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Taste-independent nutrient selection is mediated by a brain-specific Na+/solute cotransporter in *Drosophila*

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Abstract

Animals can determine the nutritional value of sugar without the influence of taste. Here, we describe a *Drosophila* mutant that is insensitive to the nutritional value of sugars, but responds only to the concentration (i.e. sweetness). The affected gene encodes a sodium/solute cotransporter-like protein, designated *dSLC5A11* (or *cupcake*), which is structurally similar to mammalian sodium/glucose cotransporters (SGLTs) that transport sugar across the intestinal and renal lumen. However, dSLC5A11 is prominently expressed in 10-13 pairs of R4 neurons of the ellipsoid body (EB) in the brain and functions in these neurons for selecting appropriate foods. We propose that *dSLC5A11* and EB R4 neurons carry out a critical signaling function in responding to internal glycemic levels.

Eating behavior is controlled by multiple factors including food palatability and nutritional needs. External chemosensory taste receptors primarily detect palatable food, but animals lacking taste receptors can still develop a preference for sugars based on their nutritional value[1-6]. *Drosophila melanogaster* can detect nutritive (i.e. metabolizable) sugars in the absence of peripheral sugar receptors[6]. This taste-independent nutrient selection pathway is activated when the internal energy reservoir is depleted. We found that food choice behavior correlates strongly with a decrease in sugar (glucose and trehalose) levels in the hemolymph (Fig. 1a). Specifically, flies that had been food-deprived for approximately 15 hours (the length of time that leads to a dramatic fall in hemolymph sugar levels) selected the nutritive D-glucose over the non-metabolizable L-glucose. This suggests that the hemolymph sugar might be the postingestive cue that drives feeding behavior independently of gustatory inputs.

To establish a causal link between hemolymph sugar levels and taste-independent food choice behavior, we investigated the possibility that blocking the entry of glucose into the hemolymph interfered with the induction of this behavior. Phlorizin, a drug that blocks the transport of glucose from the intestinal lumen into the blood in mammals[7], was used to

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Author Contributions:

G.S.B.S. and M.D. designed experiments, analyzed data and wrote the manuscript. M.D. performed all experiments except PA-GFP. M.A. performed the PA-GFP experiments. G.S.B.S., M.D., and MA edited the manuscript. G.S.B.S. supervised the project.

suppress the entry of glucose into the hemolymph in flies, thereby preventing a prandial rise in glycemia (Fig. 1b). In a two-choice assay, the "taste-blind" *pox-neuro* mutant *(poxnΔM22-B5)*[8] that is insensitive to the taste of sugars, developed a preference to glucose over plain agar after 18 hours of food deprivation, but failed to consume glucose mixed with phlorizin (Fig. 1c). This is likely due to a failure of the phlorizin-laced glucose, which is not transported into the hemolymph, to activate the taste-independent nutrient selection pathway. By contrast, because phlorizin does not inhibit fructose transport[7], the taste-blind mutants were able to detect the nutritional value of fructose mixed with phlorizin (Fig. 1c). The control flies, *pox-neuro* mutants that carry a rescue transgene *(poxnΔM22-B5;SuperA158)*[8], can distinguish glucose mixed with phlorizin from agar. Moreover, when given the choice between D-glucose mixed with phlorizin and a more concentrated (sweeter) L-glucose mixed with phlorizin, taste- blind *(poxnΔM22-B5)* and sugar-blind (*GR5a;GR64a*) mutants showed equal preference for these two enantiomers (Supplementary Fig. 1). On the contrary, control flies chose the sweeter L-glucose because blockade of glucose transport promotes food selection based on gustatory information alone. These observations suggest that a prandial rise in hemolymph glucose would trigger the taste-independent nutrient selection pathway.

