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Identification of neurons responsible for feeding behavior in the *Drosophila* brain

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Drosophila melanogaster feeds mainly on rotten fruits, which contain many kinds of sugar. Thus, the sense of sweet taste has evolved to serve as a dominant regulator and driver of feeding behavior. Although several sugar receptors have been described, it remains poorly understood how the sensory input is transformed into an appetitive behavior. Here, we used a neural silencing approach to screen brain circuits, and identified neurons labeled by three Gal4 lines that modulate *Drosophila* feeding behavior. These three Gal4 lines labeled neurons mainly in the suboesophageal ganglia (SOG), which is considered to be the fly's primary taste center. When we blocked the activity of these neurons, flies decreased their sugar consumption significantly. In contrast, activation of these neurons resulted in enhanced feeding behavior and increased food consumption not only towards sugar, but to an array of food sources. Moreover, upon neuronal activation, the flies demonstrated feeding behavior even in the absence of food, which suggests that neuronal activation can replace food as a stimulus for feeding behavior. These findings indicate that these Gal4-labeled neurons, which function downstream of sensory neurons and regulate feeding behavior towards different food sources is necessary in *Drosophila* feeding control.

feeding behavior, sugar-sensing neurons, SOG, CAFE assay, proboscis extension response (PER)

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Feeding is one of the most conserved innate activities of animals. How an animal decides what behaviors to engage in remains a major question in neurobiology, and feeding behavior provides an easily measured output for dissecting decision-making processes. Although diverse feeding habits have evolved to accommodate a complex array of food sources, animals, from flies to humans, react to taste molecules in a similar way: most are attracted to sugars and repelled by bitter and toxic compounds [1–3]. In addition to this similarity with mammals, the powerful genetic tools and many conserved metabolic elements [4,5] make *Drosophila melanogaster* a good model system to study feeding behavior [6–8]. In *Drosophila*, feeding begins with the detection of a palatable food source by the taste system. Taste perception allows flies to discriminate sweet or nutritionally rewarding food from food that is potentially contaminated or toxic, which typically tastes bitter [9–12]. Hence, the ability to taste sweetness is fundamental to survival.

The gustatory system plays a central role in the food evaluation process. Unlike humans, fruit fly taste perception

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is not restricted to one tissue. Flies sample their local chemical environment through sensilla, which are hair-like structures distributed across the proboscis (labella), internal mouthpart organs, legs, wings, and ovipositor [13–15]. On the proboscis, taste sensilla have a pore at their tip that allows chemicals to interact with gustatory receptor neurons (GRNs) [16], which reside underneath the sensilla and send axons directly to the central nervous system [2,17,18]. Taste detection is mediated by distinct sets of gustatory receptors, encoded by approximately 60 gustatory receptor (Gr) genes [19–22]. Multiple Gr genes are expressed in each GRN, which in turn can be divided into different subtypes according to certain features related to responsiveness [18,23]. For example, eight Grs (Gr5a, Gr61a and Gr64a–Gr64f) are partially co-expressed in a single GRN, which are characterized as sugar-sensing GRNs because the expressing receptors are responsive to sugars [24–26]. Specifically, in sugar-sensing GRNs, Gr5a is necessary for the response to a small subset of sugars, including trehalose [24,27,28]. In contrast, Gr64a is essential for the detection of multiple other sugars, including sucrose, glucose, and maltose [24,26,29]. More recent studies demonstrate that Gr64e is a receptor for glyce- rol [30] and Gr64f, as a co-receptor, functions with Gr5a and Gr64a to detect most sugars [25].

Feeding behaviors are highly regulated not only by the peripheral sensory system but also by neurons in the brain. For example, motor neurons drive proboscis extension and fluid ingestion [31,32]. A set of dopaminergic neurons classified as ventral unpaired medial (TH-VUM) neurons mediates increased sugar sensitivity during hunger [33,34]. In addition, various neuropeptide (Hugin, NPF, DILP, etc.)-expressing neurons modulate the initiation/termination of a meal and interact with other sensory systems to alter food odor attractiveness (reviewed in [35]). Despite a growing body of knowledge regarding feeding behavior regulation in flies, much less is known about neural processing underlying appetitive behavior. It is thought that peripheral attractants and internal metabolic cues are integrated in the brain before being output to shape feeding behavior [36].

In this study, to dissect the neural circuits involved in feeding control and to identify previously uncharacterized neurons involved in the process, we conducted a behavioral screen to identify sugar-responding neurons in the central nervous system. We identified three Gal4 lines that label groups of neurons in the central nervous system responsible for food intake. The neural activity of these neurons is necessary to generate normal feeding behavior.

1 Materials and methods

1.1 Experimental animals

Drosophila stocks were maintained on standard medium with a 12:12 h light/dark cycle [37]. Flies not expressing heat-shock inducible constructs were raised and tested at 25°C. Flies expressing UAS-dTrpA1 were raised at 18°C and heat-shock was performed by infrared laser or by raising the room temperature to 30°C during tests.

All the flies for behavioral experiments were 3–7-day-old females. Flies were first collected and fed on regular food for 1 d and then starved with access to water for 22 h. All the behavioral experiments were carried out between 10:00 a.m. to 2:00 p.m. to control for circadian influences.

The following flies were used: $poxn^{70}$ [38], $poxn^{\Delta M22}$ [39], UAS-dORK [40], Gr5a-Gal4, Gr61a-Gal4, Gr64a-Gal4, Gr64c-Gal4, Gr64d-Gal4, Gr64e-Gal4, Gr64f-Gal4 [41], UAS-dTrpA1 [42], UAS-mCD8::GFP [43], NP115-Gal4, NP883-Gal4, NP1076-Gal4 (*Drosophila* Genetic Resource Center).

1.2 CAFE assay

The capillary feeding (CAFE) assay was carried out as described by Ja et al. [44], with the following modifications. The apparatus consisted of an empty vial capped with a cotton plug. Two capillaries (length 100 mm, inner diameter 0.4 mm) were inserted into the plug via adaptors made of truncated pipette tips. Capillaries were filled with two types of liquid food by capillary action. To reduce evaporation, experiments were carried out in a small room in which the relative humidity was kept at 80%. Ten female flies were transferred to each vial by brief CO_2 anesthetization and allowed to feed on the provided food sources for 3 h. Five control vials without flies were used to determine the effects of evaporation in each experiment. Since the diameter of the capillary was constant, we measured the length of the meniscus descent to determine the evaporation rate. The length of meniscus descent in vials with flies minus the length of meniscus descent in vials without flies was calculated as food consumption. For single-type food experiments where two capillaries were filled with same liquid food, all the experimental procedures were the same as described above except that the food consumption of the two capillaries in each vial was summed to get total consumption.

