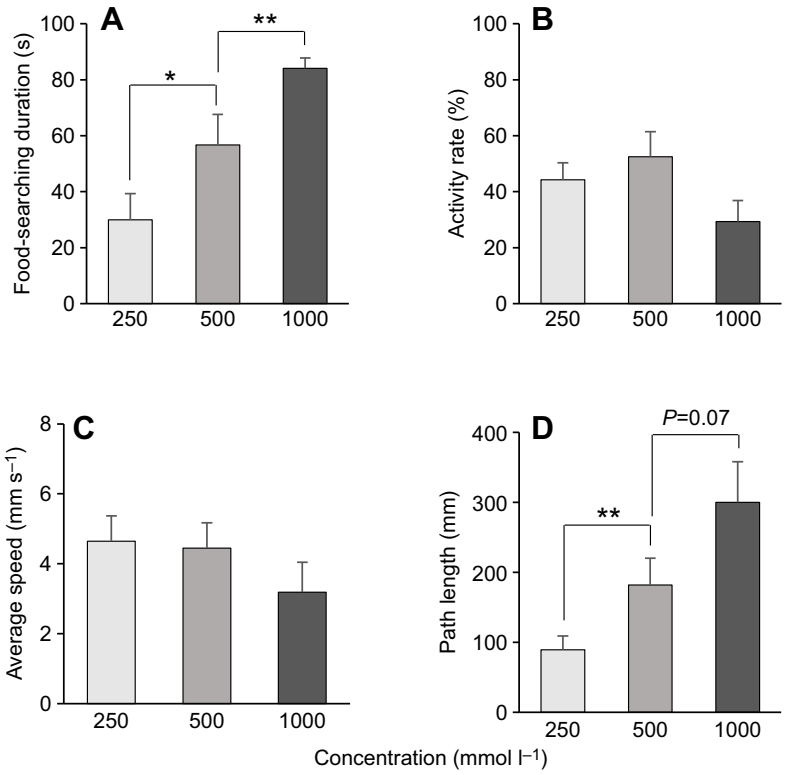

Fig. 1. Comparison of four behavioral parameters. Flies were given 0.1 µl of three different concentrations of glucose. Four parameters were calculated: (A) the duration of food search, (B) activity rate, (C) average speed and (D) path length. All data are presented as means±s.e.m. The duration of food search and path length are in proportion to the concentration of the glucose solution (ANOVA with Tukey's *post hoc* test, **P*<0.05, ***P*<0.01, *n*=10). In contrast, activity rate and average speed were not affected by sugar concentration.

1 mol l^{-1} glucose, 1 mol l^{-1} fructose, 1 mol l^{-1} arabinose, 1 mol l^{-1} trehalose and 1 mol l^{-1} sorbitol; see Fig. 2). All the sugars except sorbitol and trehalose elicited a long-lasting searching behavior. After drinking a droplet of these sugar solutions, the fly moved around and returned to the position where the sugar droplet had been placed. In contrast, after drinking water or sorbitol, the fly escaped from the arena immediately after drinking or remained motionless for a long time. These differences are evident in the temporal patterns of the fly position shown in Fig. 2B, where the radial distance from the sugar droplet is plotted for an individual fly. Sorbitol is a nutritive sugar but does not taste sweet to flies. There is a clear difference in trajectories between those induced by sweet sugar solution and by sorbitol (Fig. 2B). In fact, the path lengths of the local searching behavior caused by sweet sugar solutions were significantly larger than those caused by water and non-sweet sugar solution. When a fly found salt solutions (50 and 500 mmol l^{-1}