To investigate the genetic basis for this behavior, we screened for mutants that failed to exhibit a preference for a nutritive sugar (D-glucose) over a non-metabolizable sugar (L-glucose) when starved. Wild type flies showed no sugar preference when sated, but preferred D-glucose when starved[6]. We identified one mutant that failed to choose D-glucose when starved, and made food choice based on the palatability alone. When D-glucose was more concentrated and presumably sweeter, this mutant preferred D-glucose to L-glucose; conversely, when L-glucose was more concentrated, the mutant chose L-glucose over D-glucose (Fig. 2a). The affected gene encodes a putative sodium/solute cotransporter protein, which we named *Drosophila* sodium/solute cotransporter-like 5A11 (*dSLC5A11* and also *cupcake*). We speculated that the ability to detect sugar would be completely abolished in *GR5a*, *dSLC5A11*^{1}, and *GR64a* triple mutants. Indeed, these flies displayed equal preference for D-glucose and L-glucose (Fig. 2b), presumably because they had neither external sugar receptors that detect the palatability nor *dSLC5A11* that allows flies to respond to the nutritional value of sugar. Consistent with this result, we found that *poxnΔM22-B5; dSLC5A11*^{1} mutants were unable to develop a postingestive preference for nutritive D-glucose (Supplementary Fig. 2).

To ensure that this phenotype is caused by the mutation in the *dSLC5A11* locus, we generated fly strains carrying the *dSLC5A11*^{1} allele *in trans* to two independent deficiencies uncovering the locus. These strains were phenotypically indistinguishable from *dSLC5A11*^{1} homozygotes (Fig. 2c). By contrast, flies in which the transposable element was precisely excised from the *dSLC5A11* locus exhibited a normal preference to D-glucose when starved. We later identified another mutation, designated *dSLC5A11*^{2} that had a phenotype similar to that of *dSLC5A11*^{1} (Fig. 2c). The quantitative PCR analysis showed that the *dSLC5A11* transcript was significantly reduced in the brains of *dSLC5A11*^{1} *and dSLC5A11*^{2} homozygotes (Supplementary Fig. 3). The food-deprived *dSLC5A11* mutant also failed to exhibit the shift in preference for other nutritive sugars including sorbitol, trehalose and

galactose, which increase hemolymph glycemia upon ingestion (Fig. 2d, e and Supplementary Fig. 4, 5).

dSLC5A11 belongs to a large sodium/solute cotransporter (SLC5A) family, members of which are highly homologous to the human SLC5As such as iodide, monocarboxylate and multivitamin cotransporters (Fig. 2f). The human sodium/glucose cotransporters (SGLTs) have a distinct clade, yet hold approximately 24%-30% amino acid identities to the *Drosophila* SLC5As. Some mammalian SLC5As, including SGLT1, function in the brush-border cells of the small intestine to absorb glucose from the intestinal lumen by using the sodium electrochemical gradient[9]. We therefore hypothesized that dSLC5A11 could have a similar function and that its mutation would disrupt glucose transport; this could adversely affect circulating sugar levels that lead to a defect in taste-independent food preference. However, we found that the hemolymph glycemia as well as glycogen stores in *dSLC5A11* mutants were indistinguishable from those in controls (Supplementary Fig. 6a, b). This suggests that *dSLC5A11* regulates feeding behavior by a different mechanism.

To determine the expression pattern of *dSLC5A11*, we engineered transgenic flies that carry the *dSLC5A11* promoter, $P_{dSLC5A11}$-GAL4, driving UAS-mCD8GFP. Surprisingly, we found few labeled neurons in the brain of a transgenic fly. Notably, approximately 10-13 pairs of R4 neurons of the ellipsoid body (EB)[10] showed prominent GFP expression (Fig. 3a). To further analyze the projection patterns of these EB R4 neurons, we labeled a single R4 neuron in a fly carrying photoactivatable GFP (UAS-C3PA-GFP)[11] under the control of $P_{dSLC5A11}$-GAL4 driver. The labeled R4 neuron extended its neurite along the RF tract, formed bleb-like termini and innervated the outer ring of the EB, ramifying its processes over the entire circumference of the ellipsoid body ring (Fig. 3b).

Moreover, the transgene was expressed in a few populations of olfactory sensory neurons (Fig. 3a and Supplementary Fig. 7a). However, *dSLC5A11* in these neurons is unlikely to have a relevant role, as the olfactory organs were dispensable for taste-independent food preference[6] (also Supplementary Fig. 7b). We also observed the expression in a small number of neuronal fibers in the subesophageal ganglion, but did not find any GFP labeling in the external taste organs (Supplementary Fig. 7c, d). A subset of Prospero-positive, enteroendocrine cells in the anterior midgut[12, 13] were labeled in $P_{dSLC5A11}$-GAL4 (Supplementary Fig. 7e). However, *dSLC5A11* in these cells are not required for taste-independent food preference (Supplementary Fig. 7f). In contrast, *dSLC5A11* is required in EB R4 neurons for this behavior. Flies with *dSLC5A11* knock-down in R4 neurons selected more concentrated, non-nutritive L-glucose even after 18 hours of starvation (Fig. 3c). This defect was as strong as the *dSLC5A11* mutant phenotype, suggesting that *dSLC5A11* functions in R4 neurons to promote this behavior. Consistent with this, we found that expression of UAS-*dSLC5A11* only in R4 neurons of *dSLC5A11* mutants completely rescued the behavioral defect (Fig. 3d).

