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Report

The Molecular Basis of Sugar Sensing in *Drosophila* Larvae

Dushyant Mishra,[1] Tetsuya Miyamoto,[1] Yohannes H. Rezenom,[2] Alex Broussard,[1] Ahmet Yavuz,[1] Jesse Slone,[1,3] David H. Russell,[2] and Hubert Amrein[1,*]
[1]Department of Molecular and Cellular Medicine, College of Medicine, Texas A&M Health Science Center
[2]Department of Chemistry, College of Science, Texas A&M University
College Station, TX 77843, USA

Summary

Evaluation of food chemicals is essential to make appropriate feeding decisions. The molecular genetic analysis of *Gustatory receptor* (*Gr*) genes and the characterization of the neural circuits that they engage has led to a broad understanding of taste perception in adult *Drosophila* [1, 2]. For example, eight relatively highly conserved members of the *Gr* gene family (*Gr5a*, *Gr61a*, and *Gr64a-f*), referred to as *sugar Gr* genes, are thought to be involved in sugar taste in adult flies [3–8], while the majority of the remaining *Gr* genes are likely to encode bitter taste receptors [9–11], albeit some function as pheromone [12–14] and carbon dioxide [15, 16] receptors. In contrast to the adult fly, relatively little is known about the cellular and molecular basis of taste perception in larvae. Here, we identify Gr43a, which was recently shown to function as a hemolymph fructose sensor in adult flies [17], as the major larval sugar receptor. We show that it is expressed in taste neurons, proventricular neurons, as well as sensory neurons of the brain. Larvae lacking *Gr43a* fail to sense sugars, while larvae mutant for all eight *sugar Gr* genes exhibit no obvious defect. Finally, we show that brain neurons are necessary and sufficient for sensing all main dietary sugars, which probably involves a postingestive mechanism of converting carbohydrates into fructose.

Results

The larval taste system is compartmentalized, and taste neurons are found in five major anatomical sites (Figure 1A) [18, 19]. Two of these sites, the terminal and ventral organ (TO and VO; Figure 1A), sample soluble chemicals externally. Three internal taste organs, the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, and PPS) monitor chemicals as food passes through the pharynx (Figure 1A). About 40 *Gr* genes are expressed in neurons of both the external taste organs, as well as in internal pharyngeal sense organs; however, none of the eight *sugar Gr* genes are expressed in larval chemosensory neurons [19], even though larvae can sense most sugars [20].

Larvae Exhibit High Sensitivity to Fructose and Sucrose

To identify larval sugar receptor(s), we adapted the two-choice taste assay developed by Schipanski and colleagues [20]. w^{1118} (i.e., *Gr43a*$^{+}$ control) larvae show strong preference for fructose and sucrose across a wide concentration range (500 mM to 0.8 mM; Figure S1 available online). Larvae exhibit an immediate attraction (i.e., within 2 min) to both sugars at concentrations as low as 20 mM, while preference at even lower concentrations is delayed and not noticeable until 4 min or more. An immediate preference for glucose and trehalose is observed only at very high concentrations (1 M and 500 mM), but a preference for both sugars is also noticeable at 100 mM concentrations after 16 min (Figure S1). These observations confirm previous findings [20] and reveal that larvae exhibit a distinct attraction for sugar containing agar in a time- and concentration-dependent fashion.

Gr43a Is Expressed in Larval Taste Neurons

While the *sugar Gr* genes appear not to be expressed in larvae, *Gr43a* was found in two internal taste neurons of larvae [19]. To investigate *Gr43a* expression in more detail, we employed *Gr43a*^{GAL4}, an allele in which the coding sequence of *Gr43a* was replaced by that of *Gal4* using homologous recombination [17]. *Gr43a*^{GAL4} is expressed in approximately four neurons in the pharyngeal taste clusters (DPS, VPS, and PPS), while no expression is observed in the external taste organs (TO and VO) [19] (Figure 1B). Combining *Gr43a*^{GAL4} and *Gr66a-GFP-IRES-GFP-IRES-GFP*, a marker for bitter-sensing neurons, revealed no overlap in expression between these two genes (Figures 1C and 1D), which is consistent with a possible function of *Gr43a* in sugar sensing. We note that *Gr43a*^{GAL4} is also expressed in neurons associated with the proventriculus (Figure 1B; Figure S2). Furthermore, immunostaining identified *Gr43a*^{GAL4} expression in the brain, including neurons in the protocerebrum, the mushroom body and the ventral nerve cord (VNC; Figure 1E; Figure S2).