1.3 Proboscis extension reflex (PER)

Flies were immobilized on slides in a humidified chamber for 2 h at 18°C. Experiments were carried out at room temperature (<25°C). Flies were first fed with water to satiation then heat shocked with an infrared laser [45] aimed at the head for 5 s. Each fly was stimulated by heat three times, with water administered between each heat treatment, and the number of proboscis extensions was recorded. Three batches of 20–30 flies were tested for each genotype.

1.4 Immunohistochemistry

Three- to five-day-old female flies were collected and brain dissection was performed in cold phosphate-buffered saline

(PBS). The samples were fixed in freshly prepared paraformaldehyde (4% in PBS) for 3 h on ice, rinsed 3×15 min in PBT (PBS with 0.5% Triton X-100), followed by blocking with PNT (10% normal goat serum in PBT) for 30 min at room temperature. Next, samples were incubated with primary antibodies diluted in PNT overnight at 4°C. Samples were then washed in PBT (3×15 min at room temperature), and then incubated overnight in secondary antibody. After being rinsed in PBT for 3×15 min, samples were mounted in Vectashield Fluorescent Mounting Media (Vector Laboratories, Burlingame, USA) and observed. The following antibodies were used: mouse nc82 antibody (1:100, Developmental Studies Hybridoma Bank, University of Iowa, USA), rabbit anti-GFP antibody alexa 488 conjugated (1:400, Invitrogen, USA), goat anti-mouse antibody TRITC-conjugated (1:200, Jackson ImmunoResearch Laboratories, USA).

1.5 Imaging

Mounted whole brains were scanned with a confocal microscope (Leica TCS SP5). Stacks of optical sections at 2 μm spacing were collected by a Leica 40× objective lens with 1024×1024 pixel resolution at 200 Hz. The images were processed with ImageJ (National Institutes of Health, rsbweb.nih.gov/ij/). Figures were prepared using Adobe Photoshop (Adobe System, CA, USA).

1.6 Statistics

Student's two-tailed *t*-test was carried out to evaluate the consumption difference between two groups in Figure 1. Two-way ANOVA (analysis of variance) was carried out to analyze variation among and between groups in Figures 2 and 3. One-way ANOVA was carried out to evaluate the consumption difference among multiple groups in Figure 5. Fisher's exact test was used to analyze PER data. Statistical significance was assigned as $*P<0.05$, $**P<0.01$, $***P<0.001$; n.s., not significant.

2 Results

2.1 *Drosophila* preference for nutritious food is concentration-dependent

To test feeding preference towards sugar directly, we used a modified capillary feeding (CAFE) system as our behavior paradigm. First, several types of sugar (sucrose, fructose, trehalose) solutions were paired with pure water to test whether different foods elicit different levels of attraction. After about 22 h of starvation, flies showed a significant preference towards these sugars over pure water during a 3 h test. As shown in Figure 1A and B, when either 100 mmol L^{-1} sucrose or fructose solution was paired with water, flies

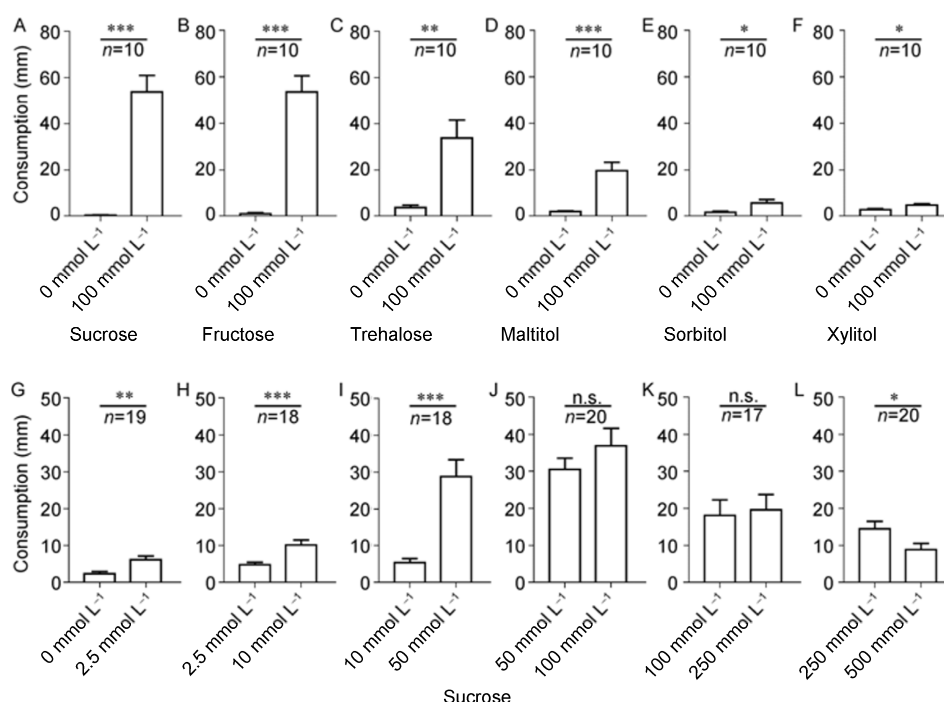

Figure 1 Preference for sweet or nutritious food in *Drosophila* is concentration dependent. A–F, Wild-type flies were given different foods to test feeding preference. Compared with pure water, flies showed robust preference towards various sweet or nutritious foods, including sucrose (A), fructose (B), trehalose (C), maltitol (D), sorbitol (E) and xylitol (F). G–L, When exposed to a gradient of sucrose concentrations, flies showed sweet preference dimorphism. They were attracted at low and moderate sucrose concentration (G–J), but avoided sucrose at very high concentration (K and L). Error bars indicate SEM. Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$; n.s., not significant.