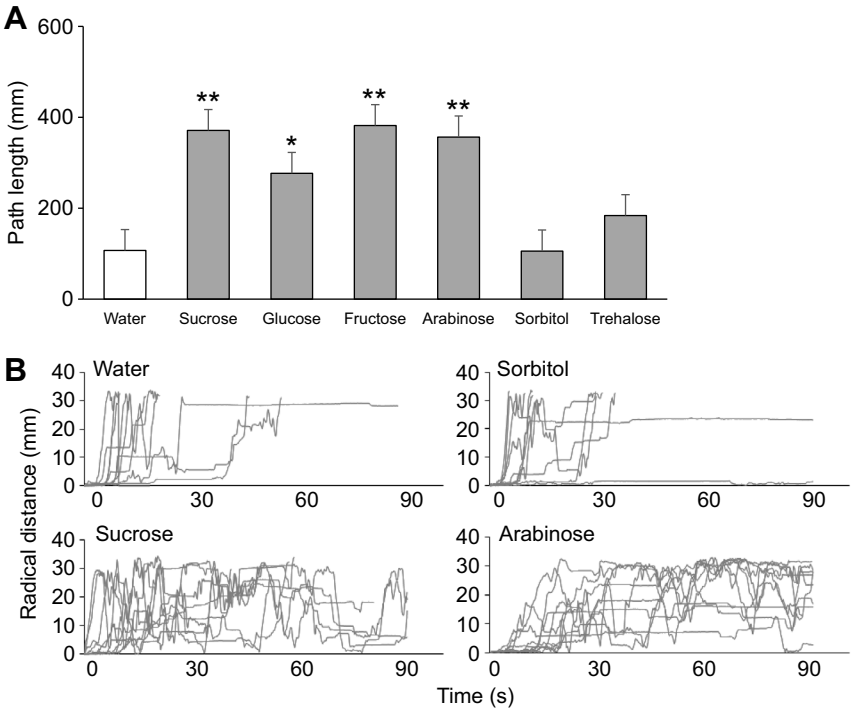

Fig. 2. Sweet sugar elicits searching behavior in flies. (A) Comparison of path length of searching behaviors induced by sweet sugar, water, sorbitol and trehalose (ANOVA with Tukey's *post hoc* test, **P*<0.05, ***P*<0.01, *n*=10). (B) Temporal changes in radial distance of searching behavior in flies fed with water, sucrose, arabinose and sorbitol. All data of an individual fly are plotted (*n*=10). Flies fed with sucrose or arabinose repeatedly returned to the position where the sugar droplet had been placed. In contrast, flies fed with water or sorbitol immediately ran away after feeding, remained motionless after a short walk or did not move from near the position of the droplet.

Journal of Experimental Biology (2017) 220, 3231-3237 doi:10.1242/jeb.161646

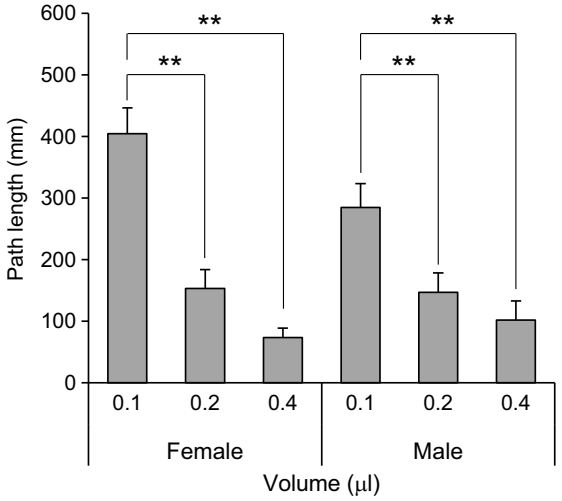

Fig. 3. Effect of volume of sugar solution on path length. A significant decrease was observed in both females and males when the fly ingested more than 0.1 µl of 200 mmol l^{-1} sucrose (ANOVA with Tukey's *post hoc* test, $^{**}P<0.01$, $n=10$). Data are means±s.e.m.