Gr43a Is the Main Sugar Receptor in Larvae

We next carried out behavioral two-choice preference assays for fructose and glucose using w^{1118} control larvae, larvae lacking all eight *sugar Gr* genes, *Gr43a*^{GAL4} mutant larvae, and larvae in which the *Gr43a*^{GAL4} null mutation is rescued by a *UAS-Gr43a* transgene (Figure 2A). The preference for fructose was abolished in *Gr43a* mutants but not in larvae lacking the *sugar Gr* genes. Importantly, *Gr43a* mutant larvae expressing a *UAS-Gr43a* transgene regained their preference for fructose (Figure 2A; Figure S3A). When larvae were tested for glucose preference, all genotypes but the *Gr43a* mutants exhibited a pronounced attraction to this sugar (Figure 2A). However, establishing this preference required prolonged assay time (16 min). Regardless, these observations indicate that the preference for glucose and fructose is dependent on a single Gr protein, Gr43a, which is surprising since this receptor is narrowly tuned to fructose and does not respond to glucose [17, 21]. We therefore tested larvae with two additional sugars, melezitose and sorbitol (Figure 2B). Melezitose is a complex nutritious sugar containing a fructose moiety and indeed was sensed immediately, just like fructose. Sorbitol, a tasteless sugar alcohol, is sensed in a moderately delayed fashion (but faster than glucose) by wild-type larvae but not sensed at all in *Gr43a* mutants. Importantly, the phenotype

[3]Present address: Department of Biology, College of Art and Sciences, Vanderbilt University, Nashville, TN 37235, USA
*Correspondence: amrein@tamhsc.edu