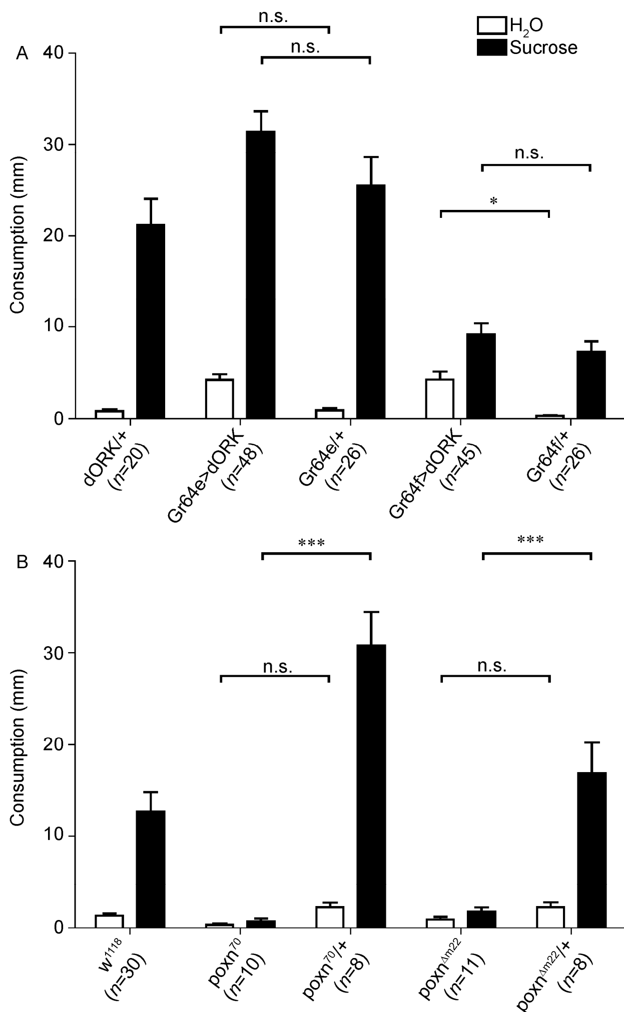

Figure 2 Blocking the taste sensory system decreases sugar preference. Flies with an impaired sugar sensory system showed abnormal feeding behavior. A, After expressing dORK in sugar-sensing neurons driven by Gr64f-Gal4, flies increased water consumption substantially, while sucrose consumption remained unchanged. Expressing dORK in other Gr-Gal4 lines (e.g., Gr64e-Gal4) had little influence on both water and sucrose solution intake. B, *poxn* null mutants showed a "refusing to eat" phenotype characterized by significantly decreased consumption of sucrose solution but not water. White bars indicate water consumption while black bars indicate sucrose consumption. Error bars indicate SEM. Two-way ANOVA, *P<0.05, **P<0.01, ***P<0.001; n.s., not significant.

consumed significantly more sugar solution than pure water (P<0.0001), and the total consumption of sugar solution and water was more than 50 mm. When 100 mmol L^{-1} trehalose solution was paired with water, flies also consumed more trehalose solution than water (P=0.0012), but total consumption decreased down to 35 mm (Figure 1C).

Several groups have reported that *Drosophila* is able to evaluate food nutrition independent of taste [9–12]. To address this, we used several types of sugar-alcohol (sorbitol, xylitol, maltitol) solutions, which have nutritional value but sorbitol and xylitol solutions lack sweet taste, to pair with water and test the fly's feeding preference. As shown in Figure 1D, when given water and 100 mmol L^{-1} maltitol solution (sweet), flies still displayed a robust preference towards maltitol (P<0.0001). However, the total consumption of sugar alcohol solution and water decreased down to 20 mm. Then, we chose to pair two tasteless but energy-rich sugar alcohol, the sorbitol and xylitol solutions, with water respectively. As we expected (Figure 1E and F), the consumption of sorbitol (P=0.0173) or xylitol (P=0.0388) was significantly higher than that of water. However, the total consumption of sugar alcohol solution and water decreased to less than 10 mm. These results indicate that *Drosophila* always preferred nutritious food over water, regardless of whether it was sweet or not. However, the amount of food intake was different in different food sources.

Although the above experiments showed an obvious inclination towards sugar or sugar alcohol compared with pure water, we found food consumption also varied considerably in a concentration-dependent manner when flies were provided with only sucrose solutions. First, at a low concentration range (0–50 mmol L^{-1}), flies always preferred the "sweeter" food (Figure 1G–I). When given pure water and 2.5 mmol L^{-1} sucrose solution, flies could discriminate sucrose solution from water (P=0.0022, Figure 1G). When given 2.5 and 10 mmol L^{-1} sucrose solution, flies still showed a significant preference towards the sweeter (10 mmol L^{-1}) sucrose solution (P=0.001, Figure 1H). The preference for sweeter food persisted in the 10 and 50 mmol L^{-1} sucrose solution group (P<0.0001, Figure 1I). When the sucrose concentration increased, the total consumption increased as well. The consumption reached its peak at 50 and 100 mmol L^{-1} sucrose solution group, however, the consumption of 100 mmol L^{-1} sucrose solution was not significantly higher than that of 50 mmol L^{-1} sucrose solution (P=0.2541, Figure 1J). Interestingly, as the sucrose concentration increased even more, the total consumption decreased (Figure 1J–L). In the 100 and 250 mmol L^{-1} sucrose solution group, starved flies consumed the two food sources equally (P=0.8087). However, the level of consumption was nearly half of the 50 and 100 mmol L^{-1} sucrose solution groups (Figure 1K). In the 250 and 500 mmol L^{-1} sucrose solution groups, the preference was clearly towards the "less sweet" one (P=0.0345, Figure 1L). When we compared solutions having a greater concentration difference (100 mmol L^{-1} and 1 mol L^{-1}), flies showed a significant preference towards the more moderate concentration. This avoidance of high sugar concentrations was also seen with other sugar solutions, such as trehalose solution (data not shown). These results indicate that *Drosophila* shows sweet preference dimorphism, attracted by a low and moderate concentration while avoiding a very high concentration.

