A single pair of interneurons commands the Drosophila feeding motor program

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Many feeding behaviours are the result of stereotyped, organized sequences of motor patterns. These patterns have been the subject of neuroethological studies1,2, such as electrophysiological characterization of neurons governing prey capture in toads3,4. However, technical limitations have prevented detailed study of the functional role of these neurons, a common problem for vertebrate organisms. Complexities involved in studies of whole-animal behaviour can be resolved in Drosophila, in which remote activation of brain cells by genetic means5 enables us to examine the nervous system in freely moving animals to identify neurons that govern a specific behaviour, and then to repeatedly target and manipulate these neurons to characterize their function. Here we show neurons that generate the feeding motor program in Drosophila. We carried out an unbiased screen using remote neuronal activation and identified a critical pair of brain cells that induces the entire feeding sequence when activated. These ‘feeding neurons’ (here abbreviated to Fdg neurons for brevity) are also essential for normal feeding as their suppression or ablation eliminates sugar-induced feeding behaviour. Activation of a single Fdg neuron induces asymmetric feeding behaviour and ablation of a single Fdg neuron distorts the sugar-induced feeding behaviour to become asymmetric, indicating the direct role of these neurons in shaping motor-program execution. Furthermore, recording neuronal activity and calcium imaging simultaneously during feeding behaviour6 reveals that the Fdg neurons respond to food presentation, but only in starved flies. Our results demonstrate that Fdg neurons operate firmly within the sensorimotor watershed, downstream of sensory and metabolic cues and at the top of the feeding motor hierarchy, to execute the decision to feed.

To identify neurons controlling feeding behaviour we have behaviourally screened flies in which randomly targeted neurons are activated to induce the feeding motor program in a small, temperature-controlled chamber (Supplementary Fig. 2). To genetically target random sets of neurons, we took advantage of the collection of NP (Nippon) lines6. Each of these lines expresses the yeast transcription factor Gal4 in a different stereotyped pattern of neurons that depends on the GAL4 insertion site7. Gal4-expressing cells were activated by mating flies of each line to flies with a transgene encoding a rat cold-activated cation channel, TRPM8 (ref. 8), or a Drosophila heat-activated-channel, TRP1 (ref. 9), under the control of upstream activating sequences (UAS) recognized by Gal4. A screen of 835 NP lines identified the GAL4 line NP883, which showed continuous feeding behaviour with TrpA1 at increased temperature. The induced behaviour was compared with natural feeding behaviour8 (Fig. 1a–d, Supplementary Figs 3, 4 and Supplementary Videos 1, 2). The natural feeding pattern, evoked by contact with food, is characterized by an initial cessation of locomotion followed by the sequential execution of eight basic motor patterns (Fig. 1a) for taking up food by repeated proboscis extension/retraction and opening/closing labellar lobes at the tip of the proboscis. This behavioural sequence was reproduced in a food-free environment by TRP1-mediated activation of neurons in the GAL4–expressing pattern (Fig. 1b and Supplementary Video 2). The TRP1-induced sequence was well-coordinated and indistinguishable from natural feeding behaviour in the duration of proboscis extension, labellar contact with the substrate and proboscis retraction (Fig. 1d). We observed repeated labellar opening even with the rostrum and haustellum immobilized (Supplementary