NaCl) or amino acid solution (500 mmol l^{-1} glycine), the fly did not drink the whole droplet and moved away from the droplet. These results indicate that, for *Drosophila*, the key stimulus for initiating local searching behavior is sweet sugar. In addition, our results indicate that high osmolarity does not act as a trigger as sorbitol failed to elicit searching behavior. To confirm this, we tested sucrose solution mixed with different, increasing concentrations of sorbitol (Fig. S1). Increasing osmolarity with sorbitol did not enhance searching behavior. Finally, trehalose did not induce searching behavior, although trehalose stimulates the labellar GRNs to a similar degree to glucose and fructose (Hiroi et al., 2002). The failure of trehalose to trigger searching behavior indicates that labellar sugar sensation might not be enough to trigger the behavior.

Crop volume and local searching behavior

We performed several experiments to verify whether crop expansion affects local searching. First, we measured the crop volume of flies that had ingested water and sugar–water solutions. In both cases the crop was expanded, indicating that both kinds of food were transferred into the crop. The crop volume did not differ between water- and sucrose-fed flies (Fig. S2). Given that water intake did not induce local searching behavior, these results strongly suggest that crop expansion does not trigger local searching behavior. In a second set of experiments, we presented flies with different amounts of sugar solutions (0.1, 0.2 and 0.4 µl; 200 mmol l^{-1} sucrose) to test whether crop volume might negatively affect searching behavior (Fig. 3). We found that flies that had ingested 0.2 and 0.4 µl droplets showed reduced local searching behavior compared with flies that had ingested 0.1 µl. The flies were able to ingest the whole sucrose droplet of 0.2 µl. However, they did not move much around the location of the droplet and quickly moved away in most cases. In contrast, flies could not ingest the whole 0.4 µl droplets. After they had stopped drinking, they showed similar movement responses to the flies that ingested 0.2 µl droplets. When the fly ingested approximately 0.3 µl of sugar solution, the crop was fully expanded and searching behavior did not occur. Both sets of experiments together indicate that crop expansion after food intake does not induce local searching behavior but full expansion of the crop suppresses local searching behavior. Thus, local searching behavior would be an adaptive behavioral strategy to search for an additional sugar droplet possibly present in the vicinity of the original droplet.

Pharyngeal GRNs are necessary for local searching behavior

Given that sweet sugars are the key stimulus for searching behavior, we investigated which specific taste organ senses the stimulus and