2.2 Blocking the taste sensory system eliminates sucrose preference

To understand the neural circuits responsible for sugar pref-

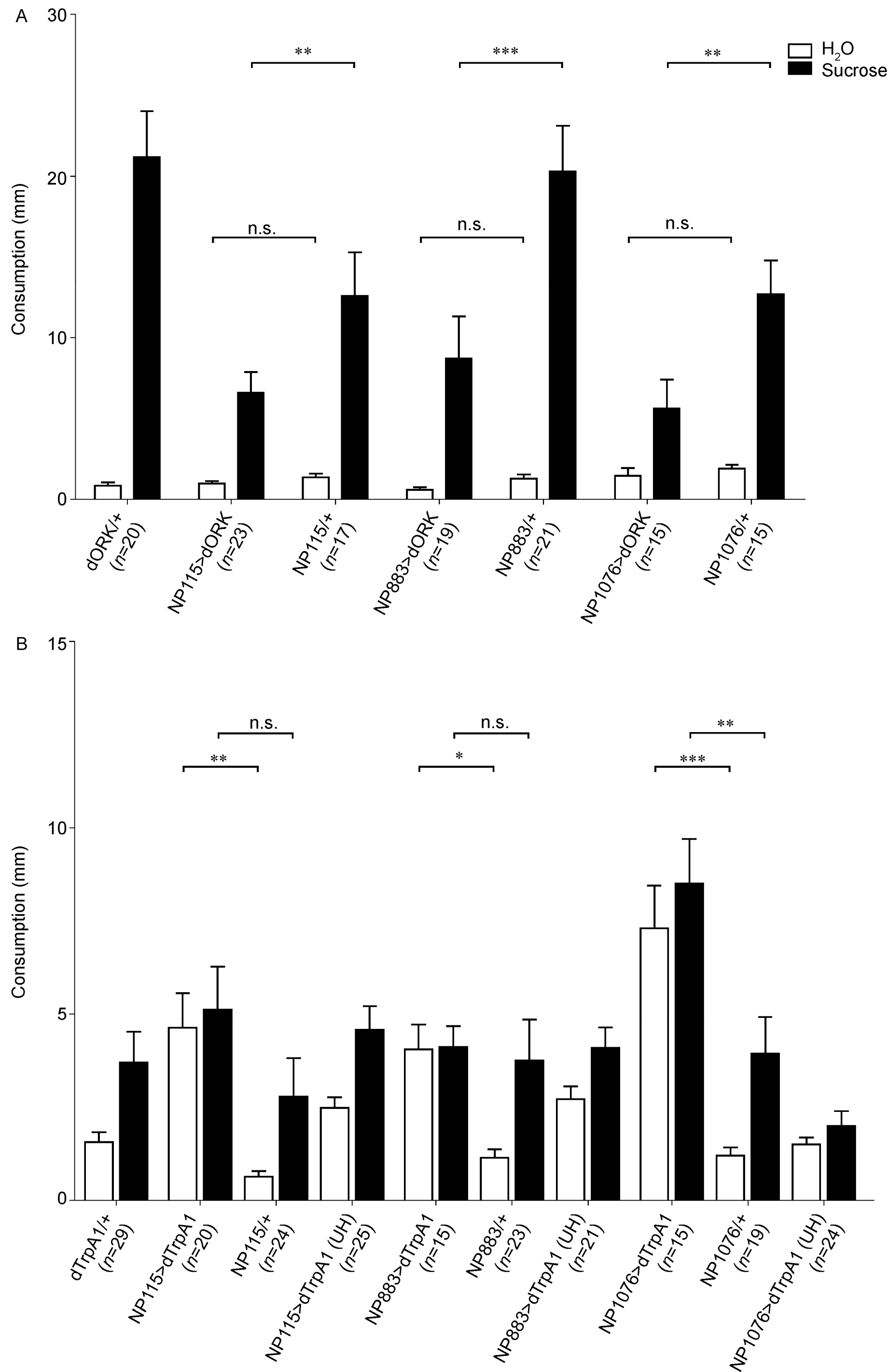

Figure 3 Neural silencing screen identifying three NP-Gal4 labeled circuits involved in feeding behavior. A, When the three Gal4 (NP115-, NP883- and NP1076-Gal4) labeled neurons were silenced, flies decreased their sucrose consumption significantly while maintaining water consumption (A). B, After activation of three NP-Gal4 labeled neurons flies increased water consumption significantly. Only NP1076-Gal4>dTrpA1 activated flies also increased sucrose solution consumption significantly. White bars indicate water consumption while black bars indicate sucrose consumption. Error bars indicate SEM. Two-way ANOVA, *$P<0.05$, **$P<0.01$, ***$P<0.001$; n.s., not significant.

erence and to identify neurons involved in taste processing, we tested the role of sugar-sensing neurons that were labeled by several Gr-Gal4 (Gr5a-, Gr61a-, Gr64a-, Gr64c-, Gr64d-, Gr64e, Gr64f-Gal4) lines. To silence neuronal activity of these taste receptors, we crossed the Gr-Gal4 lines with UAS-dORK [40], which expresses a mutant K^+ channel that opens at the resting membrane potential, causing increased K^+ efflux and therefore membrane hyperpolarization [46]. Flies were provided with water and 10 mmol L^{-1} sucrose solution (this was the food source used in the fol-