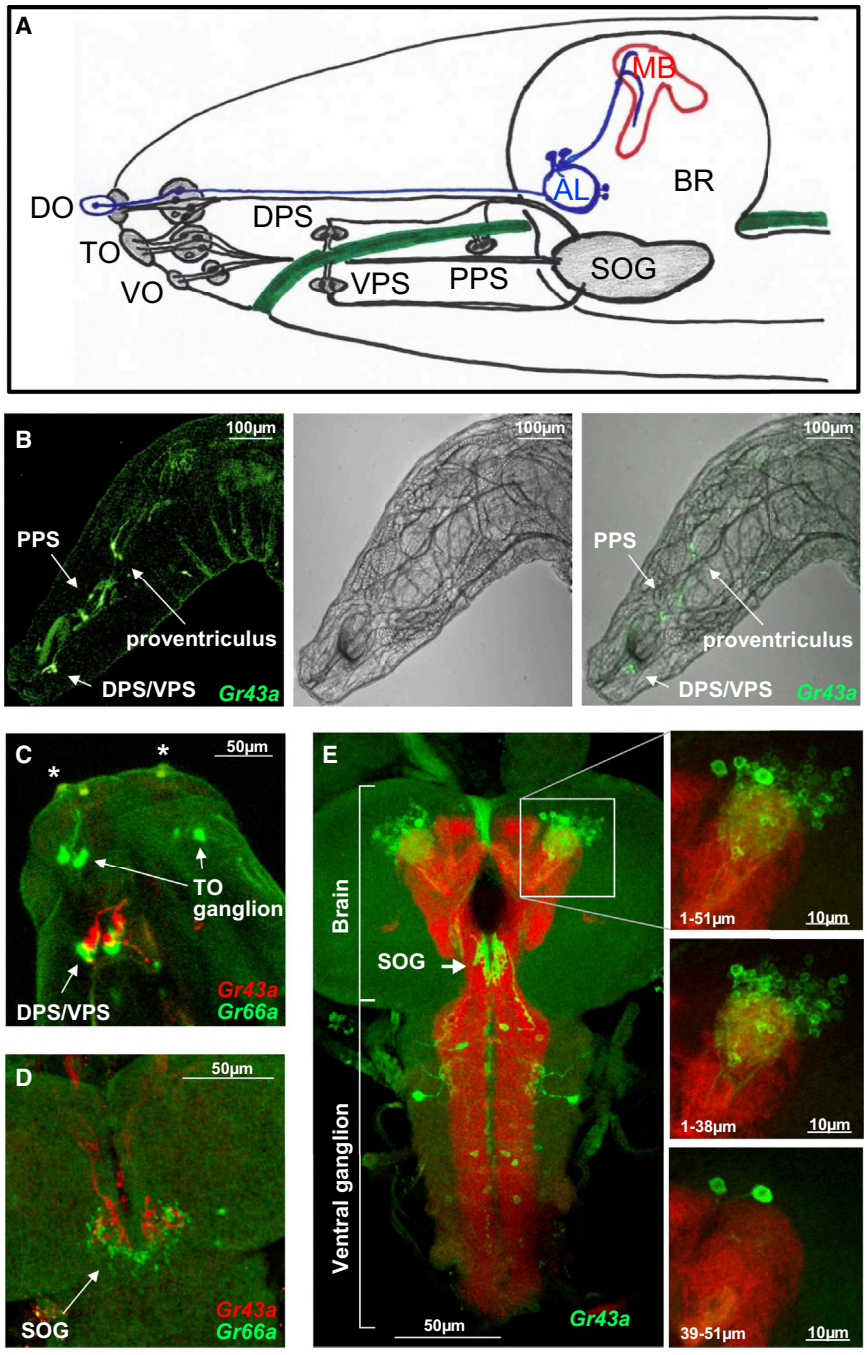


Figure 1. GR43a Is Expressed in the Larval Taste Organs, as well as the Brain and the Gastrointestinal System

(A) Diagram of larval taste organs, adapted from Stocker [18]: structures associated with taste sensing and processing are shown in gray. The cell bodies of the taste neurons are arrayed in ganglia and extend dendrites into the taste organ and axons into the subesophageal ganglion. Olfactory structures are shown in blue. Note that only three brain structures—subesophageal ganglion, the antennal lobe, and the mushroom bodies—are specifically indicated. The pharynx is shown in green. Abbreviations: AL, antennal lobe; BR, brain; MB, mushroom bodies; SOG, subesophageal ganglion; DO/TO/VO, dorsal/terminal/ventral organ; DPS/VPS/PPS, dorsal/ventral/posterior pharyngeal sense organ. (B) Overall view of *Gr43a* expression in larvae. *Gr43a*^{GAL4} drives *UAS-mCD8GFP* expression in neurons located in chemosensory organs, the DPS/VPS and the PPS, and the proventricular ganglion. The live expression of mCD8GFP is at the left, the phase-contrast image at the center, the overlaid image is at the right. (C and D) Expression analyses of *Gr43a*^{GAL4} *UAS-mCD8RFP* (red) and *Gr66a-GFP-IRES-GFP-IRES-GFP* (green) in chemosensory organs (C) and their projections to the SOG (D). *Gr43a* is expressed only in the DPS/VPS neurons, but it is not expressed in the same neurons as *Gr66a*. Note that *Gr66a* is also expressed in neurons of the TO ganglion besides of the DPS/VPS neurons. Asterisks indicate DO and TO. (E) *Gr43a*^{GAL4} drives mCD8GFP expression in the CNS. The images on the right are an enlargement view of the dorsal protocerebrum. *Gr43a*^{GAL4} is expressed in one or two big neurons per hemisphere and in many Kenyon cells. *Gr43a*^{GAL4}-expressing neurons are also located in the VNC. Neuropil was counterstained with nc82.

of *Gr43a* mutants to both melezitose and sorbitol is rescued with the expected temporally displayed preference in the presence of a *UAS-Gr43a* transgene (Figure 2B). Lastly, lack of preference is specific to sugars because *Gr43a* mutant and control larvae showed similar preference for agar containing casein peptone (an amino acid/peptide mixture), a mixture of three amino acids or low concentration of sodium chloride (Figure 2C; Figure S3B). Taken together, our analysis shows that in *Drosophila* larvae, (1) fructose is the most stimulating sugar substrate, (2) Gr43a is the main sugar receptor and acts independently of the sugar Gr proteins, (3) the sugar Gr proteins play no significant role in sugar sensing, and (4) nonfructose-containing sugars, such as glucose and trehalose, are probably sensed by a postprandial mechanism that relies on conversion of (a fraction of) these sugars into fructose ([22], see below).

Sugar Sensing by the Larval Brain

The two-choice feeding assay reveals a time-dependent component for establishing a food preference. This is evident when comparing the response for six different sugars versus agar alone at 100 mM concentration over a 16 min assay period. Larvae show an immediate preference (i.e., within the first 2 min) for fructose and sugars containing a fructose moiety (sucrose, melezitose), while they fail to establish such a preference for glucose, trehalose, or sorbitol (Figures 2A and 2B; Figures S1 and S3A). Notably, after 16 min, wild-type larvae, but not *Gr43a* mutant larvae, exhibit a robust, late preference for all these sugars as well (Figures 2A and 2B). We propose that the late preference for glucose, trehalose, and sorbitol is mediated by internal *Gr43a*-expressing neurons, probably those in the brain. In adult flies, a fraction of ingested nutritious carbohydrates are converted into fructose, leading to transient fructose increase in the hemolymph, which in turn mediates satiation-dependent feeding activity [17].