To test whether the EB R4 neurons are required for taste-independent food preference, we used a variety of other R4-specific drivers that are not expressed in the antennae, the subesophageal ganglion or the anterior midgut in order to specifically inactivate R4 neurons and tested the flies in the two-choice assay. We found that conditional inactivation of this

subset of EB R4 neurons by UAS-Kir2.1, P_{tub}-GAL80^{ts} [14, 15] under the control of three different R4-GAL4 drivers abolished the selection of nutritive D-glucose, whereas silencing of the neighboring R1 and R3 neurons had no effect on this behavior (Fig. 3e and also Supplementary Fig. 8). Notably, the R1 and R3 neurons are required for spatial memory, but R4 neurons are dispensable for this associative behavior[16, 17]. These findings suggest that visual place learning unlikely plays a role in the taste-independent food preference. Consistent with this, we found that foraging sugar-blind flies in the 2-choice arena were still able to choose D-glucose over L-glucose in the dark (Supplementary Fig. 9).

The SLC5A11-expressing EB R4 neurons possibly act as a cellular substrate that detects the nutritional value of food through direct activation by nutritive sugar during the prandial rise in the hemolymph sugar levels. Alternatively, the function of EB R4 neurons may be to monitor the internal energy status of flies and stimulate feeding behavior upon starvation while the nutrient sensing is mediated by other neurons. In the mammalian brain, glucose excited or inhibited currents that are predominantly modulated by SGLTs[18] might play a key role in the detection of nutrients, but their function is unclear due to the lack of functional analyses. Our studies using the genetically amenable *Drosophila* model provide a foundation for understanding interoceptive nutrient sensing in humans.

Online Methods

Fly strains

Flies were reared in standard cornmeal-molasses medium at 25°C with 12:12 D:L cycles. The standard laboratory line Canton-S (CS) was used as wild-type control. d*SLC5A11*^{1} (CG8451, *cupcake*, stock #22498: y^{1} w^{67c23}; P{EPgy2}CG8451^{EY21708}), *dSLC5A11*^{2} (stock #6768: y w; P{Mae-UAS.6.11}CG8451^{UY1824}), and deficiencies (stock #9705 and #9706) uncovering the *dSLC5A11* locus were obtained from the Indiana Bloomington stock center. *dSLC5A11*$^{1\ revertant}$ flies were generated by mobilizing the P element with Δ*2-3* transposase. Precise excision lines were identified by absence of mini-white+ eye color and confirmed by PCR genotyping and sequencing. GAL4 and enhancer trap lines labeling the ellipsoid body neurons were kindly provided by Dr. Paul Taghert, Washington University, St Louis, MO, Dr. Vivek Jayaraman, Janelia Farm, VA, and Dr. Michael Reiser Janelia Farm, VA. *R38H02* labels a subset of R4 cells[17], *R28D01* the R1[17], *R15B07* the R1/R4[17], *c232* the R3/R4d[10, 16], 189y the R3[10]. $P_{prospero}$-GAL4 was kindly provided by Dr. Benjamin Ohlstein, Columbia University, NY. A fly line carrying UAS-Kir2.1, $P_{tubulin}$-GAL80^{ts} was kindly provided by Dr. David Anderson, Caltech, CA; UAS-CD8-GFP by Indiana Bloomington stock center. UAS-*dSLC5A11 RNAi* lines were obtained from the Vienna *Drosophila* RNAi Center (stock #48984 and #3424). *Gr5a* and *Gr64a*^{1} mutants were from Dr. John Carlson, Yale, CT. *pox-neuro* mutant and rescue flies were provided by Dr. Ulrike Heberlein, Janelia Farm, VA.

Transgenic lines

$P_{dSLC5A11}$ -GAL4 was made by cloning a 1.1-kilobase (kb) portion of DNA sequence upstream of *dSLC5A11* into pCasper4-AUG-GAL4X. UAS-dSLC5A11 was generated by cloning the cDNA sequence of *dSLC5A11* into pUAST. Transgenic flies were generated by Bestgene, Inc.