**Figure 1** | Thermogenetic activation reproduced coordinated natural feeding behaviour. a, Natural feeding behaviour of a starved wild-type (WT) fly on normal food at 21 °C, consisting of eight basic motor patterns: (1) all main joints of the fly’s forelegs (black arrowheads) bend to bring the head closer to the food (dashed horizontal lines for comparison of head heights); (2) the rostrum (magenta arrowheads) projects forward (a1 to a3) while (3) the haustellum (blue arrowheads) extends downward (a3 to a5), resulting in protrusion of the proboscis; (4) the paired lobes at the tip of the proboscis, called labella (green arrowheads), open upon touching the food to take up food (a2 to a3); (5) taking food, the labella close (a3 to a4) and (6) the rostrum and (7) the haustellum retract, returning the entire proboscis to its original position while (8) the forelegs (black arrowheads) raise the body to its original position (a3 to a5). b, TRP1-induced proboscis extension in a satiated NP883 > TrpA1 fly at 31 °C with the eight basic motor patterns indistinguishable from a. c, Schematic drawings depict unfolding sequence of major segments of proboscis. d, Comparison of time taken for each step in proboscis extension in c. n = 22 for each genotype. e, Proboscis-extension rate of free-running, satiated flies observed singly in an arena without food at 31 °C for each genotype (see Methods for description of the other line, NP5137, with a similar expression pattern to NP883 Supplementary Fig. 8c)). Magenta bars denote mean values. ***P < 0.001 (see Methods for statistics). n = 40 for each genotype. f, Temperature dependence of proboscis-extension rate without food for free-running, satiated flies for each genotype. n = 40 for each genotype at each temperature. Error bars in all figures are s.e.m.

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The induced feeding thus represents a ‘fixed action pattern’, which is completely executed without food or substrate, although the natural feeding must be coordinated with sensory stimuli as well. To quantify the feeding behaviour induced by stimulation of neurons in the NP883 pattern we adopted the extension and retraction of the rostrum and haustellum (numbers 2, 3, 6 and 7 in the sequence outlined in the legend of Fig. 1a), referred to as ‘proboscis extension’. Measured by this index, the induced feeding behaviour observed in NP883 > TrpA1 flies at increased temperature required both the NP883-GAL4 insertion and the TrpA1 transgene (Fig. 1c) and exhibited a temperature dependence consistent with the activation properties of the Drosophila TRPA1 channel (Fig. 1f). Feeding was acutely induced in both sexes (Supplementary Fig. 6).

An essential component of feeding behaviour not measured by proboscis extension is the rhythmic activity of the pharyngeal pump, which is used for swallowing food. To assess whether the behaviour induced in NP883 > TrpA1 flies included activation of the pharyngeal pump, we fluorescently labelled the pharyngeal muscles using green fluorescent protein expressed under control of the enhancer in the myosin heavy chain (Mhc) locus (Mhc–GFP) (Fig. 2a, b) so that they could be observed through the cuticle. These muscles, m11, m12-1 and m12-2, (Fig. 2b–d) are attached to the upper sclerotized plate out of two sclerotized plates, which lie on top of one another (Fig. 2e and Supplementary Fig. 7a). Observing the action of the pump using a dye-coloured sugar solution to visualize fluid flow upon presentation to a starved fly revealed the dynamics of pump movement shown in Fig. 2a–e, Supplementary Fig. 7a–c and Supplementary Video 3. In brief, m12-1, m12-2 and m11 sequentially contract and relax in an alternating manner to lift first the anterior and then the posterior parts of the upper plate to generate rhythmic peristaltic waves of the upper plate, which move ingested material from the mouth to the esophagus between the two plates. Counting of individual pump cycles under m12-1 and m12-2 (Fig. 2f) revealed that sucrose-induced feeding in a starved fly was mediated by vigorous pumping at 6–8 Hz when the temperature was set at 29 °C, with the rate declining gradually as the fly became satiated, then leading to a fully swollen crop in 2 min (Fig. 2g). When we tested NP883 > TrpA1 flies in the Mhc–GFP background we found that temperature increase induced intermittent pumping that was indistinguishable from natural pumping (Supplementary Video 3). Satiated NP883 > TrpA1 flies showed only occasional pumping at 21 °C, but pumped the sugarless dye solution at 6–8 Hz at 29 °C in a pattern indistinguishable from that of wild-type starved flies with sucrose solution (magnified plots in Fig. 2g). Satiated wild-type flies used as controls showed much less pumping of the dye solution, even at 29 °C, compared to NP883 > TrpA1 flies at the same temperature (Fig. 2g), as total pumping pulses were quantified to show a sevenfold difference (Supplementary Fig. 7d). Although the induced total pumping during 2 min was 40% lower than the sucrose-induced pumping of starved wild-type flies (Supplementary Fig. 7d), comparison of rates (Fig. 2h) showed that the NP883 > TrpA1 flies maintained pumping at the same steady-state rate as starved wild-type flies, although the latter initially pumped more vigorously in response to sucrose. By measuring the net amount of ingested fluid, we observed that, in the first 2 min, induced pharyngeal pumping led to ingestion of 4.7-fold more fluid than wild-type controls (Supplementary Fig. 7e), and after approximately 5 min led to a fully swollen crop, although the crop was not filled at 2 min (Fig. 2g and Supplementary Video 3). Taken together, our results demonstrate that the activation of Gal4-expressing cells in...
NP883 produces the complete feeding motor program consisting of all essential motor patterns, including pharyngeal pumping.