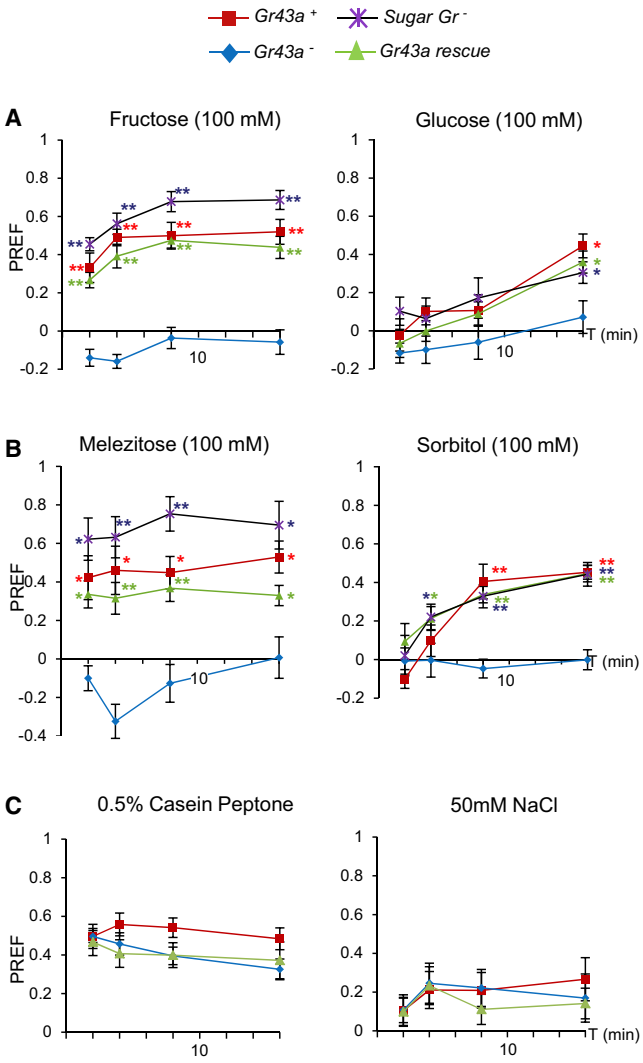


Figure 2. *Gr43a* Is Required for Fast and Slow Preference to Fructose and Nonfructose-Containing Sugars

Time-dependent taste preference of w^{1118} (*Gr43a*$^{+}$), *w; Gr43a*$^{GAL4/GAL4}$ (*Gr43a*$^{-}$), *w R1 Gr5a*^{LexA}*; R2/R2; Gr61a*$^{-}$*ΔGr64/Gr61a*$^{-}$ *ΔGr64* (*sugar Gr*$^{-}$); and *w; Gr43a*$^{Gal4/Gal4}$*;UAS-Gr43a/+* (*Gr43a rescue*) to 100 mM sugars with and without a fructose moiety. (A) Gr43a was necessary for both the immediate and late preference response to fructose and the late preference for glucose; $12 \leq n \leq 30$, Mann-Whitney U test was used to compare samples to *Gr43a*$^{-}$; *p < 0.05, **p < 0.001. Negative values for fructose at 2′ and 4′ and melezitose at 4′ (see B) by *Gr43a* mutant larvae (one-sample sign test, p < 0.05) probably reflect avoidance of high osmolarity in the absence of any sugar taste capability. (B) Response to melezitose and sorbitol recapitulate the response to fructose and glucose, both in *Gr43a* mutant and control larvae, implying a fructose moiety to be necessary for the immediate but not the late preference; $6 \leq n \leq 12$, Mann-Whitney U test was used to compare to *Gr43a*$^{-}$; *p < 0.05, **p < 0.001. (C) Larval preference for agar containing 0.5% casein peptone (mixture of amino acids and peptides) and 50 mM NaCl is not affected by the *Gr43a*^{GAL4} mutation. Mann-Whitney U test was used to compare genotypes to w^{1118} (*Gr43a*$^{+}$); $18 \leq n \leq 27$ for casein peptone, $12 \leq n \leq 18$ NaCl; *p < 0.05, **p < 0.001. All data are presented as mean and error bars as ±SEM.