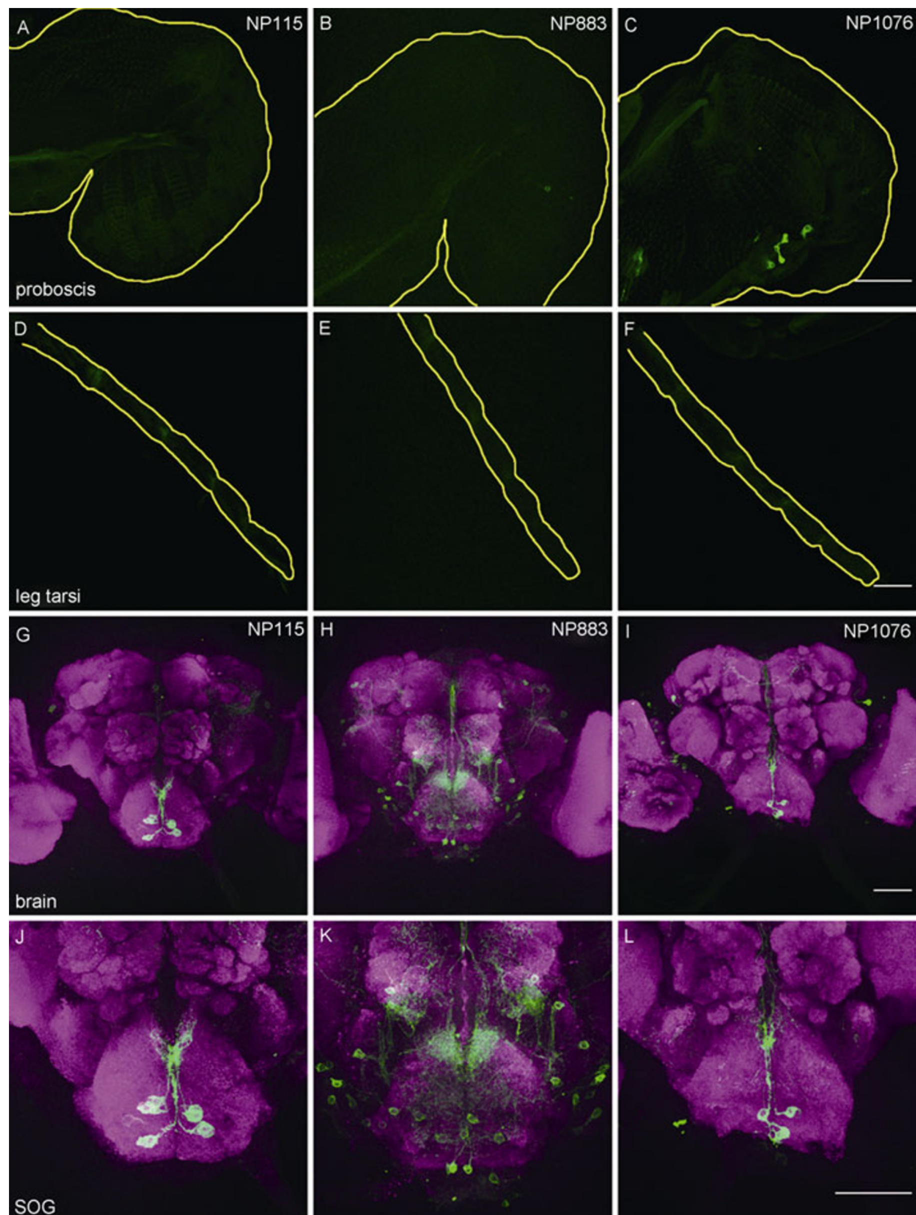

Figure 4 The three NP-Gal4 lines are expressed primarily in the SOG. Membrane tethered GFP was used to report Gal4 expression. A–F, The expression patterns of the three Gal4 lines in periphery sensory organ. No obvious sensory neurons were labeled in either proboscis (A–C) or leg tarsi (D–F). G–L, The expression patterns of the three Gal4 lines in central nervous system. Three Gal4 lines express sparsely across the brain (G–I) but primarily in the SOG (J–L, larger images of G–I SOG region respectively). Green, GFP signal; magenta, nc82 signal; scale bar, 50 μm.

lowing experiments unless otherwise noted). Among the seven groups of sugar-sensing neurons that were silenced, only Gr64f-Gal4>UAS-dORK flies decreased their sugar preference, by increasing water consumption substantially. These flies showed increased water consumption up to 4.3 mm, which was significantly higher than controls ($P<0.05$). However, their sugar consumption was not significantly changed (Figure 2A). Likewise, Gr64e-Gal4>UAS-dORK flies also consumed more water (as much as 4.3 mm), which was high but not significant compared with the control group. Similar to Gr64f, the sugar consumption difference between Gr64e-Gal4>UAS-dORK flies and control flies was also not changed (Figure 2A). In contrast, silencing the other five taste receptors revealed normal sugar preference coupled to a small amount of water consumption (less than 3 mm, data not shown). These results showed that neuronal silencing in most of the Gr lines results in a normal sugar preferences, except for one line (Gr64f) showing significantly increased water consumption when given a choice of food sources, which suggests that these sugar-sensing neurons may compensate for each other in sugar detection, and silencing one at a time cannot eliminate sugar preference completely.

To determine whether eliminating a suite of taste receptors might produce a stronger sugar preference defect, we next tested the taste-blind mutants, $poxn^{70}$ and $poxn^{\Delta M22}$, in