To identify the specific neurons within the NP883 expression pattern (Fig. 3a and Supplementary Figs 8a, b, 9) that activate feeding behaviour, we used the ‘flip-out GAL80’ technique\(^\text{15}\), in which the initially ubiquitous expression of Gal80, an inhibitor of Gal4-mediated transcription, is eliminated in small numbers of neurons by the flippase-mediated random removal of the GAL80 gene (see Methods). By this means, we simultaneously expressed TRPA1 and GFP in small subsets of the NP883 pattern and identified the GFP-labelled neurons, whose presence we could correlate with TRPA1-induced feeding. From screening 1,243 flies (Supplementary Fig. 10) we dissected the flies that showed a proboscis-extension rate of six times per min or above, a value not observed in non-flipped-out specimens (Supplementary Table 1).

Examination of the 40 proboscis-extension-positive flies led to the identification of one type of interneuron, the GFP expression of which correlated with flies having a higher proboscis-extension frequency as seen in the histogram of Fig. 3b. We termed this pair of interneurons Fdg neurons. They possess a distinct and stereotypical morphology with extensive arborization (Fig. 3d, Supplementary Figs 10c and 11, Supplementary Video 5), which can be unambiguously identified with a fast neuronal marker antibody, nc82 (yellow, on the basis of synaptic marker analyses in Supplementary Fig. 12).

Interestingly, we observed an unusual directionality to the proboscis extension-positive flies (Supplementary Video 4). These Fdg neurons more closely mimicked natural feeding behaviour. To determine whether Fdg neuron activity is required for natural feeding, we first suppressed activity of all neurons in the NP883-GAL4 pattern by the expression of an inward rectifier potassium channel, Kir\(^\text{10}\), leading to abolishment of natural feeding behaviour in response to sucrose (Supplementary Fig. 14 and Supplementary Video 6). The main sugar-sensing neurons of the labellum, which express the gustatory receptor GR5A\(^\text{16,17}\), terminate in the vicinity of the dendrite of the Fdg neuron, but careful confocal analysis revealed no direct contact between the processes of the two types of neuron (Supplementary Fig. 15). To determine whether the Fdg neurons receive indirect input from the sugar-sensing neurons, we assayed their response to gustatory stimuli by calcium imaging using a genetically encoded Ca\(^{2+}\) indicator, GCaMP3.0 (ref. 20), driven by NP883-GAL4. To achieve this, we used a specially designed setup (that is, the feeding circuit/fly brain live imaging and electrophysiology stage, or FLIES) to visualize SEG neurons through an opening in the head (Fig. 4a, b). As shown in Fig. 4c, stimulation of the labellar lobes of a starved fly with 400 mM sucrose resulted in brief lobe opening, and a simultaneous, large increase in GCaMP3.0 fluorescence in the cell body of the Fdg neuron (Supplementary Video 7). This response was specific insofar as an adjacent neuron, which we called LPE (lateral peri-esophageal) (Supplementary Figs 13 and 16a), showed no increase in GCaMP3.0 fluorescence, even in starved flies (Supplementary Fig. 17a). Furthermore, red fluorescent protein (RFP) fluorescence did not change with the sucrose stimulus (Fig. 4c and Supplementary Fig. 17a). Interestingly, neither labellar opening nor Ca\(^{2+}\) increase in the Fdg neuron was observed in satiated flies (Supplementary Fig. 17). Our results thus indicate that sucrose acutely activates the Fdg neurons, and that this response is contingent on the metabolic state of the animal.