To address the role of the brain neurons in fructose sensing, we investigated the temporal preference dynamics of *Gr43a*^{GAL4} mutant larvae expressing a *UAS-Gr43a* transgene with and without *Cha*$^{7.4kb}$*-GAL80* (Figure 3). GAL80 binds to and suppresses transcriptional activity of GAL4 [23]. *Cha*$^{7.4kb}$*-GAL80* suppresses GAL4 expression almost completely in the two internal taste clusters and the neurons associated with the proventriculus but not the neurons in the brain (Figure 3A). As expected, "*Gr43a* brain-only larvae" showed the same preference dynamics for glucose and sorbitol as control larvae; however, their immediate (2 min) preference to fructose was severely reduced, albeit not completely abolished (Figure 3B). This may be explained by residual expression of *Gr43a* in proventricular neurons of some larvae (Figure 3A) or by rapid uptake of fructose into the hemolymph. We then tested sugar preference in larvae in which *Gr43a* function was restricted to peripheral neurons by expressing tetanus toxin (TNT) in the brain (*Gr43a*$^{GAL4/+}$*; UAS-TNT/ Cha*$^{7.4kb}$*-GAL80*; TNT inhibits neural transmission [24]). Indeed, these larvae exhibited an immediate preference for fructose but failed to generate a late preference for glucose and sorbitol (Figure 3C).

In adults, feeding of all nutritious sugars, such as fructose, sorbitol, or glucose, leads to a significant increase of hemolymph fructose [17]. To examine the effects of dietary sugars on larval hemolymph fructose, we let larvae feed on agarose only (control) or agarose containing 100 mM sugars (fructose, sorbitol, or glucose) before their hemolymph was collected and examined for the three major hemolymph sugars. As expected, hemolymph fructose levels increased significantly when fed fructose or sorbitol, while hemolymph glucose/trehalose levels did not change (Figure 4). When larvae were fed glucose, hemolymph glucose and trehalose levels increased, the latter significantly, while no change in hemolymph fructose was observed.

Discussion

The sweet taste of sugars is crucial for providing animals a hedonic stimulus for feeding on nutritious carbohydrates. Here, we have shown that the *sugar Gr* genes mediating sweet taste in adult flies are dispensable for sugar sensing during the larval stage. Instead, Gr43a is the major sweet taste receptor in larvae. Gr43a is narrowly tuned to fructose and the fructose-containing disaccharide sucrose [17, 21] found in most fruit. In contrast to adult flies, in which Gr43a has only an auxiliary role in tasting dietary sugars and in which its main role is to sense fructose in the brain hemolymph [17], larvae appear to rely almost exclusively on this receptor for carbohydrate sensing.

Why does *Drosophila* employ distinct sugar receptors during the different life stages? Larvae and adults face different sets of challenges to satisfy similar dietary needs of carbohydrates and proteins. Larvae are highly restricted in their choice of carbohydrates, which is determined by where they have been deposited as eggs. Thus, they consume a relatively homogenous source of sugar. The major gustatory challenge, therefore, is not to locate and identify sugars but to verify its presence and assure that it is void of hazardous chemicals that may be produced by microorganisms colonizing ripening and decaying fruit. Indeed, at least 40 putative bitter receptors are expressed in the larval taste sensory neurons [19], some of which may sense such harmful chemicals. In contrast, adults explore diverse habitats to locate appropriate food sources, find mates, escape predators, and lay eggs. Thus, a set of *Gr* genes encoding different sugar receptors capable of detecting a variety of sugars is better suited to identify appropriate food sources in the complex environment of adult *Drosophila*.