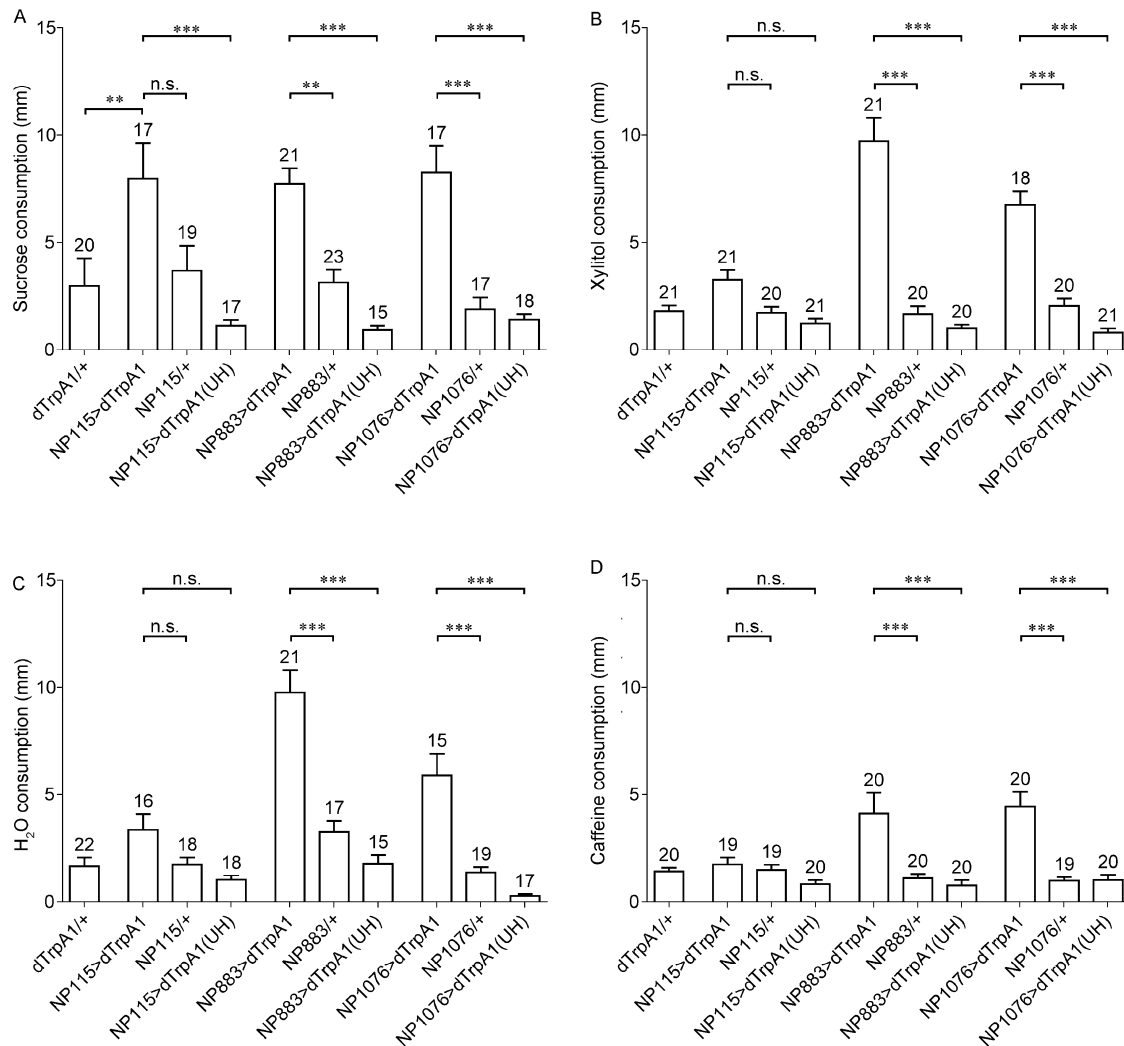

Figure 5 Increasing the activity of the three Gal4 labeled neurons enhanced food intake in different food conditions. Flies were subjected to the CAFE apparatus with only one kind of solution in heated or unheated conditions (UH). The activated NP115-Gal4>UAS-dTrpA1 flies showed increased consumption only in sucrose solution condition, whereas the other two activated lines showed increased consumption in all food conditions, including sucrose solution (A), xylitol solution (B), water (C), caffeine solution (D). Error bars indicate SEM. One-way ANOVA, *P<0.05, **P<0.01, ***P<0.001; n.s., not significant.

which taste bristles are transformed into mechanosensory bristles lacking gustatory receptors [38,47,48]. As a result of the absence of gustatory attraction to sucrose, *poxn* mutant flies displayed no preference in the two-choice CAFE assay (Figure 2B), as evident by their dramatically lowered consumption of sucrose solution (P<0.001). In fact, they ate little of either food, showing a "refusal to eat" phenotype. These results indicated that gustatory receptors are indeed needed in normal feeding behavior and blocking the taste sensory system could decrease or even eliminate sugar preference.

2.3 Three Gal4 lines were identified in a screen for altered feeding behaviors

To find additional neurons that affect feeding behavior, we screened 456 Gal4 lines crossed with UAS-dORK to silence neurons, and then tested the flies' performance in CAFE system. We found three lines with significantly decreased sucrose consumption when neuronal activity was silenced in these neurons (NP115-, NP883- and NP1076-Gal4; Figure 3A). NP115-Gal4>UAS-dORK flies consumed 6.6 mm sucrose solution which was significantly less compared with control NP115-Gal4/+ flies (P<0.01). NP883-Gal4>UAS-dORK flies consumed 8.7 mm sucrose solution, which was also significantly less compared with control NP883-Gal4/+ flies (P<0.001). NP1076-Gal4>UAS-dORK flies consumed 5.6 mm sucrose solution, which was, again, significantly less than control NP1076-Gal4/+ flies (P<0.01, Figure 3A). Water consumption was not different from controls for all three transgenic lines (Figure 3A). These neural silencing experiments suggest that neurons marked by the

three NP-Gal4 lines are necessary for sugar intake.

Since silencing these neurons decreased sucrose intake, we questioned whether activating the neurons might have the opposite effect. We monitored the behavioral effect during acute activation of these three Gal4 labeled neurons by using the temperature-inducible activated cation channel dTrpA1 [42]. Interestingly, activating these three circuits primarily increased water consumption, relative to sucrose (Figure 3B). NP115-Gal4>UAS-dTrpA1 activated flies consumed 4.6 mm of water, which was significantly higher than 0.6 mm in NP115-Gal4/+ flies ($P<0.01$). NP883-Gal4>UAS-dTrpA1 activated flies consumed 4.0 mm water, which was also significantly higher than NP883-Gal4/+ flies (1.1 mm, $P<0.05$). Finally, NP1076-Gal4>UAS-dTrpA1 activated flies consumed as much as 7.3 mm water, significantly higher compared to the 1.2 mm water consumption in NP1076-Gal4/+ flies ($P<0.001$). In contrast, sucrose solution consumption was not elevated to the same degree as that of water. Only NP1076-Gal4>UAS-dTrpA1 activated flies displayed significantly elevated sucrose solution consumption after neuronal activation (Figure 3B). These flies consumed 8.5 mm sucrose solution, which was significantly higher compared to 3.9 mm in NP1076-Gal4/+ flies ($P<0.01$). NP115-Gal4>UAS-dTrpA1 and NP883-Gal4>UAS-dTrpA1 activated flies consumed the same amount of sucrose solution as the control groups (Figure 3B). These neuronal activation experiments showed that when the activity of three Gal4 labeled neurons was acutely increased, flies increased liquid consumption significantly, with a preference for water consumption. Taken together, these results indicate that the activity of the three NP-Gal4 labeled neurons in this study is involved in feeding behavior.

