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Pharyngeal sense organs drive robust sugar consumption in *Drosophila*:

Pharyngeal taste in adult *Drosophila*

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Abstract

The fly pharyngeal sense organs lie at the transition between external and internal nutrient sensing mechanisms. Here, we investigate the function of pharyngeal sweet gustatory receptor neurons (GRNs), demonstrating that they express a subset of the nine previously identified sweet receptors and respond to stimulation with a panel of sweet compounds. We show that *pox-neuro* (*poxn*) mutants lacking taste function in the legs and labial palps have intact pharyngeal sweet taste, which is both necessary and sufficient to drive preferred consumption of sweet compounds by prolonging ingestion. Moreover, flies putatively lacking all sweet taste show little preference for nutritive or non-nutritive sugars in a short-term feeding assay. Together, our data demonstrate that pharyngeal sense organs play an important role in directing sustained consumption of sweet compounds, and suggest that post-ingestive sugar sensing does not effectively drive food choice in a simple short-term feeding paradigm.

Keywords

Chemosensation; nutrient sensing; feeding; sweet taste

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Author contributions

Y-C.C. performed the wild-type *Gr-GAL4* expression analysis. E.E.L. and M.D.G. performed the experiments to examine *Gr-GAL4* expression in *poxn* mutants and the GCaMP imaging. E.E.L. performed proboscis extension experiments and the majority of binary feeding assays. A.Y.J. performed initial binary feeding assays. E.E.L. and Y-C.C. performed temporal consumption assays. E.E.L., Y-C.C., A.Y.J., A.D., and M.D.G. planned experiments and analyzed the data. M.D.G. and A.D. conceived of and supervised the project. M.D.G. wrote the paper with A.D., E.E.L., and Y-C.C.

Competing financial interests

The authors declare no competing financial interests.

Introduction

Sweet taste plays a key role in promoting ingestion of nutritionally rich sources of carbohydrates. Adult *Drosophila* express sweet taste receptors in gustatory receptor neurons (GRNs) located in the legs, labellum, and a set of three pharyngeal sense organs collectively referred to as the internal mouthparts¹. Sweet GRNs in the legs and labellum are broadly tuned to sugar stimuli, and their activation initiates feeding behaviours including the proboscis extension reflex (PER)²⁻⁷. However, neither the physiology nor behavioural roles of pharyngeal GRNs have been described.

The pharyngeal sense organs consist of the dorsal and ventral cibarial sense organs (DCSO, VCISO) and the more distal labral sense organ (LSO)^{1,8}. The DCSO has two gustatory sensilla on each side of the midline, each housing three GRNs. Each side of the VCISO and LSO has three gustatory sensilla housing a total of eight and ten GRNs, respectively^{8,9}. Axons from pharyngeal GRNs project via the pharyngeal nerve to the subesophageal zone (SEZ) of the brain, where they target an area that is distinct from the projections of leg and labellar GRNs^{1,3}. Mapping of body parts to different areas of the SEZ raises the possibility that taste detection by the legs, labellum, and pharyngeal sense organs may each have distinct ethological importance.

The *Drosophila* genome encodes 68 members of the gustatory receptor (Gr) family, with nine classified as sweet receptors^{2-7,10-13}. Eight of these, Gr5a, Gr61a, and Gr64a-64f, are closely related in sequence and are the defining members of a clade of insect sweet taste receptors¹⁴. Both expression and functional analyses suggest that sweet GRNs co-express multiple sweet receptors^{4,10,11,13,15}. Furthermore, mapping of *Gr promoter-GAL4* expression patterns to identified sensilla in the labellum and tarsi suggests that individual sweet Grs may be expressed in overlapping but distinct subsets of sweet GRNs^{8,9,16,17}. In addition to the sweet clade, a highly conserved receptor, Gr43a, also functions as a sugar receptor^{5,13,18}. Interestingly, Gr43a, which is expressed in a few neurons in the protocerebrum, appears to be restricted to some tarsal and pharyngeal GRNs in the gustatory system^{13,16,17}.

In addition to sweet taste, mounting evidence suggests that the caloric content of sugars can drive feeding preferences in both flies and mammals¹⁹⁻²⁶. To distinguish between the nutritional and gustatory effects of various sugars, it would be beneficial to examine animals that completely lack taste sensory input^{19,21,22}. One proposed means of achieving this effect in flies has been to use *pox-neuro (poxn)* mutants, in which external taste bristles are transformed into mechanosensory bristles^{21,22,27,28}. However, there is evidence that the pharyngeal sense organs of *poxn* mutants retain expression of at least some gustatory genes²⁹. If *poxn* mutants have functional pharyngeal taste sensilla, this could account for their observed preference for caloric sugars^{21,22}.

Here, we attempt to parse out the distinctions between external and internal sugar sensing by examining the role of pharyngeal taste in the fly. We show that the pharyngeal sense organs include several GRNs that express sweet taste receptors and respond to sweet compounds. We also demonstrate that *poxn* mutants have functional pharyngeal sweet GRNs, and use

silencing of these neurons in the *poxn* mutant background to examine the effects of taste and nutrient sensing on feeding decisions. We find that flies lacking all identified sweet GRN function lose most of their preference for sweet compounds in a simple short-term binary choice assay. Our results suggest that taste is the primary influence on short-term feeding decisions and that post-ingestive sugar sensing plays at most only a minor role in this context.