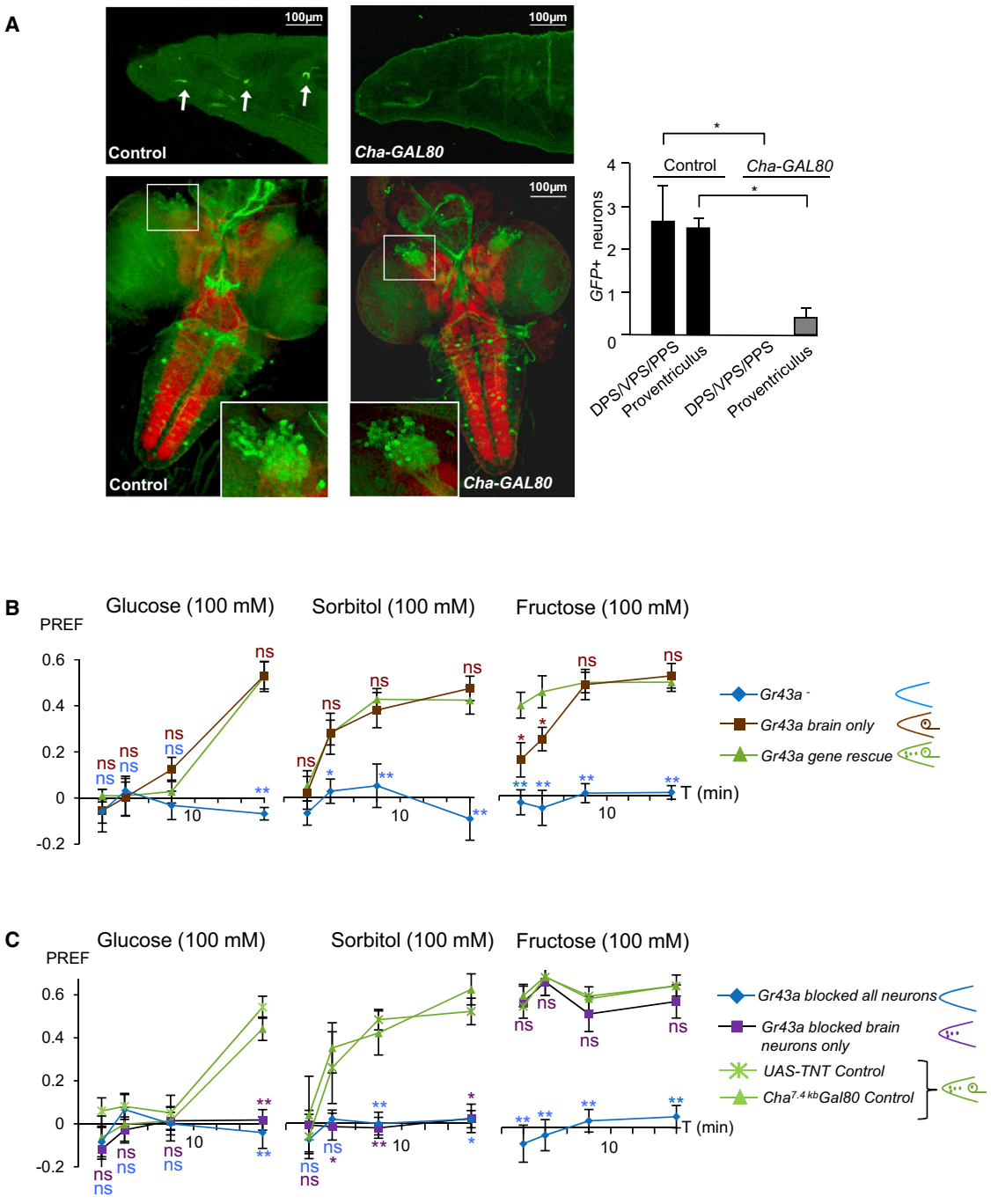
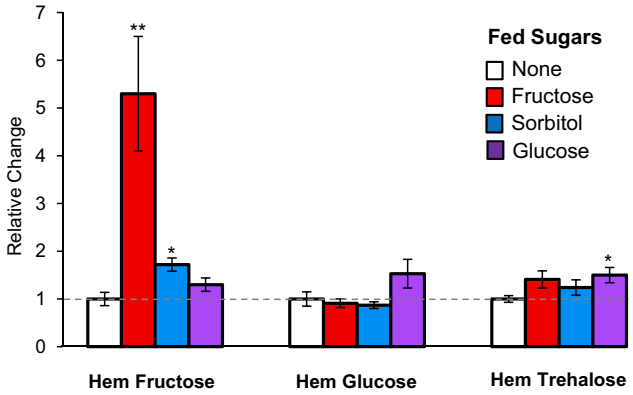