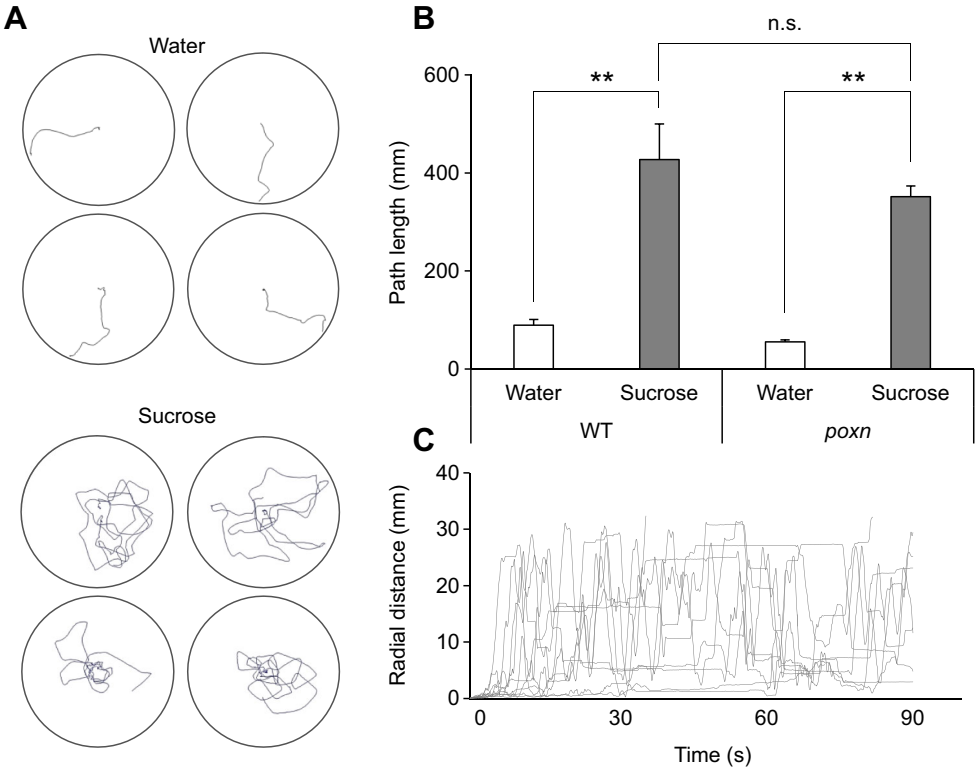

Fig. 4. Tarsal or labellar chemosensory reception is not needed to trigger the sugar-elicited searching behavior. Starved *poxn* flies were tested with water or 200 mmol l^{-1} sucrose. (A) Typical trajectories of *poxn* flies fed with water or sucrose. (B) Mean±s.e.m. path length and (C) radial distance of individual *poxn* flies fed with sucrose are shown. There were no significant differences in path length for sucrose between wild-type and *poxn* flies. Significant differences between the total path length for water and sucrose were observed both for wild-type and *poxn* flies (ANOVA with Tukey's *post hoc* test, $^{**}P<0.01$, $n=10$). *poxn* flies fed sucrose moved around and repeatedly returned to the position where the sugar droplet had been placed in a similar way to wild-type flies.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 3231-3237 doi:10.1242/jeb.161646

Table 1. Expression of gustatory-receptor–*GAL4* lines and their activation effect on searching behavior

	Expression			
		Pharynx		
GAL4 lines	Labellum	LSO	VCSO	Effect on searching behavior
Gr43a^{GAL4}	–	+	+	+
Gr64a^{GAL4}	–	+	–	+
Gr64e-GAL4	+	+	+	+
Gr5a-GAL4	+	–	–	–

Expression refers to reported expression profiles, according to Miyamoto et al. (2012), Fujii et al. (2015) and LeDue et al. (2015). LSO, labral sense organs; VCSO, ventral cibarial sense organ.

induces local searching behavior. First, we tested whether external taste sensilla are involved in local searching behavior using *poxn* mutants (Fig. 4). Adult *Drosophila* have taste organs located on the legs, labellum and pharynx, whereas *poxn* mutants lack all external tarsal and labellar taste sensilla, because these have been developmentally transformed into mechanosensory bristles. In our experiments, *poxn* flies needed a long time to find and ingest the sugar droplet, but once they had found and drunk it, they initiated the local searching behavior.

Path length was not different between *poxn* mutants and the wild-type control (Fig. 4B). Likewise, *poxn* flies did not initiate searching after ingesting distilled water. The path length of the local searching behavior induced by water was significantly shorter than that induced by sugar water. These results indicate that *poxn* mutants, although they do not have any external sugar-sensitive sensilla, still respond to sensing sweet sugar by initiating search behavior. Thus, our results suggest that the sugar-responsive GRNs in the pharyngeal taste organ are highly likely to be the receptors necessary to induce local searching behavior. In addition, if local searching behavior were triggered by tarsal sugar stimulation, flies would start moving around just after sensing a sugar solution. We never observed such behavior.

We next investigated whether the experimental activation of pharyngeal sugar-responsive GRNs could induce local searching behavior. To investigate the importance of pharyngeal sugar-responsive GRNs in local searching behavior, we used *Gr43a*^{GAL4}, *Gr64a*^{GAL4}, *Gr64e-GAL4*, *Gr5a-GAL4* and *UAS-VR1E600K* flies (Fig. 5). VR1 is the mammalian capsaicin receptor channel belonging to the TRP family, and the variant *VR1E600K* gene encodes receptors with higher channel activity (Marella et al., 2006). *Gr43a* is expressed in both pharyngeal GRNs and a single tarsal GRN. *Gr64a* and *Gr64e* are expressed in pharyngeal, labellar and tarsal GRNs. *Gr5a* is expressed in labellar and tarsal GRNs (Fujii et al., 2015). *GAL4* lines of these gustatory receptors allow us to activate particular sugar-responsive GRNs by feeding capsaicin.