Results

Pharyngeal GRNs express sweet receptors

Previous studies have suggested that sweet receptors are expressed in internal pharyngeal GRNs^{4,13,30}. To assign selected sweet receptors to identified pharyngeal GRNs, we used seven previously reported *Gr promoter-GAL4* transgenes^{16,17} and a LexA knock-in allele of *Gr64f*¹³. Mapping was based on examination of GFP expression in the LSO, VCSO, and DCSO of flies carrying each *Gr-GAL4* or *Gr^{LexA}* driver, as well as analysis of GFP expression in flies carrying two different drivers. Six of the seven sweet *Gr-GAL4* drivers, as well as *Gr64f^{LexA}*, showed expression in the pharynx and suggest that the LSO and VCSO are innervated by sweet GRNs (Figure 1). We did not detect any *Gr5a-GAL4* expression in the pharynx, and none of the sweet *Gr-GAL4* lines tested showed expression in the DCSO (not shown). The map identifies two candidate sweet GRNs per side of the LSO, which co-express Gr43a and Gr64e with other members of the sweet clade. The VCSO also contains two candidate sweet GRNs per side, both of which express *Gr43a-GAL4* and *Gr64e-GAL4*. Thus, *Gr43a-* and *Gr64e-GAL4* offer two tools to explore the physiological and behavioural roles of the pharyngeal sense organs. Both are expressed in all identified candidate pharyngeal sweet neurons as well as GRNs in the legs; however, *Gr64e-GAL4* is also expressed in the taste hairs and taste pegs of the labellum, while *Gr43a-GAL4* lacks labellar expression, but is expressed in sugar-sensing neurons in the protocerebrum^{13,16,17,30}.

Pharyngeal GRNs detect a variety of sugars

To examine the role of sweet taste detected by the pharyngeal sense organs, we began by measuring the response properties of pharyngeal neurons expressing Gr43a. We expressed GCaMP3 under the control of *Gr43a-GAL4* and used an *in vivo* imaging preparation to measure the calcium responses of GRN axon terminals in the SEZ during consumption of sweet stimuli (Figure 2). Pharyngeal Gr43a⁺ GRNs exhibited broad tuning to sweet compounds, with responses to both nutritive (sucrose, fructose, glucose) and non-nutritive (arabinose, L-fucose) sugars, as well as the sweet sugar alcohol glycerol (Figure 2e). Consistent with the reported lack of *Gr5a* expression in the pharyngeal sense organs, we did not observe responses to the Gr5a ligand trehalose³⁻⁵. We also saw no response to the nutritive sugar alcohol sorbitol, which is generally considered tasteless to the fly. Taken together these data confirm that, as predicted by their gustatory receptor expression, a subset of pharyngeal GRNs is activated by the ingestion of sweet compounds. Additionally, it is notable that calcium responses in pharyngeal GRNs were sustained much longer than those previously observed from stimulation of labellar GRNs^{6,31}.

***poxn* mutants retain functional pharyngeal sense organs**

To investigate the behavioural role of internal pharyngeal GRNs, we began by examining null mutants for the transcription factor *poxn*. *poxn* mutants lack external taste bristles, which are instead transformed into mechanosensory bristles²⁸. This has resulted in the use of *poxn* mutants as taste-blind flies^{21,22}. However, the pharyngeal sense organs of *poxn* mutants have been reported to express a reporter for the apparently gustatory-specific odorant binding protein *OBP56b*, raising the possibility that taste sensilla may be intact in these tissues²⁹. Moreover, many of the neurons in the pharyngeal organs of the adult originate in the embryo and persist through the larval stages and metamorphosis, which contrasts with the general principle that adult sensory structures are born during metamorphosis and suggests that the pharyngeal organs may depend on an entirely distinct developmental program⁹. We therefore began by asking whether *poxn* mutants have functional pharyngeal taste sensilla.

Transheterozygotes for two *poxn* null alleles (*poxn*⁷⁰ and *poxn*^{M22-B5}) showed normal expression of *Gr43a-GALA* in GRNs of the LSO and VCSO (Figure 3a,b). Additionally, brains from *poxn* null mutants had morphologically normal projections from pharyngeal GRNs, while they lacked the leg projections seen in otherwise wild-type flies (Figure 3e,f). Examining *Gr64e-GALA* expression in the *poxn* background confirmed these results and additionally demonstrated that labellar taste peg GRNs are also present in *poxn* mutants (Figure 3c,d,g,h). To ask whether the pharyngeal GRNs of *poxn* mutants are functional, we expressed GCaMP3 under the control of *Gr43a-GALA* in the *poxn* null mutant background, and measured calcium responses during ingestion of sweet compounds. We observed robust activation of Gr43a⁺ pharyngeal GRNs upon ingestion of fructose and glycerol but not sorbitol (Figure 3i). Due to the technical difficulties in stimulating flies lacking external taste sensation to ingest sweet tastants during calcium imaging, we did not expand our analysis to a larger panel of compounds. However, it is very likely that *poxn* Gr43a⁺ pharyngeal neurons retain the same receptive fields seen in a wild-type background (Figure 2e). By contrast, Gr64e⁺ taste peg GRNs did not respond to any of the sweet compounds tested but were activated by carbonated water (Figure 3j), as previously reported for taste pegs in a wild-type background³². Together, these data demonstrate unequivocally that *poxn* mutants retain functional pharyngeal taste sensilla that are capable of responding to sweet compounds. Moreover, while functional taste peg GRNs also exist in these mutants, they do not respond to sweet compounds and thus are unlikely to affect our subsequent behavioural analyses of sweet taste preferences driven by the pharyngeal sense organs.

***poxn* mutants prefer sweet compounds**

Given that *poxn* mutants have functional pharyngeal sweet taste, but apparently lack all peripheral sweet taste sensation, we asked whether pharyngeal taste is sufficient to direct consumption of a variety of sweet compounds. First, we verified that *poxn* null mutants lack peripheral sweet taste responses by performing the proboscis extension reflex (PER)^{7,33,34}. We used ribose as a negative control stimulus, as it evokes no significant response from L-type taste sensilla on the labellum⁴. However, our observation that ribose elicits a mean PER response of ~20% in control flies suggests that it may stimulate some appetitive taste neurons at a low level (Figure 4). Alternatively, these responses could be the result of

osmotic differences between the ribose solution and water used to water satiate the flies prior to testing^{35,36}. Nevertheless, control flies (*w¹¹¹⁸*) showed robust PER to all sweet compounds tested, responding at frequencies significantly higher than those elicited by ribose (Figure 4a,c). By contrast, PER was entirely abolished in *poxn* mutants following stimulation of either the labellum or the tarsal segment of the legs (Figure 4b,d).