2.4 The expression pattern of three NP-Gal4 lines was mainly in SOG

To identify the neurons that are labeled by the three Gal4 lines identified in our screen, we initially used CD8::GFP to characterize the expression pattern of those lines in the peripheral taste organ (proboscis, leg tarsi) as well as in the central nervous system. No obvious GFP signals were observed in peripheral taste organs (Figure 4A–F), which suggested that the neurons in the central nervous system rather than those of the peripheral sensory system were responsible for the food intake phenotypes we uncovered. As shown in Figure 4G–L, GFP signals of three Gal4 lines distribute sparsely across the brain, but intense labeling is seen in SOG, including in cell bodies and neural projections throughout this structure. Although the Gal4 lines had these characteristics in common, they did have some distinct expression patterns. For example, NP115-Gal4 labeled four bundles of neural fibers that converge onto the top of the SOG, with two localizing in the anterior part and the other two in the posterior part. Within one bundle, more than one cell body was detected. A similar expression pattern was observed in NP1076-Gal4. However, it labeled a few more neurons than NP115-Gal4 line did, including six bundles of fibers that converged together in a pair along the midline, located from anterior to posterior in SOG. NP883-Gal4 showed a more extensive expression pattern compared with the other two Gal4 lines, labeling cell bodies sparsely at the bottom of the SOG, and intense projective arborization at the top of the SOG. These results indicate that among our Gal4 candidates, two label similar neurons while the other one labels a distinct group of neurons (Figure 4J–L). Taken together with our behavioral data, our imaging results suggest that the SOG neurons labeled by these three Gal4 lines might be involved in the regulation of food intake.

2.5 Increasing the activity of neurons labeled by the three NP-Gal4 lines promotes feeding behavior

To investigate whether acute neuronal activation of the three Gal4 circuits promotes feeding in general, we provided transgenic flies with a single type of food in the CAFE assay. In this scenario, rather than presenting competing food sources, only a single type of food was presented (10 mmol L^{-1} sucrose solution, 10 mmol L^{-1} xylitol solution, pure water or 2.5 mmol L^{-1} caffeine solution). As shown in Figure 5, when fed with 10 mmol L^{-1} sucrose solution, all three heat-activated flies consumed significantly more sucrose solution than the unheated (UH) controls ($P<0.001$ for each group), as well as the heated parental controls ($P<0.01$; Figure 5A). When fed with 10 mmol L^{-1} xylitol solution, which is reported as tasteless but nutritious to flies [9], NP883-Gal4>UAS-dTrpA1 flies consumed as much as 9.7 mm when heated, in comparison to 1.0 mm when unheated and 1.7 mm for heated NP883-Gal4/+ control flies ($P<0.001$). NP1076-Gal4>UAS-dTrpA1 activated flies also significantly enhanced their xylitol solution consumption up to 6.7 mm compared with two control groups (UH control 0.8 mm, NP1076-Gal4/+ control 2.1 mm, $P<0.001$; Figure 5B). Only NP115-Gal4>UAS-dTrpA1 activated flies displayed similar xylitol solution consumption (3.3 mm) as two control groups (1.2 and 1.7 mm, respectively). When flies were fed water, heat activated NP883-Gal4>UAS-dTrpA1 and NP1076-Gal4>UAS-dTrpA1 flies also consumed more: 9.7 and 5.9 mm, respectively, both of which were significantly higher than corresponding controls ($P<0.001$). However, NP115-Gal4>UAS-dTrpA1 activated flies did not increase their consumption as the other two activated lines (Figure 5C). In addition to these positive or neutral substances, we also tested caffeine, which tastes bitter to flies, as a negative stimulator to test whether flies still increase consumption after activation of three Gal4

labeled neurons. Interestingly, when fed with 2.5 mmol L^{-1} caffeine solution, although all the lines consumed less, heat activated NP883-Gal4>UAS-dTrpA1 and NP1076-Gal4> UAS-dTrpA1 flies still consumed more than 4 mm caffeine solution, which was significantly higher than inactivated controls and heated parental controls ($P<0.001$). Caffeine consumption in heat activated NP115-Gal4>UAS-dTrpA1 flies was not significantly different from controls (Figure 5D). These results indicate that activation of the neurons labeled by the three Gal4 lines promotes food intake in different manners. The activation of NP115-Gal4 labeled neurons promoted feeding only in food condition of sucrose solution, which is sweet and energy-supportive. The activation of NP883- and NP1076-Gal4 labeled neurons promoted feeding in all four food conditions: sweet and nutritious, tasteless and nutritious, bitter, or neutral.

In the preceding experiments, a food stimulus was always present when we tested feeding behavior. To investigate whether the neurons labeled by the three Gal4 lines affect feeding behavior independent of food stimulation, we measured their proboscis extension reflex (PER) upon transient stimulation, in the absence of a food stimulus. We subjected flies to the PER paradigm by directly activating the Gal4 neurons with temperature sensitive dTrpA1. In the PER assay, the flies, which expressed dTrpA1 driven by the three NP-Gal4 lines, were focally heated with an infrared heat pulse directed to the head. NP883-Gal4>UAS-dTrpA1 flies displayed a 79% proboscis extension frequency upon application of the laser heat (Figure 6A), which was dramatically enhanced as compared to all the control flies ($P<0.0001$; Figure 6B). Heat activated NP115-Gal4> UAS-dTrpA1 flies (35%) and NP1076-Gal4>UAS-dTrpA1 flies (31%) showed modest but significant enhancement compared with the same flies without heat stimulation or the heated parental controls ($P<0.0001$ except for the comparison of heat activated NP1076-Gal4>UAS-dTrpA1 and NP1076-Gal4/+ flies, $P=0.0032$; Figure 6B). These results showed that induced activation of the three Gal4 labeled neurons can, to some extent, replace the attractive cues represented by foods, which suggests that these neurons might be involved in feeding control as well as motivation to feed.