Figure 3. *Gr43a*^{GAL4} Brain Neurons Are Sufficient for Sugar Sensing

(A) *Gr43a*^{GAL4} expression was repressed by *Cha*$^{7.4kb}$*-Gal80* in taste neurons DPS/VPS/PPS and neurons in the gastrointestinal tract but not in the brain neurons. Image of the taste organs and quantification data were obtained from live GFP microscopy, and image of the brain was from immunostained preparations of third-instar larvae using anti-GFP antibody. Genotypes were the following: *w; Gr43a*^{GAL4}*;UAS-mCD8GFP* (control) and *w; Gr43a*^{Gal4}*; UAS-mCD8GFP/Cha*$^{7.4kb}$*-Gal80*. Number of GFP-expressing cells in taste organs and proventriculus was determined using live GFP microscopy. Weak expression in at most one proventricular neuron was observed in ~40% of larvae. Mann-Whitney U test was used to compare genotypes. (B) The slow response (16 min) to glucose and sorbitol was rescued in *Gr43a* mutant larvae in which *Gr43a* function was restricted to the brain. Immediate, but not slow, response to fructose was reduced in these larvae. Genotypes were the following: *w; Gr43a*$^{GAL4/GAL4}$*; Cha*$^{7.4kb}$*-Gal80/+* (*Gr43a* mutant control), *w; Gr43a*$^{Gal4/Gal4}$*;UAS-Gr43a/+* (*Gr43a* rescue), and *w; Gr43a*$^{Gal4/Gal4}$*;UAS-Gr43a/Cha*$^{7.4kb}$*-Gal80* (*Gr43a* "brain only"). Mann-Whitney U test was used to compare genotypes to *w; Gr43a*$^{Gal4/Gal4}$*;UAS-Gr43a/+*; $12 \leq n \leq 18$; *p < 0.05, **p < 0.001. ns, no statistical difference between *Gr43a rescue* and *Gr43a* "brain only" groups. (C) Larvae in which the brain neurons were inactivated by expression of TNT showed an immediate response to fructose (2 min), but their late response (16 min) to glucose or sorbitol was completely abolished. Genotypes were the following: *w; Gr43a*$^{GAL4/+}$*; Cha*$^{7.4kb}$*-Gal80/+* (*Cha-GAL80* control), *w; UAS-TNT/+* (*UAS-TNT* control), *w; Gr43a*^{Gal4}*/UAS-TNT* (all *Gr43a* neurons inactivated), and *w; Gr43a*^{GAL4}*/UAS-TNT; Cha*$^{7.4kb}$*-Gal80/+* (only brain *Gr43a* neurons inactivated). Mann-Whitney U test was used to compare genotypes to controls. Note that significance at 4′ time point in sorbitol versus agar was attained only when compared to *Cha-GAL80* control; $6 \leq n \leq 12$; *p < 0.05, **p < 0.001. ns, no statistical difference between control groups and "only brain *Gr43a* neurons inactivated" group. All data are presented as mean and error bars as ±SEM.



Fed sugars	Hemolymph sugars		
	Fructose (μg/mg)	Glucose (μg/mg)	Trehalose (μg/mg)
none	0.07±0.01	0.64±0.1	5.4±0.4
Fructose	0.37±0.09**	0.58±0.06	7.6±1.0
Sorbitol	0.12±0.01*	0.56±0.05	6.7±0.9
Glucose	0.09±0.01	0.98±0.2	8.1±0.9*

Figure 4. Concentration of Hemolymph Sugars after Glucose, Sorbitol, or Fructose Feeding

Larvae from the w^{1118} strain were fed on agarose containing 100 mM of each sugar and compared to larvae kept on plain agarose. The amount of each sugar (in μg) was normalized to larval weight (in mg). Data are presented as mean and error bars as ±SEM. Student's t test was used to compare sugar fed groups to agar fed group; $4 \leq n \leq 8$ for all groups; *$p < 0.05$, **$p < 0.001$.

Fructose as in Internal Nutrient Signal

The two-choice feeding assay reveals two temporally distinct phases while acquiring a sugar preference, both of which are dependent on Gr43a. We propose that if a sugar can be sensed by taste neurons (such as fructose-containing sugars), a preference is established within 2 min (Figures 2 and 3C; Figure S1). The second phase in larval sugar perception is characterized by the establishment of a delayed preference, is dependent on *Gr43a* function in the brain (Figure 3), and most likely is mediated by fructose in the hemolymph, converted from ingested sugar (Figure 4) [17]. The observation of a slow developing sugar preference is surprising, given the elapsed time from food intake and actual activation of brain neurons by fructose. Preliminary experiments suggest that crawl speed of larvae is dependent on the nutritious content of the agar, but more extensive behavioral analyses that consider turn frequency, digging activity, etc. will be necessary to uncover the rationale for the observed late sugar preference.

In adult flies, only about six brain neurons express *Gr43a*; they were shown to sense circulating fructose and regulate consumption of nutritious carbohydrate [17]. Larvae have several groups of $Gr43a^{GAL4}$-expressing neurons, one probably corresponding to the neurons in the posterior superior lateral protocerebrum of adult flies, a group of mushroom body Kenyon cells, and about 20 to 30 neurons dispersed along the VNC. The Kenyon cells are unlikely to be involved in this process, as they are not necessary to mediate the late sugar preference (Figure S4), but they may play a role in associative learning during feeding. Regardless, our analysis suggests that some of the brain neurons in the larvae function as nutrient sensors by detecting hemolymph fructose, derived from dietary fructose or fructose converted from other nutritious carbohydrates. Measurements of hemolymph fructose in flies show significant increases after glucose-, sorbitol-, or fructose-based meals (3-, 5-, and 10-fold, respectively) [17]. These values are higher than those observed in larvae, in which only feeding on fructose and sorbitol, but not on glucose, raises hemolymph fructose levels (Figure 4). Two scenarios may account for this difference, both implying a role of the blood-brain barrier (BBB): first, conversion of fructose may be restricted to the brain or, second, the conversion into fructose may be ubiquitous, but accumulation in the brain may be regulated by selective transporters for fructose across the BBB [25]. The specificity of Gr43a for fructose and the location of this sensor in the brain, therefore, invoke a postingestive mechanism requiring conversion of dietary sugars into fructose. Whatever the mechanism, the brain neurons are both necessary and sufficient to mediate the late sugar preference in larvae.