Next, we subjected *poxn* mutants and controls to a variation of the binary feeding choice paradigm in which groups of food-deprived flies were allowed to feed for two hours on drops of 1% agar with or without a given test compound. Again we used ribose as a negative control because it has little to no taste response or nutritional value^{4,26}. Control flies showed a small preference for ribose (PI = 0.23 +/- 0.09) over plain agar, which may be due to weak taste responses or the osmolarity differences discussed above (Figure 4e). This effect was reduced in *poxn* mutants (PI = 0.06 +/- 0.06).

As expected, *poxn* mutants and controls displayed robust preference for sucrose, fructose, glucose, and glycerol (Figure 4f). All of these compounds are nutritional^{23,25,26} and activate Gr43a⁺ pharyngeal GRNs (Figure 2e). The potential role of nutritional content in guiding feeding decisions is supported by the observation that *poxn* mutants preferred two compounds, trehalose and sorbitol, that are caloric but did not stimulate pharyngeal GRNs in our experiments (Figure 4f). Importantly, *poxn* mutants also strongly preferred arabinose and L-fucose, both of which stimulate sugar taste but offer no caloric value^{4,23,25,26}. These data strongly suggest that activation of pharyngeal taste neurons is sufficient to drive consumption behaviour, and that this activation accounts for at least part of the previously reported preference of *poxn* mutants for caloric sugars^{21,22}.

To further examine the role of pharyngeal taste in driving the preference of *poxn* mutants for sweet compounds, we silenced Gr64e⁺ pharyngeal GRNs in the *poxn* mutant background through expression of the inward rectifying potassium channel KIR2.1³⁷. Importantly, the insertion of *Gr64e-GAL4* used (*Gr64e-GAL4^{II}*) lacks expression in the taste pegs (Figure 5a), meaning that silencing specifically affects pharyngeal GRNs. Silencing of Gr64e⁺ GRN function in *poxn* mutants resulted in a complete loss of preference for arabinose and L-fucose over agar alone, indicating that Gr64e⁺ pharyngeal GRNs are necessary for the preference for non-caloric sweet sugars in *poxn* mutants (Figure 5b). This suggests that *Gr64e-GAL4* likely labels the complete set of pharyngeal sweet GRNs, and that *poxn* mutants lacking Gr64e⁺ pharyngeal GRN function may be sweet-blind.

Since *poxn*, Gr64e-silenced flies lack both peripheral and identified pharyngeal sweet taste, we used them to re-evaluate the role of post-ingestive nutrient sensing in driving preference for a number of sugars. Silencing of pharyngeal taste caused only a mild, insignificant decrease in the preference of *poxn* mutant flies for sorbitol (Figure 5b), consistent with previously reported post-ingestive mechanisms promoting consumption of this compound, and its reported tastelessness¹³. However, it's worth noting that the preference for sorbitol in these experiments was weak, so it is difficult to confidently ascribe a taste-independent effect. In contrast to sorbitol, the preference for trehalose was significantly reduced following silencing of Gr64e⁺ pharyngeal GRNs, suggesting that this sugar may stimulate

pharyngeal GRNs at a level below the sensitivity of our calcium imaging but enough to affect behaviour.

Strikingly, silencing Gr64e⁺ pharyngeal GRNs dramatically reduced the preference of *poxn* flies to all four sweet and nutritive compounds tested (Figure 5b). These data suggest that taste is the dominant driver of feeding preference in our short-term binary choice assay, and, in contrast to previous reports^{21,22}, taste-independent post-ingestive sugar sensing has little, if any, effect on consumption behaviour in this context.

To further probe the factors influencing sugar preference, we repeated the silencing experiment using a GAL4 knock-in allele of *Gr43a* that is expressed in the same complement of pharyngeal GRNs as *Gr64e-GAL4^{II}* but also shows additional expression in identified sugar-sensing neurons in the protocerebrum and a population of neurons in the proventricular ganglion¹³. Consistent with a role for Gr43a⁺ brain neurons in promoting the ingestion of sorbitol, we observed a complete lack of sorbitol preference in Gr43a-silenced *poxn* mutants (Figure 5b). Notably, this was a significant reduction compared to both genetic controls and Gr64e-silenced flies. Interestingly, while the behavioural assay used may lack the resolution to tease out small differences between Gr64e and Gr43a silencing, we observed a trend towards increased preference for sweet and nutritive sugars in Gr43a-silenced mutants compared to Gr64e-silenced flies. This trend, which was insignificant for sucrose and fructose but significant for glycerol, could reflect weaker silencing with the *Gr43a^{GAL4}* driver, a difference in genetic background, or the previously reported role for Gr43a⁺ brain neurons in promoting feeding termination of nutritive sweet sugars in some contexts¹³. Nevertheless, overall our silencing results strongly support the conclusion that short-term sugar preferences are primarily taste-mediated, even in *poxn* mutants lacking external gustatory sensilla.

Pharyngeal sweet GRNs sustain ingestion

Based on the anatomical position of pharyngeal GRNs, we wondered whether they might affect food preference by preferentially sustaining the ingestion of sweet compounds. To test this we subjected *poxn*, Gr64e-silenced flies to a temporal consumption assay³⁸ and compared their behaviour to that of *poxn* controls with intact pharyngeal GRN function (Figure 5c). We found that *poxn* flies with silenced pharyngeal GRNs displayed a dramatic reduction in the duration of consumption during the first bout of feeding on a solution of sweet, non-nutritive arabinose compared to non-silenced controls, as well as a reduction in total feeding time over multiple bouts and an elevated number of feeding bouts prior to “satiety” (defined here as the refusal to initiate further feeding). These data support the notion that pharyngeal GRNs indeed function to sustain ingestion of sweet, appetitive food sources.

Discussion

Much is known about sugar sensing through peripheral sweet taste neurons in the fly's legs and labellum, and there is growing interest in mechanisms that sense dietary sugars following ingestion^{39,40}. Sugar sensing by the pharyngeal sense organs, however, has remained virtually unexplored due to their inaccessibility to electrophysiology and the lack

of genetic tools to specifically manipulate their function. This represents an important gap in our understanding of how sugar feeding is regulated, since the pharyngeal sense organs must operate following the initiation of feeding, but prior to ingestion. They are therefore poised to provide feedback during feeding, contributing to the ongoing decision of whether to maintain or terminate ingestion.