Two-choice assay

Feeding assays were performed as previously described[6] using approximately 50 flies per two-choice arena to avoid crowding. Briefly, the 50 4-8 days old male flies were starved in an empty vial with wet Kim wipe for 5 or 18 hours, and then given a choice between two sugars, or a sugar and plain agar for 2 hours. Food preference was determined as percent preference index (PI%) by scoring the abdomen color of each fly:

$$\%PI = \frac{(\#\text{eaten food1} + 0.5\text{X}\#\text{eaten both}) - (\#\text{eaten food2} + 0.5\text{X}\#\text{eaten both})]}{(\text{total}\#\text{flies eaten})}$$

All sugars, except for L-glucose (Carbosynth), were from Sigma. For Fig. 1c, 10mM phlorizin (Sigma) was added to warm agar with or without sugar. For the experiments in the dark, individual plates were wrapped in aluminum foil and then placed in a dark drawer while tested flies were making a choice between the two substrates. For the neural silencing experiments, flies bearing UAS-Kir2.1, $P_{tubulin}$-GAL80^{ts}, and a GAL4 driver were treated as described[17]. These flies were raised at 18°C, incubated at 29°C for 40 hours, starved at 29°C for next 18 hrs and then tested in the two-choice assay at room temperature. For a negative control, the flies were raised and starved at 18°C, and tested at room temperature. Because metabolism is slower at 18°C than at 25-29°C, the flies at 18°C needed to be starved for a longer period. Thus, we determined that the 18 hours of starvation at 25-29°C is equivalent to the 50-60 hours of starvation at 18°C. Also, see Supplementary Fig. 8.

Hemolymph glycemia and glycogen measurements

Glycogen and glycemia were measured as previously described[6]. For the prandial rise of hemolymph glycemia, flies were starved for 18 hours, and then fed with 100mM glucose (with or without 10mM phlorizin) for 15-20 minutes. Their hemolymph was immediately collected and measured.

Immunohistochemistry

Brains were fixed and stained with addition of a permeabilization step (10 mins with 0.5% Triton-X 100, 0.5% BSA in Phosphate buffered saline, PBS) for better antibody penetration into the central brain. Guts were immunostained as previously described [12, 13] The following antibodies were used: mouse anti-bruchpilot at 1:20 (Developmental Studies Hybridoma Bank, NC82), mouse anti-Prospero at 1:10 (Developmental Studies Hybridoma Bank, MR1A) and rabbit anti-GFP IgG at 1:500 (Invitrogen, A-6455). Secondary antibodies were Alexa Fluor 555 goat anti-mouse IgG at 1:500 (Invitrogen, A-21425), Alexa Fluor 488 goat anti-rabbit IgG at 1:500 (Invitrogen, A-11070) and TO-PRO3 at 1:500 (Invitrogen, T3605) used for DNA labeling. Images were acquired using a Zeiss LSM 510, at 1.5μm intervals with 1024×1024 or 512×512 resolution.

Photoactivatable GFP (PA-GFP)

Brains from <1 day old $P_{dSLC5A11}$-GAL4; UAS-C3PA homozygotes were dissected in the adult hemolymph buffer[11], immobilized onto a silicone gel plate with pins and visualized under a two-photon microscope using a 40× water immersion lens. Prior to photo-

conversion, the low intensity fluorescence of the PA-GFP protein was visualized by the two-photon laser at 925nm to identify the cells of interest. For photoconversion, the two-photon laser at 715nm was applied repeatedly to their cell bodies for 60 cycles with 30-second intervals between cycles to allow diffusion of the photo-converted GFP protein. The converted PA-GFP protein was then visualized at 925nm.

qPCR

Thirty brains of starved adult CS flies were dissected in PBS, and RNA was isolated with Trizol reagent (Invitrogen). 1μg RNA was used to make cDNA using oligo dT primers. The expression of *dSLC5A11* and *GAPDH* transcript was assayed by qPCR with a Biorad C1000 thermal cycler (CFX96 Real-Time System). Primers for *dSLC5A11* were TGCTTCAAGATGCAACCAAG (forward) and TTGAAGTGCAAATGCTCAGG (reverse) and for *GAPDH* GAAATCAAGGCTAGGTCG (forward) and AATGGGTGTCGCTGAAGAAGTC (reverse). cDNA dilutions of 1/10, 1/100, and 1/1000 were used for each primer set to calculate the qPCR efficiency.