$Gr43a^{GAL4}$ is also expressed in the larval proventriculus, reiterating the adult expression profile in which this structure separates the foregut from the midgut [17]. Interestingly, the *Gr43a* orthologs of the cotton bollworm *Helicoverpa armigera* and the silkworm *Bombyx mori* are expressed in the gut, indicating an important role for this fructose sensor in the gastrointestinal tract [21, 26]. Sensing of dietary fructose may induce expression/secretion of carbohydrate-modifying enzymes and/or regulate peristaltic movements of the midgut to aid in digestion. Future studies in *Drosophila* and other insects will illuminate the function of this unique gustatory receptor both in the gastrointestinal system and the brain.

Experimental Procedures

Genetics

For behavioral analysis, w^{1118} larvae were used as wild-type controls, $Gr43a^{Gal4/Gal4}$ larvae as null mutants for *Gr43a*, *R1* $Gr5a^{LexA}$/*R1* $Gr5a^{LexA}$; *R2/R2; Gr61a*$^{-}$*ΔGr64/Gr61a*$^{-}$ *ΔGr64* larvae as triple mutant lacking all eight *sweet Gr* genes, and $Gr43a^{Gal4}/Gr43a^{Gal4}$;*UAS-Gr43a/+* larvae as *Gr43a* rescue controls. For larvae with $Gr43a^{GAL4}$ expression restricted to the brain, we used $Gr43a^{Gal4}/Gr43a^{Gal4}$;*UAS-Gr43a/*$Cha^{7.4kb}$*-Gal80* larvae, while $Gr43a^{Gal4}/Gr43a^{Gal4}$*; UAS-Gr43a/+* and $Gr43a^{Gal4}/Gr43a^{Gal4}$*;* $Cha^{7.4kb}$*-Gal80/+* were used as controls. Expression analysis was carried out on $Gr43a^{Gal4}$*/+; UAS-mCD8GFP/+* and $Gr43a^{GAL4}$ *UAS-mCD8RFP/Gr66a-GFP-IRES-GFP-IRES-GFP*. *R1* and *R2* are rescue transgenes containing non-*Gr* genes deleted in *ΔGr64* [6].

Behavioral Assays

Two-choice preference assay: flies were kept in mass culture under standard conditions. Third-instar, feeding-stage larvae (4 days after egg laying) of the indicated strains were used for all assays. Larvae were collected from culture tubes and briefly washed in water to remove food particles. We used 15 larvae for each preference assay and placed on the midline separating the two agar media (one containing sugar and the other lacking sugar) on a 55 mm plastic Petri dish. Images were taken after the indicated time points (2, 4, 8, and 16 min) to establish larval distribution and to calculate preference indices. Larvae that dug in the agarose or crawled onto the lid were excluded. A preference index (PREF) was then calculated as PREF = [number of larvae on sugar/agar − number of larvae on agar only] / total number. A positive PREF score indicates a preference for the sugar-containing agar, while a negative PREF score would indicate a preference for plain agar.

Preparation of media is as follows: 55 mm Petri dishes are filled with a solution of 1% agarose. After the agarose has solidified, the agar is split into two identical halves using a razor blade, and one half is discarded. The emptied half of the dish is then replaced with agar containing the sugar at the indicated concentration. Plates were used within an hour after preparation.

Hemolymph Collection

Third-instar larvae were kept on agar overnight, collected, rinsed in water, briefly dried, and weighted in batches of five. A batch of larvae was then transferred on plates for 16 min of either plain agarose or agarose plates with 100 mM glucose, sorbitol, or fructose. Each batch was rinsed to remove food particles, briefly dried off, and transferred to a clean dissection dish, filled with ~50 μl of distilled water. Larvae were carefully torn apart using forceps to drain hemolymph into the water, and 40 μl of the hemolymph-containing solution was collected. Protein and cellular debris were removed by treatment with Barium Hydroxide and Zinc sulfate (0.3 N).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.028.

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