Our calcium imaging data demonstrated that the receptive fields of pharyngeal sweet GRNs are in line with predictions based on receptor expression and specificities^{4,5,10,11,13,15,41}. All putative pharyngeal sweet GRNs co-express Gr43a and Gr64e, which are receptors tuned to fructose and glycerol, respectively, and we observed robust calcium responses to those two compounds. Expression of Gr61a in the LSO likely accounts for the response to glucose, since Gr61a-dependent glucose responses are seen in tarsal GRNs lacking Gr5a⁴¹. Likewise, sucrose and L-fucose responses can be accounted for by the expression of Gr64a in the LSO^{4,5,10}. Notably, we did not observe responses to trehalose, consistent with the lack of Gr5a expression, which is necessary for trehalose responses in the labellum⁴. However, our behavioural data suggested that Gr64e⁺ pharyngeal GRNs mediate some attraction to trehalose. This apparent discrepancy could be explained if trehalose excites sweet pharyngeal GRNs at a level too low to observe significant calcium responses in our preparation, a possibility supported by previous reports that overexpression of Gr43a or Gr64e confers trehalose sensitivity to tarsal GRNs or ab1C olfactory receptor neurons, respectively^{5,13}.

Using *poxn* mutants lacking peripheral taste, we demonstrated that pharyngeal sweet GRNs are sufficient to drive preference for sweet compounds in binary choice feeding assays. These results indicate that appetitive taste is not absolutely necessary for feeding initiation, and that flies must “sample” potential food sources in the absence of sweet taste input from the legs and labellum. Moreover, we provide evidence that activation of pharyngeal sweet GRNs prolongs feeding by providing a positive feedback signal to sustain ingestion. This proposed role for pharyngeal GRN function is also consistent with their physiology, which exhibits prolonged activation during consumption of sweet compounds compared to previously reported responses in the labellum^{6,31}. It is likely that this prolonged activation functions to maintain ingestion during feeding bouts lasting up to several seconds. In the future, it will be interesting to examine whether pharyngeal sweet taste plays any specific roles outside of feeding. For example, pharyngeal bitter GRNs appear to be important in selection of egg laying substrates⁴².

A key component of our work is the demonstration that *poxn* mutants have a functional pharyngeal taste system that is critical in guiding their preference for sweet compounds, which therefore precludes their experimental use as “taste-blind” flies. The behavioural preference we observed of *poxn* mutant flies for the non-nutritional sweeteners L-fucose and arabinose contrasts with the conclusion reached by Dus and colleagues (2011), who performed a similar experiment with the artificial sweetener sucralose. One possible explanation for this difference is that arabinose and L-fucose may activate pharyngeal sweet GRNs more potently than sucralose. Another possible source of the discrepancy is that Dus and colleagues analyzed their feeding data by plotting the total proportion of flies to eat each option in the binary choice assay, while we analyzed relative preference between the

options. Nevertheless, the strong dependence of *poxn* mutants on Gr64e⁺ GRNs for preferred consumption of both nutritive and non-nutritive sweeteners demonstrates that the majority of the preference of these mutants for sweet compounds is mediated by pharyngeal taste sensitivity.

By silencing Gr64e⁺ GRNs in a *poxn* mutant background, we created for the first time a fly that may completely lack sweet taste, allowing one to reevaluate the taste-independent role for nutrient sensing mechanisms in behaviour. While we did observe some evidence for taste-independent selection of nutritive carbohydrates, the observed effect was much weaker than previously suggested^{21,22}. Although ample additional evidence supports the existence of taste-independent carbohydrate sensing^{13,23-26}, its specific role in regulating feeding remains unclear. Why do we observe such weak preference of putatively sweet-blind flies for nutritive sugars? One possibility is the particular behavioural assay used, which operates over only two hours. We have previously reported increasing effects of caloric content on feeding preferences over the course of a 16-hour assay²⁶. It will be instructive to reevaluate the feeding behaviour of *poxn*, sweet GRN-silenced flies over longer time periods using different behavioural paradigms.

Our newly established putatively sweet-blind flies also afforded the opportunity to examine potential nutrient sensing mechanisms in a taste-independent context. For example, by comparing Gr43a⁺-silenced to Gr64e⁺-silenced flies in a *poxn* mutant background, we were able to observe effects for non-taste Gr43a⁺ populations that are largely consistent with those previously reported¹³. Further evaluation of these and other putative nutrient-sensing cell populations in a sweet taste-blind background will continue to shed light on the apparently complex interactions between the taste-dependent and -independent mechanisms that ultimately guide critical feeding decisions.

Methods

Fly stocks

Flies were raised on standard cornmeal fly food at 25°C and 70% relative humidity. The following fly lines were used: *Gr5a-GAL4*, *Gr43a-GAL4*, *Gr64a-GAL4*, *Gr61a-GAL4*, *Gr64c-GAL4*, *Gr64d-GAL4*, and *Gr64e-GAL4* (ref¹⁶); *Gr64^fLexA* and *Gr43a^{GAL4}* (ref¹³); *UAS-GCaMP3* (ref⁴³); *UAS-KIR2.1* (ref³⁷); *UAS-TdTomato* and *UAS-GFP* (Bloomington stock center); *poxn^{M22-B5}* (ref⁴⁴); *poxn⁷⁰* (ref²⁸).

Immunohistochemistry

Immunohistochemistry was carried out as previously described³³. The primary antibodies used were rabbit anti-GFP (1:1000; Invitrogen) and mouse nc82 (1:50, DSHB). The secondary antibodies used were goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-568 (Invitrogen). Images are maximum intensity projections of confocal z-stacks acquired using a Leica SP5 II confocal microscope with 25x water immersion objective.

Gr expression mapping

Expression of sweet *Gr promoter-GAL4* lines was mapped in the three pharyngeal sense organs by visualizing *UAS-mCD8::GFP* fluorescence in live tissue. Proboscis tissue was dissected and mounted in 80% glycerol in 1X PBS before imaging with a Leica SP5 laser scanning confocal microscope. For double driver analysis, the *UAS-mCD8::GFP* transgene was under the control of two different *Gr promoter-Gal4* transgenes and the number of GFP-labeled neurons were compared to those in flies containing a single Gal4 driver alone.