Dendrogram

We used the dSLC5A11 sequence to blast the *Drosophila* and human genomes, and identified 14 *Drosophila* genes and 12 human genes that are homologous to dSLC5A11. We used clustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to conduct sequence alignment with the 15 dSLC5A and 12 hSLC5A, and formatted the guide tree file. We then used FigTree (http://tree.bio.ed.ac.uk/software/figtree/) to plot the dSLC5A/hSLC5A radial tree.

Statistics

GraphPad Prism software was used for all graphs and statistical analysis. All experiments were done with experimental and control genotypes in parallel. Data represent multiple independent experiments. Error bars are SEM. One-way or two-way ANOVA with Tukey or Bonferroni posthoc-test or student t-test (for qPCR) were used according to the number of conditions and genotypes. In Fig. 2a (left), 2b-c, 3c-d the food choice behavior of *dSLC5A11* mutant and knock-down flies was significantly different from that of controls, $P<0.0001$. In Supplementary Fig 6a and b, there is no significant difference in hemolymph glycemia between *dSLC5A11* mutant and control flies according to ANOVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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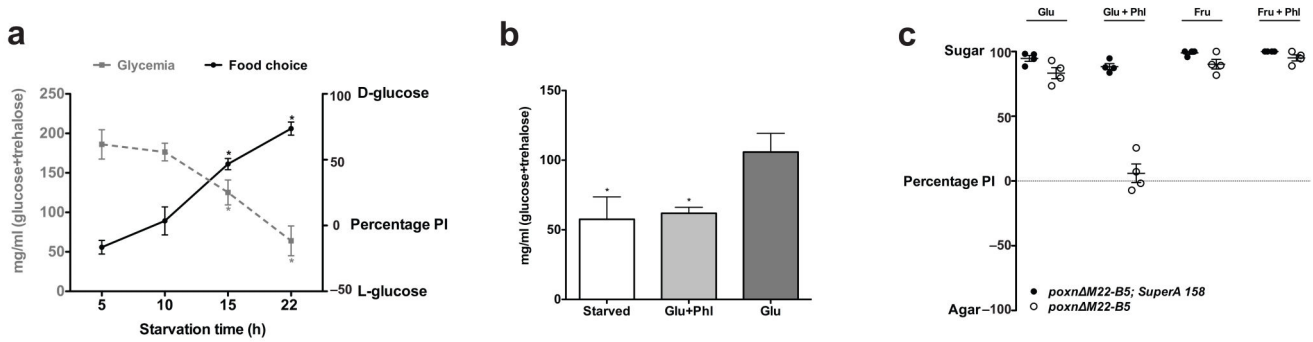

Figure 1. A prandial rise in hemolymph glycemia is required for appropriate food choice behavior in starved flies

(a) The hemolymph glycemia (grey, n=10-12) and food choice (black, 50mM D-glucose versus 200mM L-glucose, n=4-8) of starved wild-type (Canton-S) were measured. **(b)** The hemolymph glycemia of 18-hour starved flies was measured after feeding 100mM glucose with or without 10mM phlorizin for 20 minutes. n=10-12. **(c)** The food choice behaviors of 18-h food deprived *poxnΔM22-B5* (unfilled circles) and *poxnΔM22-B5; SuperA158* control (filled circles). These flies were given a choice between agar containing 100mM sugars with or without 10mM phlorizin and plain agar (Glu, Glucose; Fru, Fructose; Phl, Phlorizin). n=4. Asterisk, $P<0.001$ (one-way ANOVA, Tukey test).