The Fdg neuron, as shown in Fig. 3d and Supplementary Fig. 10c, is responsible for the feeding behaviour with higher proboscis-extension rates (see also Supplementary Note 1).

Supplementary Fig. 10d shows the induced behaviour (Supplementary Video 4) of a fly that had strong GFP expression only in a single Fdg neuron, as shown in Fig. 3d and Supplementary Fig. 10c. The behaviour induced by TRPA1 in this fly clearly included all eight motor patterns of the natural feeding program following the initial cessation of locomotion, indicating that activation of a single Fdg neuron can induce the entire sequence of feeding behaviour. It should be noted that the feeding behaviour observed in Fdg-neuron-positive flies contrasted with that of NP883 \(\bowtie\) TrpA1 flies in that it included more walking (Supplementary Video 4) and lacked the leg tremors observed in NP883 \(\bowtie\) TrpA1 flies (Supplementary Video 2), probably owing to suppression of TRPA1 expression in other cells by Gal80. In these respects, the behaviour resulting from the activation of individual Fdg neurons more closely mimicked natural feeding behaviour. Interestingly, we observed an unusual directionality to the proboscis extensions produced by flies in which single Fdg neurons were activated. As shown in Supplementary Fig. 10e, Supplementary Video 4 and Supplementary Table 2, proboscis extension was consistently directed towards the side the GFP-expressing Fdg neuron was on. This asymmetric regulation of proboscis extension by the Fdg neuron suggests that each Fdg neuron may selectively regulate the strength of proboscis muscle contraction on the same side of the body, consistent with the observation that presentation of food to gustatory receptors on one side of the body leads to proboscis extension on that side (Supplementary Video 1).

Figure 3 | Identification of the Fdg neuron. a, Full expression pattern of NP883 in the SEG as a confocal section, which covers both Fdg neurons (arrowheads). b, c, A histogram of proboscis-extension rate in 40 proboscis-extension-positive (proboscis extension > five times per min) flies, with GFP detected in Fdg neuron (b) or ALLH neuron (c) filled in black. **P < 0.01, Mann–Whitney’s U-test between ‘with GFP’ and ‘no GFP’. d, The Fdg neuron, labelled with anti-GFP antibody (green). A confocal montage of the SEG (lower half) and antennal lobes (upper half), with the neuropil marker antibody, nc82 (magenta). e, Fdg neuron with the presumptive axon digitally traced in deep yellow, on the basis of synaptic marker analyses in Supplementary Fig. 12. Arrow indicates position where the axon posteriorly branches off from cell body fibre (CBF), then, the sub-branches travel dorsally and ventrally as axon terminals (Ax, arrowheads). CB, cell body. All scale bars, 30 µm.

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Laser ablation parts of proboscis immobilized. Arrow, sucrose wick for stimulation.

**Figure 4 | Functional analyses of Fdg neuron.** a, Experimental design with FLIES chamber for experiments in this figure. c, Ca\(^{2+}\) imaging of Fdg neuron when NP883 > GCaMP3.0, mCD8–rfp flies were stimulated with 400 mM sucrose. Top, a representative GCaMP3.0 fluorescence at the cell body of Fdg neuron (arrowheads) in a starved fly. Dashed circle denotes quantified area outlining an Fdg neuron cell body. Scale bar, 10 μm. Middle, a time course of GCaMP3.0/RFP fluorescence as ratios to the initial fluorescence in a representative example. Bottom, labellar lobe opening (arrowheads) with other parts of proboscis immobilized. Arrow, sucrose wick for stimulation. Quantification and statistics are given in Supplementary Fig. 17. d, Laser activation of a single Fdg neuron in a satiated fly. Left panels, proboscis extension to the fly’s right side in response to laser stimulation of the fly’s right Fdg neuron cell body to activate TRP1 under a two-photon microscope. Right panels, pump movement induced by laser activation of a single Fdg neuron on either side. Dye solution was applied through a capillary tube as shown in Fig. 2f, to see ingestion through the pharyngeal pump (white arrow) and the osphagophagus (arrowhead) immediately after laser illumination. e, Laser ablation of a single Fdg neuron. A cell body of a single Fdg neuron was intensely illuminated under a two-photon microscope. Blue arrows denote sucrose-induced proboscis-extension directions before (left panels) and after (middle panels) ablation. Right panels, abolishment of sucrose-induced proboscis extension after ablation of both Fdg neurons. Chain lines denote fly’s midline. White arrows denote sucrose wick.