GCaMP imaging

Female flies aged 2-12 days were used for calcium imaging. To prepare flies for imaging, they were briefly anesthetized and all legs were removed to allow unobstructed access to the proboscis. Using a customized chamber, each fly was mounted by inserting the cervix into individual collars. To further immobilize the head, nail polish was applied in a thin layer to seal the head to the chamber. Melted wax was applied using a modified dental waxer to adhere the fully extended proboscis to the chamber rim. The antennae and associated cuticle covering the SEZ were removed and AHL buffer with ribose⁴⁵ was immediately injected into the preparation to cover the exposed brain. A coverslip was inserted into the chamber to keep the proboscis dry and separated from the bath solution.

GCaMP3 fluorescence was viewed with a Leica SP5 II laser scanning confocal microscope equipped with a tandem scanner and HyD detector. The relevant area of the SEZ was visualized using the 25x water objective with an electronic zoom of 8. Images were acquired at a speed of 8000 lines per second with a line average of 4, resulting in collection time of 60 ms per frame at a resolution of 512 × 200 pixels. The pinhole was opened to 2.68 AU. Stimuli were applied to the proboscis using a pulled glass pipette, and flies were allowed to ingest solutions during imaging. The maximum change in fluorescence ($\Delta F/F$) was calculated as the peak intensity change divided by the average intensity over 10 frames prior to stimulation.

Behavioural assays

For PER, adult female flies were aged 3-10 days and starved on 1% agar at room temperature (~22°C) for 24 hours before testing. For tarsal PER, flies were mounted on glass slides using nail polish. For labellar PER, flies were placed inside a pipette tip cut to size so that only the head was exposed. Flies were then sealed into the tube with tape, and then adhered to a glass slide with double-sided tape. Flies were allowed 1-2 hours to recover before testing began. Flies were stimulated with water on their front tarsi or labella for tarsal and labellar PER, respectively, and allowed to drink until satiated. Each fly was then stimulated with a tastant on either the tarsi or labella, and responses to each of three trials were recorded. Flies were provided with water between each tastant. All stimuli were delivered with a 1 mL syringe attached to a 20 μ L pipette tip. For statistical purposes, each trial was treated as an independent unit of analysis.

Binary choice preference tests were performed similarly to previous descriptions^{16,21,46}. Female flies aged 3-8 days were sorted into groups of 10 at least two days prior to the experiment, and starved on 1% agar at room temperature (~22°C) for 24 hours before

testing. For the assay, flies were transferred into standard vials containing six 10 μ L dots of agar that alternated in color. The agar substrates were 1% agar with or without the test stimulus at a concentration of 100 mM. Each choice contained either 0.125 mg/mL blue (Erioglaurine, FD&C Blue#1) or 0.5 mg/mL red (Amaranth, FD&C Red#2) dye, and half the replicates for each stimulus were done with the dyes swapped to control for any dye preference. Flies were allowed to feed for 2 hours in the dark at 25°C and then frozen and scored for abdomen color. Preference index (PI) for sugar was calculated as ((# of flies labeled with the stimulus color) – (# of flies labeled with the plain agar color))/(total number of flies that fed).

The temporal consumption assay was performed on flies deprived of food for 24 hours³⁸. As described above for PER, flies were mounted on glass slides using nail polish and allowed to recover for 1-2 hours in a humidified chamber. Water satiated flies were then offered 50 mM arabinose on their labella. Once they initiated feeding, the time between starting and stopping their first feeding bout was recorded (first bout length). The fly was then offered arabinose again and if they failed to reinitiate feeding for three consecutive stimulations, the assay was terminated for that fly. If flies began a new bout of feeding upon stimulation, the time of the subsequent bouts was added to the first bout to determine total consumption time, and number of bouts was recorded.

Statistical analyses

For PER analyses, the 95% binomial confidence interval was calculated using JavaStat (<http://statpages.org/confint.html>). Fisher's exact tests were calculated using Graphpad QuickCalcs (<http://www.graphpad.com/quickcalcs/>). All other statistical tests performed using GraphPad Prism 6 or SPSS software. No statistical test was used to predetermine sample size. Sample size ranges were chosen based on previously published examples of the same assays.