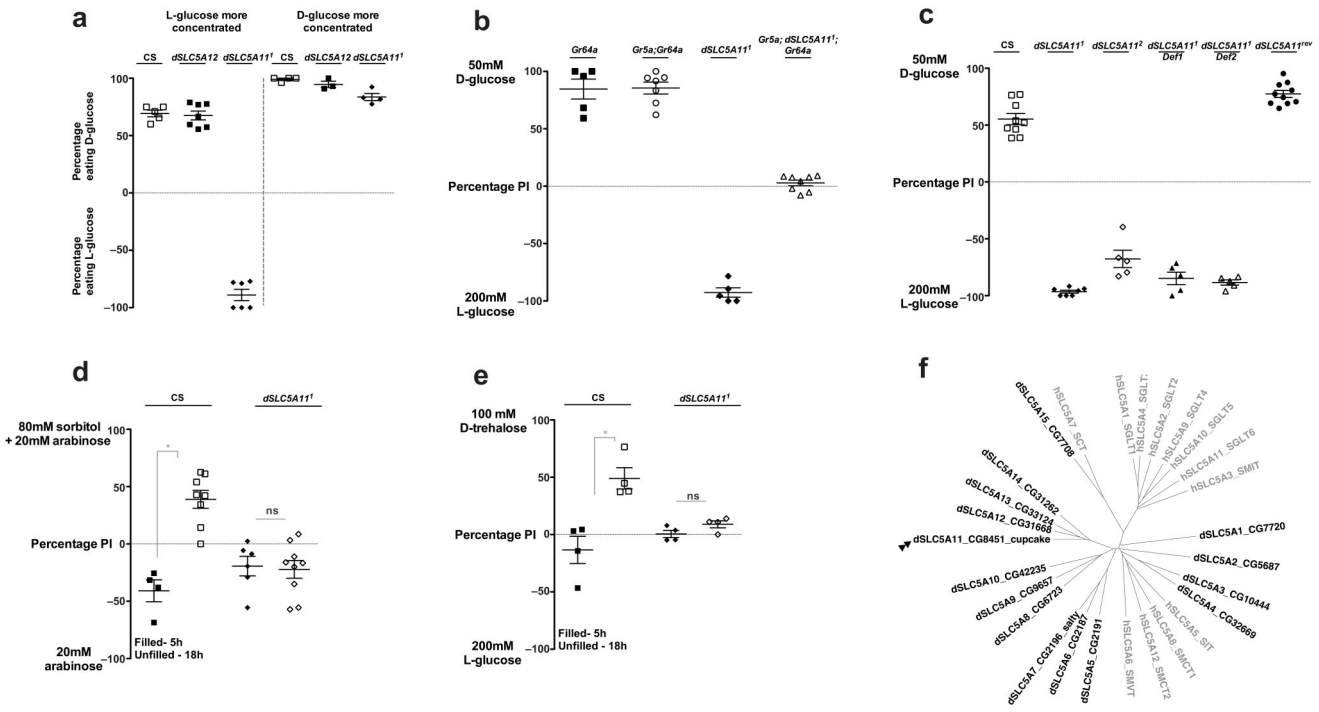

Figure 2. *dSLC5A11*, a member of the *Drosophila* sodium/solute cotransporter family, is required for taste-independent nutrient selection

(a) 18-hour starved *dSLC5A11*^{1} mutants and controls were given a choice of 200mM L-glucose v. 50mM D-glucose (left), or 200mM D-glucose v. 50mM L-glucose (right) in the two-choice assay. n=3-7. **(b)** The food choice behaviors of 18-h starved *Gr5a; dSLC5A11*^{1}*; Gr64a* triple mutants and controls were determined. n=5-8. **(c)** The food choice behaviors of different *dSLC5A11* allelic combinations and controls were measured after 18 hours of starvation. n=5-10. **(d-e)** The food choice behaviors of *dSLC5A11*^{1} and CS control were measured with **(d)** sorbitol(tasteless)+arabinose v. arabinose (non-nutritive), n=4, **(e)** D-trehalose v. L-glucose, n=9. **(f)** Dendogram of the *Drosophila* and human sodium/solute cotransporter families. Double arrowheads indicate *dSLC5A11 (CG8451, cupcake).* Asterisk, P<0.001 (one-way ANOVA, Tukey test).