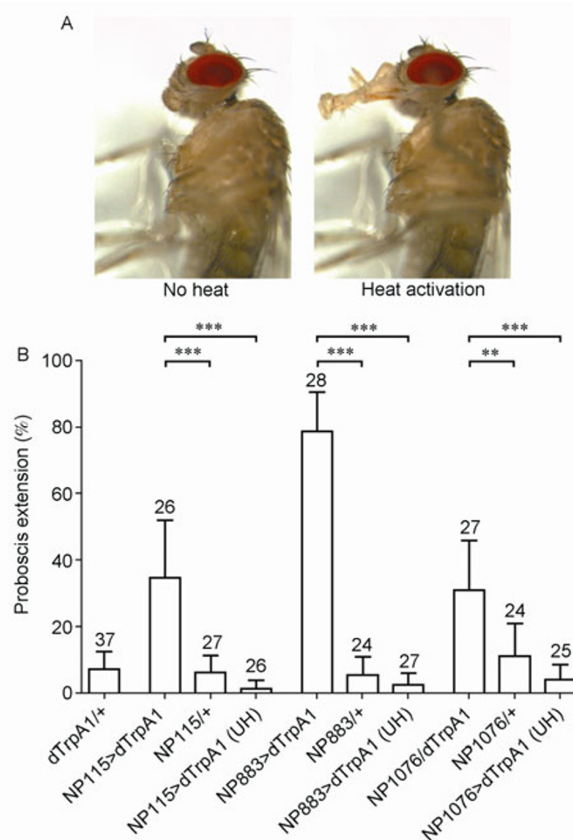

Figure 6 Activation of the three NP-Gal4 labeled neurons promotes feeding independent of food stimulation. A, Example images of NP883-Gal4>UAS-dTrpA1 flies without (left) and with (right) infrared heat stimulation. These flies did not extend their proboscises at room temperature. However, they extended their proboscises once they received a 5 s laser pulse. B, Quantification of PER experiments. All three heat activated flies showed an increased fraction of extension upon heat stimulation, which was not observed in the unheated condition (UH). Error bars indicate 95% CI (confidence interval). Fisher's exact test, $^{**}P<0.01$, $^{***}P<0.001$.

3 Discussion

Various animals show innate attraction to sweet or nutritious food while avoiding bitter or toxic food. This natural feeding behavior is a complex process that depends on both external and internal cues. Understanding how the brain integrates sensory information with internal needs, to direct appropriate feeding behaviors, requires a thorough knowledge of the entire process, from the sensory level to higher-order components in taste circuits. In this study, we have identified three Gal4 lines (NP115-, NP883- and NP1076-Gal4) that label groups of neurons in the central nervous system responsible for feeding behavior. Flies decrease their sugar solution intake when these Gal4 labeled neurons are inhibited, and conversely, enhance their feeding behavior when these neurons are activated, suggesting these neurons might be involved in feeding control.

Because feeding behavior of *Drosophila* is initiated by stimulation of peripheral sensory organs, flies respond to "sweet" food vigorously by consuming large amounts of it (Figure 1A–D). These "sweet" foods include sugars (sucrose, fructose and trehalose) as well as sugar alcohol (maltitol), which have been shown to elicit electrophysiological and behavioral responses in flies [24]. In comparison, tasteless but energy supportive sugar alcohols (sorbitol and xylitol) [9,24] were consumed much less although the preference over water remained (Figure 1E and F). We surmise

that at the beginning of the experiment, flies chose randomly between the two kinds of food. The flies provided with water and a sweet food (sugars or maltitol) rapidly recognized the sweet food and demonstrated a strong preference for it. However, those flies provided with water and a metabolizable but tasteless food (sorbitol or xylitol) required time to evaluate the nutritional value by an internal sensor after ingestion [10,12]. Hence, flies in our experimental conditions used both their taste and post-ingestive system to evaluate food sources.

Regarding the consumption discrepancies in the sucrose concentration gradient experiment, flies were indifferent to both lower and higher concentrations, but displayed an obvious preference at intermediate concentrations (Figure 1G–L), which is in agreement with preference of egg-laying site selection on sugars [49]. This behavioral dimorphism, reminiscent of preference towards salt, which is preferred at low and disliked at high concentrations [50–52], might result from the balance between the attractive (palatability, nutrition) and unattractive characteristics (viscosity, stickiness) of the solution [53]. Therefore, in our neural modulating experiments, we mainly used 10 mmol L^{-1} sucrose solution, which is sweet enough to induce feeding but not too sweet to inhibit feeding.

To establish the criterion for a larger genetic screen, sugar-sensing mutants and Gr mutants were used to test sugar preference. In previous studies, *poxn* null mutants were found to be completely devoid of sweet sensory input [38,39], whereas the GRN blocking flies may have partially retained the ability to sense sweetness because not all of the sugar sensing neurons were blocked by using one Gr-Gal4 line [24,30]. This led to the enhanced water consumption (Figure 2A). Nevertheless, *poxn* null mutants that lost the motivation to feed barely ingested either solution. This finding, which does not agree with taste-independent nutrition recognition, might be due to a developmental defect of the *poxn* homozygous mutants we used.

The SOG region is widely regarded as the primary taste center in flies, and receives information from the sensory neurons and sends signals to control feeding movement after local circuit processing [31,54]. In recent years, although much progress has been made in identifying first-order sensory neurons and motor neurons controlling feeding movement, a direct connection between the two neuronal populations has not been reported, which suggests that there is at least a one-step relay mediated by interneurons in the SOG [31,32]. Recently one group reported that a single pair of NP883-Gal4 labeled neurons acts as interneurons and directs the feeding motor program [55]. They named this pair of neurons "Fdg neurons" and showed that these neurons are essential for normal feeding behavior through activation and ablation experiments [55]. Their results are consistent with ours in that activation of these neurons triggers PER and food ingestion. However, we also showed that activation of NP883-Gal4 labeled neurons could enhance food ingestion towards many different solutions other than sugar (Figure 5). The simplest interpretation of our results is that certain neurons labeled by NP883-Gal4 function directly in feeding circuits that are hardwired in the brain. These neurons reside downstream of the first order sensory neurons. On the other hand, activation of these neurons also elicited whole movements required for proboscis extension, which is controlled by a group of motor neurons. Therefore, there is a good likelihood that these candidate neurons labeled by NP883-Gal4 are interneurons, which function as an information relay from sensory neurons to motor neuron. With respect to NP115- and NP1076-Gal4, our loss-of-function and gain-of-function studies argue that although the neurons labeled by these two Gal4 lines also play a role in feeding behavior, their function may be different from NP883-Gal4 labeled neurons. When NP115- and NP1076-Gal4 labeled neurons were activated, flies showed a relatively mild PER; their total consumption (especially NP115-Gal4>UAS-dTrpA1 flies) in single type food conditions varied according to food palatability. These results together with the similar expression pattern of NP115- and NP1076-Gal4 lines indicate that those neurons may not be a core part of taste circuits, but might instead be involved in regulating the sensation of starvation or satiety.

To test all of these possibilities and get a deeper understanding of the neural mechanism underlying feeding behavior, further studies are necessary to test and demonstrate directly the functions of the neurons. Furthermore, it will be interesting to narrow down these candidate neurons to more defined neuronal populations, and to determine their specific role in the whole circuit. Overall, our work has identified three groups of brain neurons positively controlling *Drosophila* feeding behavior, and paves the way for the future investigation of neural mechanism underlying information processing from sensory input to motor output.

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