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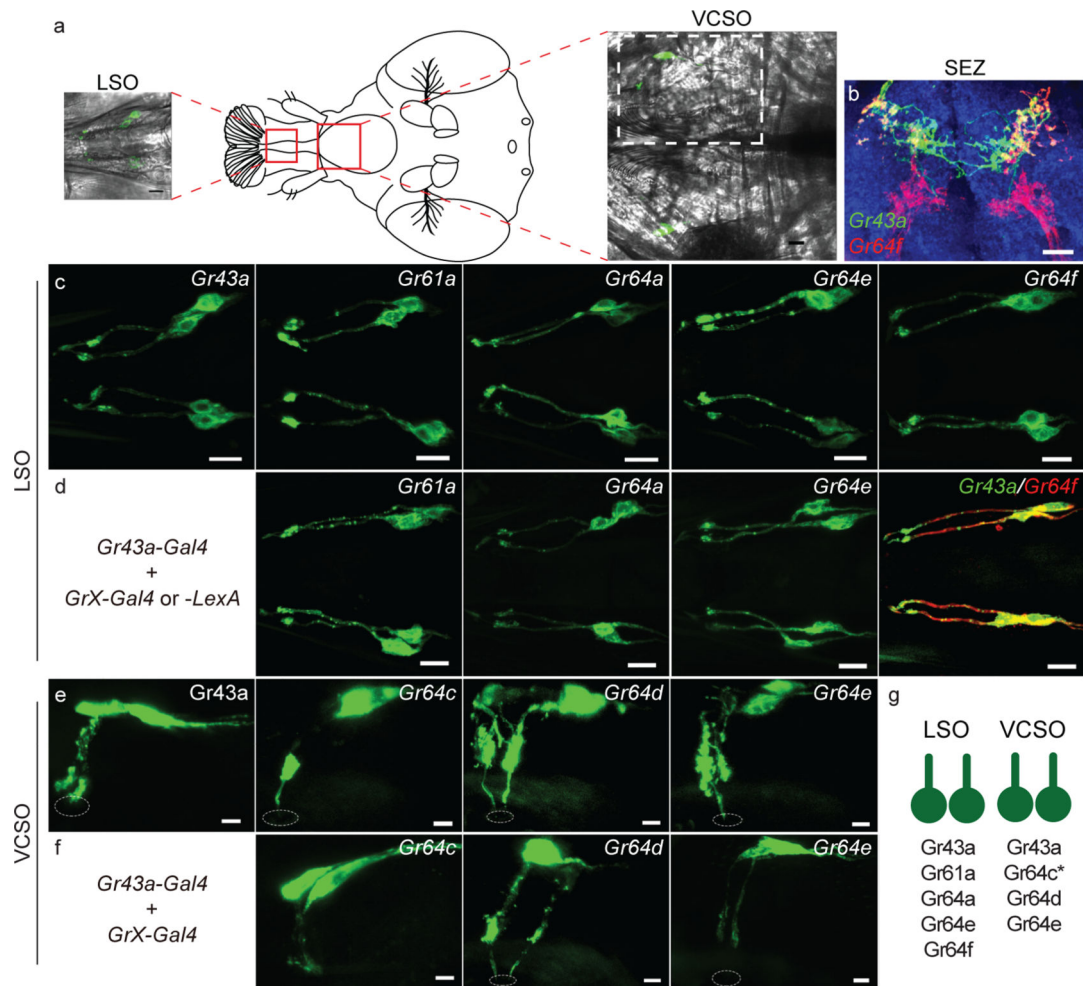
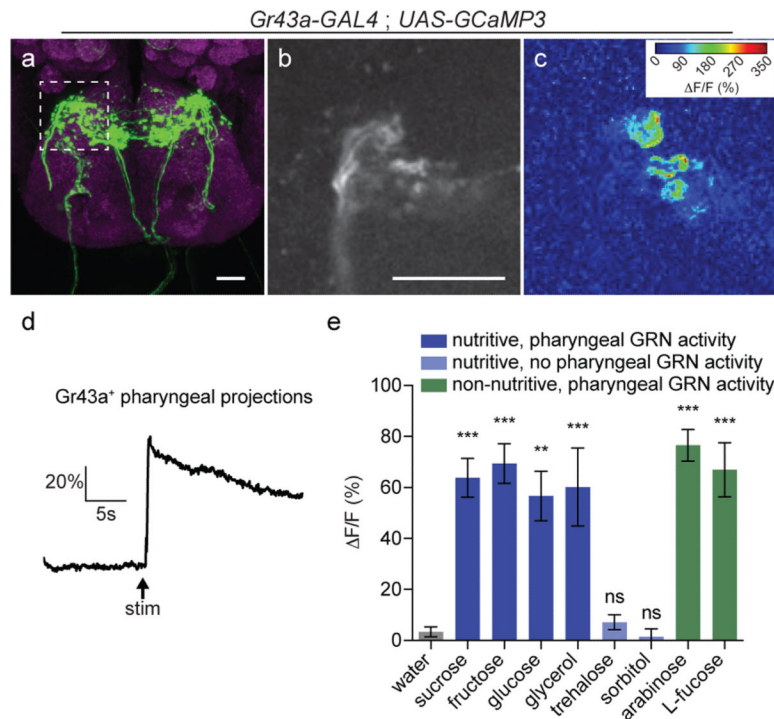


Figure 1.

Pharyngeal GRNs express sweet Grs (a) Cartoon showing the positions of the LSO and VCISO, with associated images of each structure from a fly expressing GFP under control of *Gr43a-GAL4*. Dotted white box indicates area shown in e-f. (b) Axonal projections of *Gr43a-GAL4* (green) and *Gr64f^{LexA}* (red) to the SEZ. Overlapping regions are from LSO projections. (c) *Gr-GAL4*-driven GFP expression in LSO. Scale bars are 10 μ m in c-d. (d) LSO GFP expression from flies carrying *Gr43a-GAL4* and indicated second *Gr-GAL4* or *Gr64f^{LexA}*. (e) *Gr-GAL4*-driven GFP expression in VCISO. (f) VCISO GFP expression from flies carrying *Gr43a-GAL4* and indicated second *Gr-GAL4*. Scale bars are 5 μ m in e-f. Dotted circles indicate the cuticular pore of sensilla. (g) Schematic of observed sweet Gr expression in LSO and VCISO GRNs. Asterisk indicates that *Gr64c-GAL4* expression is seen in only one neuron per side of the VCISO.

**Figure 2.**

Pharyngeal GRNs respond to sweet compounds (a) Immunofluorescence of anti-GFP (green) and nc82 (magenta) in the SEZ of flies expressing GCaMP3 under control of *Gr43a-GAL4*. Dotted line shows area imaged in panel (b). (b) Single optical section of baseline GCaMP3 fluorescence in pharyngeal GRN axon terminals. Scale bars are 20 μ m in a-b. (c) Heat map showing change in GCaMP3 fluorescence during ingestion of 1 M fructose. (d) Representative trace of fluorescence change of GCaMP3 in Gr43a axon terminals during ingestion of 1 M fructose. Arrow indicates time at which stimulus is applied to the proboscis to initiate feeding. (e) Peak fluorescence changes of GCaMP3 in Gr43a axon terminals during ingestion of 1 M solutions of the indicated compounds. Values represent mean \pm s.e.m. for $n = 5$ flies per stimulus ($n = 4$ for sorbitol), with data collected over at least 2 days. Asterisks indicate significant difference from water by one-way ANOVA with Bonferroni correction for multiple comparisons: ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