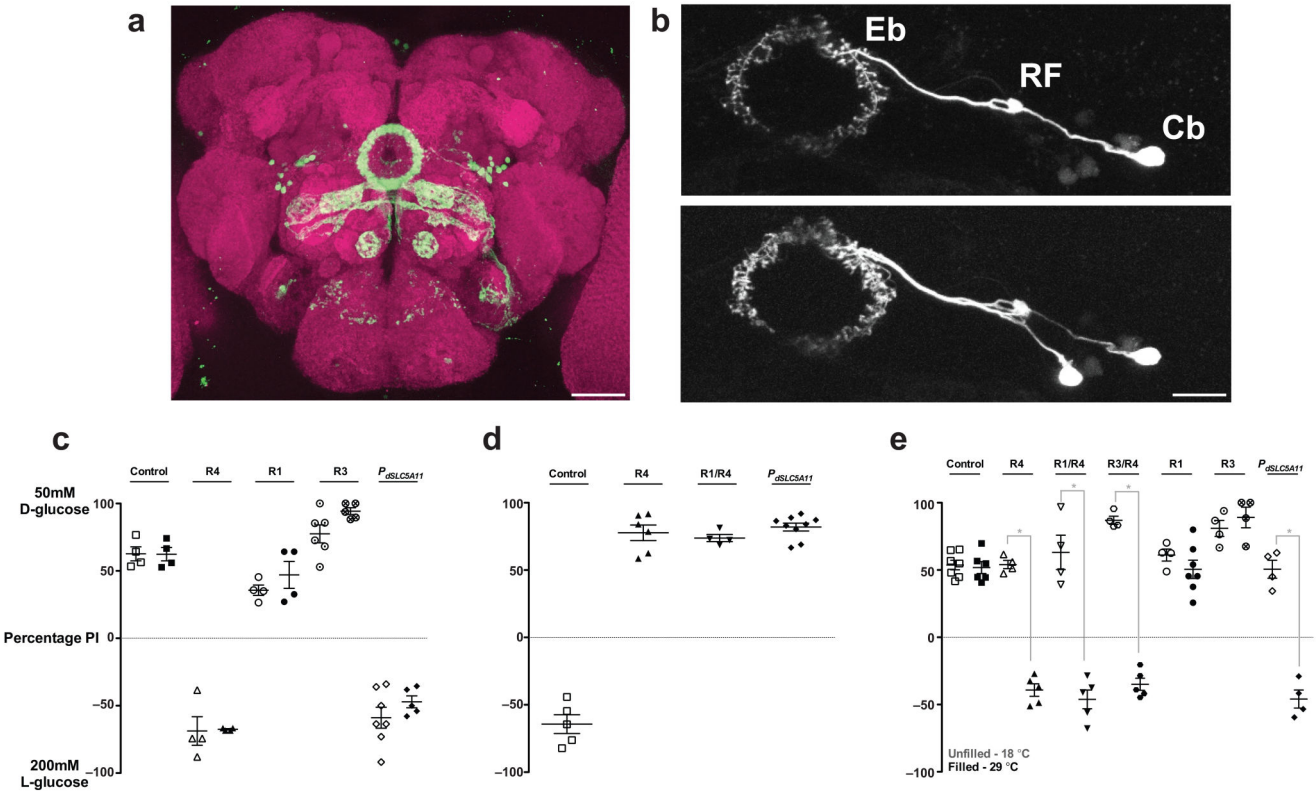

Figure 3. A subset of EB R4 neurons is required for taste-independent nutrient selection

(a) Z-stack image of the adult brain. $P_{dSLC5A11}$-GAL4::UAS-mCD8GFP stained with anti-GFP antibody in green and the neuropil marker, nc82, in magenta. Scale bar: 50μm. **(b)** Single- (*top*) or two-cell (*bottom*) labeling of the EB R4 neurons of $P_{dSLC5A11}$-*GAL4*::UAS-PA-GFP. Cb, cell body; RF, large-cell/fan-shaped body tract. Scale bar: 20μm. (**c**) The food choice behaviors of 18-hr starved flies in which d*SLC5A11* was knocked-down in different subsets of R-cells by using two independent UAS-*dSLC5A11* RNAi lines and R-cell specific drivers. Flies carrying UAS-*dSLC5A11* RNAi line alone were used as a control. n=4-7. (**d**) The food choice behaviors of 18-hr starved $dSLC5A11^1$ mutants carrying UAS-*dSLC5A11* in different subsets of EB neurons were measured. $dSLC5A11^1$ mutants bearing UAS-*dSLC5A11* alone were used as a control. n=4-9. **(e)** The food choice behaviors of 18-hr starved flies in which different EB R neurons were silenced by incubating them at 29°C (filled) or were left active at 18°C (unfilled) were measured. Flies carrying UAS-Kir2.1; $P_{tubulin}$-GAL80^{ts} without a driver were used as a control. n=4-7. Asterisk, $P<0.0001$ (Two-way ANOVA, Bonferroni test).