To evaluate the effect of capsaicin on preference in these transgenic flies, a two-choice preference test between sucrose solution and the mixture of sucrose and capsaicin was performed (Fig. S3). *Gr43a*^{GAL4}*>UAS-VR1E600K*, *Gr64a*^{GAL4}*>UAS-VR1E600K*, *Gr64e-GAL4>UAS-VR1E600K* and *Gr5a-GAL4>UAS-VR1E600K* flies preferred the mixture of 50 mmol l^{-1} sucrose and 1 mmol l^{-1} capsaicin to 50 mmol l^{-1} sucrose solution. On the other hand, there is no significant difference between the preference for the mixture and that for 100 mmol l^{-1} sucrose solution. Thus, we performed a sugar-elicited search assay using 50 mmol l^{-1} sucrose solution, 100 mmol l^{-1} sucrose solution and the mixture of 50 mmol l^{-1} sucrose and 1 mmol l^{-1} capsaicin as a droplet. We used a mixed solution of capsaicin with sucrose, because 1 mmol l^{-1} capsaicin solution acted as a moderate trigger for searching behavior. We found that flies with pharyngeal sweet GRNs activated by being fed capsaicin engaged in a long-lasting searching behavior. In *Gr43a*^{GAL4}*>UAS-VR1E600K*, *Gr64a*^{GAL4}*>UAS-VR1E600K* and *Gr64e-GAL4>UAS-VR1E600K* flies, the path lengths of the local searching behavior caused by the capsaicin mixture were significantly larger than those caused by 50 mmol l^{-1} sucrose. Conversely, we did not observe a significant difference between stimulation with 50 mmol l^{-1} sucrose and the mixture in *Gr5a>UAS-VR1E600K* flies. *Gr5a* is expressed in labellar GRNs,

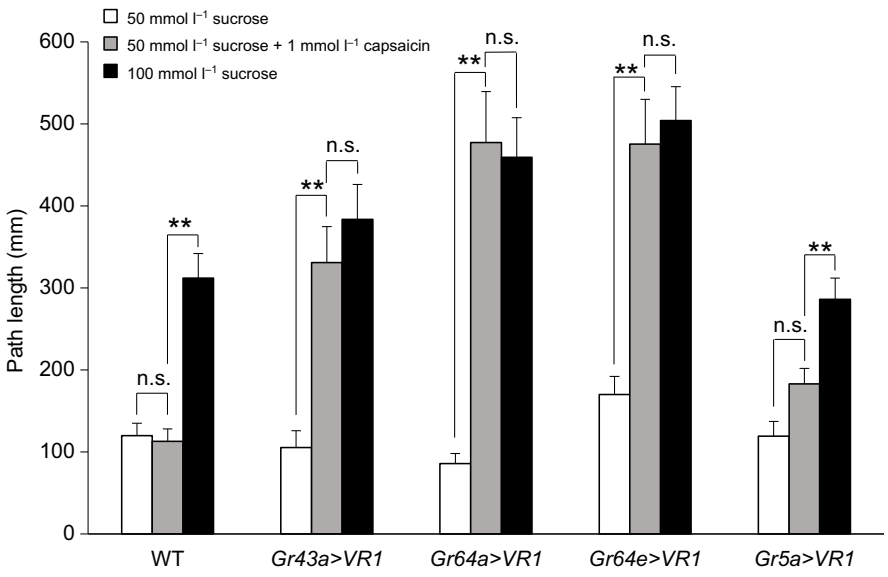

Fig. 5. Effect on path length by additive activation of gustatory-receptor-expressing gustatory receptor neurons (GRNs) by capsaicin, using Gal4/UAS. A significant increase was noted in *Gr43a*^{GAL4}*>UAS-VR1E600K*, *Gr64a*^{GAL4}*>UAS-VR1E600K* and *Gr64e-GAL4>UAS-VR1E600K* flies, whereas activation of *Gr5a* had no significant effect (ANOVA with Tukey's *post hoc* test, $^{**}P<0.01$, $n=10$). WT, wild type.