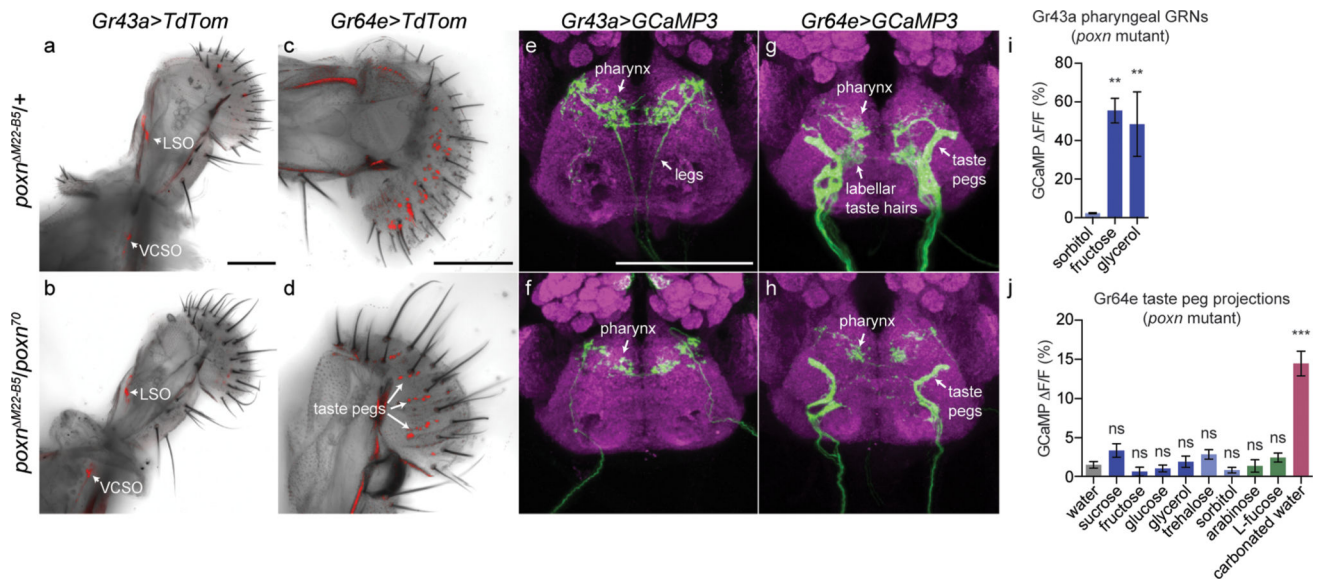


Figure 3. *poxn* null mutants retain functional pharyngeal sense organs (a,b) Pharyngeal GRNs labeled by *Gr43a-GAL4* driving *UAS-TdTomato* in *poxn*^{M22-B5/+} heterozygotes (a) and *poxn*^{M22-B5/poxn70} null mutants (b). Arrows point to GRNs in the LSO and VCISO. (c,d) Labellar GRNs labeled by *Gr64e-GAL4* driving *UAS-TdTomato* in *poxn*^{M22-B5/+} heterozygotes (a) and *poxn*^{M22-B5/poxn70} null mutants (b). Arrows point to taste peg GRNs in d. (e-h) Immunofluorescence of anti-GFP (green) and nc82 (magenta) in the brains of *poxn*^{M22-B5/+} heterozygotes (e,g) and *poxn*^{M22-B5/poxn70} null mutants (f,h) expressing GCaMP3 under control of *Gr43a-GAL4* (e,f) or *Gr64e-GAL4* (g,h). Arrows point to GRN projections originating from the various body locations. (i-j) Peak fluorescence changes of GCaMP3 in *Gr43a-GAL4* pharyngeal (i) or *Gr64e-GAL4* taste peg (j) axon terminals in *poxn*^{M22-B5/poxn70} null mutants during ingestion of the indicated compounds. Values represent mean \pm s.e.m. for $n = 5$ flies, with data collected over at least 2 days. Asterisks indicate significant difference from sorbitol (i) or water (j) by one way ANOVA with Bonferroni correction for multiple comparisons: ** $p < 0.01$, *** $p < 0.001$, ns = not significant. Scale bars are 100 μ m.