Journal of Experimental Biology (2017) 220, 3231-3237 doi:10.1242/jeb.161646

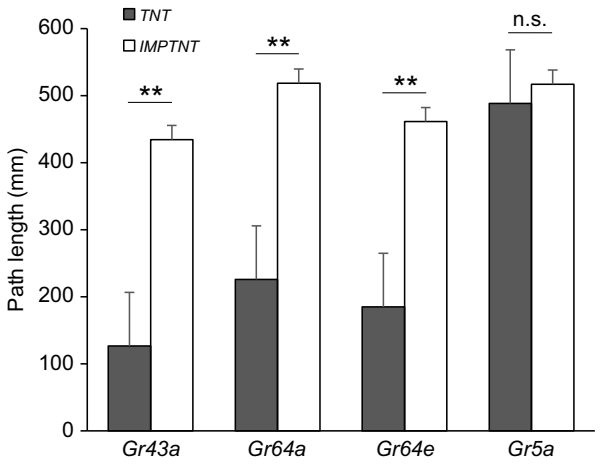

Fig. 6. Effect on path length by silencing of gustatory-receptor-expressing GRNs. Mean±s.e.m. path lengths are shown. A significant difference between tetanus toxin (*TNT*) and impotent tetanus toxin (*IMPTNT*) was noted in $Gr43a^{GAL4}$, $Gr64a^{GAL4}$ and *Gr64e-GAL4* flies, whereas silencing of *Gr5a* had no significant effect. Tests were performed using 200 mmol l^{-1} sucrose (Student's *t*-test, $^{**}P<0.01$, $n=10$).

but not in pharyngeal GRNs. Finally, in wild-type flies, the addition of capsaicin to sucrose solution had no effect on the path length, suggesting that capsaicin at the used concentration does not inhibit sugar sensation nor activate other sensory pathways. These results indicate that the pharyngeal sugar-responsive GRNs are the sensory input channel that triggers local searching behavior (Table 1).

We next used $Gr43a^{GAL4}$, $Gr64a^{GAL4}$, *Gr64e-GAL4* and *Gr5a-GAL4* to drive expression of tetanus toxin (*TNT*) to genetically disrupt the neuronal transmission of pharyngeal sweet-sensitive GRNs (Fig. 6). Flies with pharyngeal sweet-responsive GRNs silenced by *UAS-TNT* did not show searching behavior after being fed with 200 mmol l^{-1} sucrose solution. Control $Gr43a^{GAL4}$-*IMPTNT*, $Gr64a^{GAL4}$-*IMPTNT* and *Gr64e-IMPTNT* flies showed searching behavior similar to that of wild-type flies. Conversely, *Gr5a-TNT* flies showed similar searching behavior to *Gr5a-IMPTNT* flies. These results further corroborate our conclusion that pharyngeal sugar-responsive GRNs mediate the trigger signal for local searching behavior.

DISCUSSION

Local searching behavior is an adaptive foraging strategy. Flies and other animals that have found rewarding food initiate a search in the vicinity of the original spot to find more food. To identify the key stimulus for local searching behavior in *Drosophila*, we compared the path length of local searching behavior after feeding water and six different sugar solutions. We found that flies that had ingested sweet sugar solutions engaged in long-lasting local searching behavior. Arabinose, which is sweet tasting but non-nutritious for *Drosophila*, induced searching behavior as strongly as glucose. In contrast, sorbitol, a non-sweet but nutritious sugar, did not induce searching behavior. NaCl and glycine also failed to trigger the behavior. Together, these results indicate that sweet-tasting sugars and not the key satiety signal, osmolarity, are the key stimulus initiating local searching behavior in hungry *Drosophila*. At the same time, for such searching behavior to occur, the flies still need to be hungry after drinking a sugar droplet.

GRNs located in taste organs had been shown to play an important role in the finding and evaluation of food, whereas crop expansion following food intake regulates the duration and cessation of feeding behavior (Gelperin, 1971). Thus, the question arose as to which of the processes involved in feeding behavior actually initiates the sugar-elicited local searching behavior. First, we demonstrated that crop expansion does not induce local searching behavior, as a similar expansion of the crop with water never induced the searching behavior. Then, we showed that *poxn* flies with no external taste sensilla still initiated a searching behavior similar to that of wild-type flies. In addition, flies did not start searching after only tarsal stimulation with sugar (results not shown). These findings strongly suggest that the excitation of sugar-responsive GRNs of internal taste organs is necessary to induce local searching behavior.

GRNs of the pharyngeal taste organ respond to sweet sugars and influence short-term feeding decisions (LeDue et al., 2015). To examine the importance of pharyngeal sugar-responsive GRNs in local searching behavior, we performed genetically mediated stimulation of pharyngeal GRNs using the GAL4/UAS system. Transgenic flies carrying the *UAS-VR1E600K* transgene were crossed to $Gr43a^{GAL4}$, $Gr64a^{GAL4}$, *Gr64e-Gal4* and *Gr5a-Gal4* flies to generate flies expressing *VR1E600K* in different sets of GRNs. These flies were used to activate a specific taste receptor by feeding them capsaicin (Gordon and Scott, 2009; Marella et al., 2006). We found that the activation of pharyngeal sugar-responsive GRNs induced searching behavior by increasing the effect of low-sugar concentrations. In turn, when the same *Gr43a*-, *Gr64a*- and *Gr64e*-expressing GRNs were silenced, flies did not initiate searching behavior. By contrast, the *Gr5a-Gal4* driver that is expressed only in labellar GRNs failed to enhance searching behavior. The findings that the *Gr43a* and *Gr64a* drivers that we used in this study are expressed only in pharyngeal sugar-responsive GRNs and that external tarsal and labellar chemosensory reception did not trigger the sugar-seeking behavior clearly indicate that the pharyngeal GRNs are necessary to trigger searching behavior. Our results also suggest that stimulation of the labral sense organ is sufficient to induce searching (Table 1). *Gr43a* is expressed in a set of neurons in the protocerebrum and acts as an internal sugar sensor (Miyamoto et al., 2012). We do not think that the brain's fructose-sensing neurons are involved, because the non-nutritional sugar D-arabinose can trigger searching behavior. Further studies will be needed to define the relationship between ligand specificities and GRNs for individual gustatory receptors (Dahanukar et al., 2007; Freeman et al., 2014). We are also interested in testing whether this searching behavior can be induced by optogenetic stimulation of the pharyngeal gustatory neurons.

Previous studies have shown that pharyngeal GRNs project to a distinct region of the subesophageal ganglion, the primary gustatory brain center in insects, which is spatially separated from the projection area of the labellar and tarsal GRNs (Freeman and Dahanukar, 2015; Inoshita and Tanimura, 2006; Thoma et al., 2016). Now, our findings raise the interesting question whether and how this region might be connected to higher brain areas such as the central complex that are involved in initiating and regulating complex locomotion (Strauss, 2002).

In the searching behavior, flies returned repeatedly to the position where the sugar droplet had been placed and sometimes they extended their proboscis there. It will be interesting to see the relationship between searching behavior and positional memory. Searching behavior has been studied in honeybee and ant using a behavioral approach (von Frisch, 1967; Wehner and Srinivasan, 1981), but the neural circuits and molecular mechanisms are not completely clear. Our study has identified the key-stimulus input pathway of searching behavior and provides a cue to reveal the

Journal of Experimental Biology

central brain system triggering the behavior. These studies should help to unveil the neural network involved in local searching behavior.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.B., T.T.; Methodology: S.M., T.T.; Software: S.M.; Investigation: S.M.; Data curation: S.M., T.T.; Writing - original draft: S.M., T.T.; Writing - review & editing: A.B., T.T.; Supervision: T.T.; Project administration: T.T.; Funding acquisition: T.T.

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