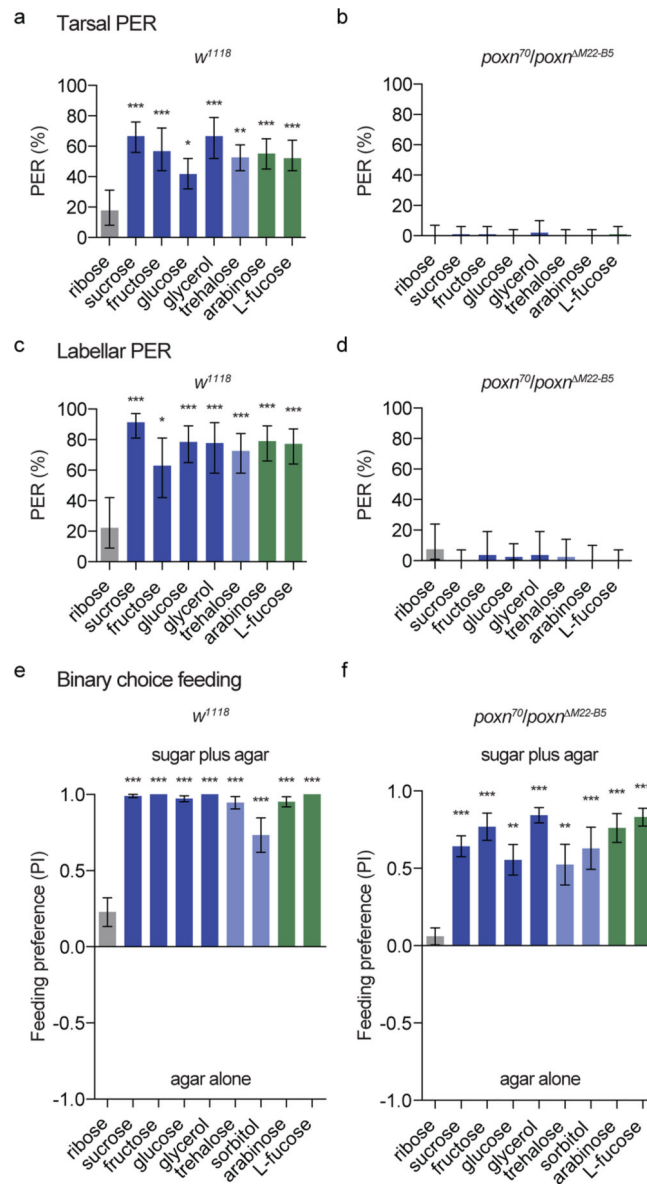


Figure 4. *poxn* null mutants lack peripheral taste responses but prefer sweet compounds (a-d) PER responses of *w¹¹¹⁸* (a,c) and *poxn^{M22-B5}/poxn⁷⁰* null mutant (b,d) flies following stimulation of the tarsi (a,b) or labellum (c,d) with the indicated compounds. Values represent percentage of stimulations resulting in a positive response; error bars show 95% binomial confidence interval, and asterisks indicate significant difference from ribose stimulation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Fisher's exact test. $n = 17-50$ flies for *w¹¹¹⁸* tarsal PER, $n = 17-33$ flies for *poxn* tarsal PER, $n = 9-19$ flies for *w¹¹¹⁸* labellar PER, and $n = 9-17$ flies for *poxn* labellar PER. (e,f) Preference of *w¹¹¹⁸* (e) and *poxn^{M22-B5}/poxn⁷⁰* null mutant flies (f) for 100 mM solutions of the indicated compounds in 1% agar versus agar alone. Values represent mean \pm s.e.m. for $n = 10$ groups of 10 flies each, with independent replicates performed over at least 2 days. Asterisks indicate significant

difference from ribose preference by one-way ANOVA with Bonferroni correction for multiple comparisons: **p < 0.01, ***p < 0.001.

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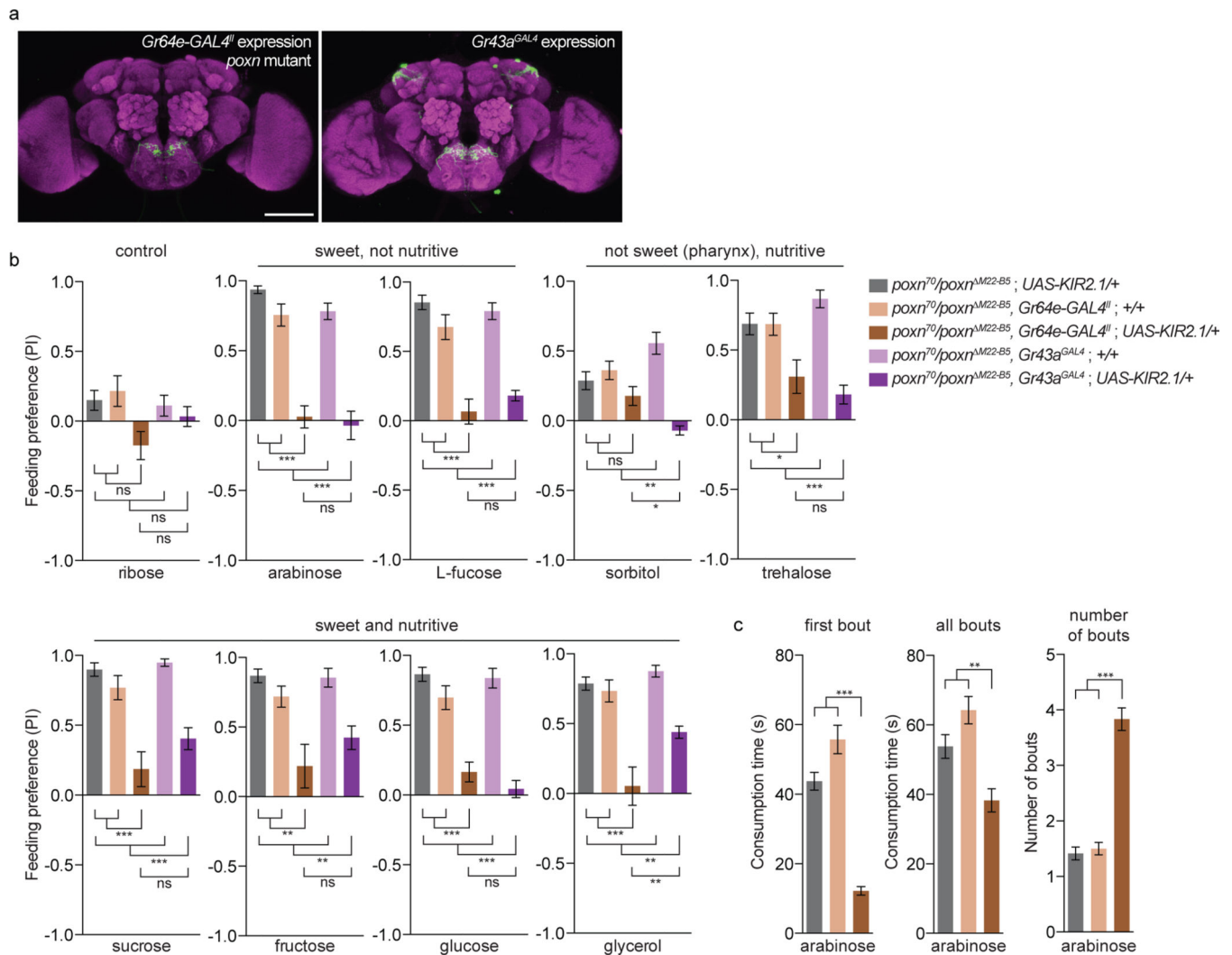


Figure 5.

Pharyngeal GRNs are necessary for the preference of *poxn* mutants for sweet compounds (a) Immunofluorescence of anti-GFP (green) and nc82 (magenta), showing expression of the *Gr64e-GAL4^{II}* and *Gr43a^{GALA}* drivers used in the behavioural experiments shown. *Gr64e-GAL4* is shown in a *poxn* null mutant background, while *Gr43a^{GALA}* is in a *poxn*/+ heterozygous background. Scale bars are 100 μ m. (b) Preference of indicated genotypes for 100 mM solutions of the specified compounds in 1% agar (positive) versus agar alone (negative). (c) Temporal consumption characteristics of the indicated genotypes in response to stimulation with 50 mM arabinose. Values represent mean \pm s.e.m. for $n = 10$ groups of 10 flies each in b and $n = 29-60$ flies in c, with independent replicates performed over at least 2 days. Asterisks indicate significant difference by one-way ANOVA with Bonferroni correction for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.