The FLIES setup allowed us to image and focally heat the cell body of a single Fdg neuron expressing TRP1 and GFP using limited illumination of the infrared laser of a two-photon microscope for activation of the Fdg neuron (see Methods and Supplementary Fig. 16b). As expected from the results of our flip-out GAL80 studies, this stimulus caused immediate asymmetric proboscis extension to the side of the stimulated neuron (Fig. 4d and Supplementary Video 8). It also induced pump movement (Fig. 4d and Supplementary Video 8). By contrast, selective illumination of an LPE neuron, located only 10–20 μm from the Fdg neuron (Supplementary Figs 13 and 16a, c), failed to induce proboscis extension or pump movement at the same stimulus level (see Methods and Supplementary Video 8). These results directly confirmed that activation of a single Fdg neuron can trigger feeding behaviour in a specific manner.

To test the requirement for the Fdg neuron in natural feeding behaviour, we selectively ablated Fdg neurons in starved NP883 > gfp flies using stronger laser illumination (see Methods and Supplementary Fig. 16d). Ablation of the Fdg neuron on one side, followed by stimulation with 400 mM sucrose, triggered proboscis extension in the direction opposite to the ablated side, whereas ablation of the Fdg neurons on both sides completely eliminated the response to sucrose (Fig. 4e and Supplementary Video 9). In control experiments, ablation of the nearby LPE neuron did not affect the proboscis-extension response, again demonstrating the specificity of the manipulation. Consistent with the results of Kir suppression (Supplementary Fig. 14), these results demonstrate that Fdg neurons are essential for natural feeding in the fly and demonstrate the absence of neurons with redundant function.

The induction of the entire feeding program by Fdg neuron activation contrasts with the effects of activating motor neurons that innervate muscles of the proboscis or the pharyngeal pump. The induction of feeding by Fdg neurons is likewise distinct from that produced by stimulation of neurons that co-express neuropeptide Y and agouti-related protein in the mammalian hypothalamus, which has long latencies (that is, minutes versus seconds) and involves indirect regulation of motor output. Their activity encodes metabolically derived motivational cues and contrasts with that of the Fdg neurons, which clearly encode integrated information of both gustatory and metabolic origin, and drive motor output in a manner that is perhaps most reminiscent of the ‘command neurons’ (interneurons whose natural activity triggers a specific motor program) first described in the crayfish. The motor—as opposed to motivational—function of the Fdg neurons is evident in their asymmetric control of proboscis extension, which indicates a specific role of each Fdg neuron in contraction of a subset of the proboscis musculature. How the Fdg neurons coordinate the various motor patterns involved in feeding remains to be determined.

Pump rhythms (Fig. 2), like the well-characterized movements of the crustacean stomatogastric nervous system, may result from the action of a central pattern generator governed by intrinsic membrane properties and inhibitory interactions of the component neurons. Recently, co-activation of motoneurons controlling m11 and m12-1 has been shown to generate rhythmic contractions of the pharyngeal pump, and activation of these neurons by the Fdg neurons might be the source of the pump central pattern generator. As seen in Supplementary Video 5, the large dendritic arborization of the Fdg neuron, which is reminiscent of the putative feeding neurons of toads and courtship neurons of *Drosophila*, suggest a role in integrating information beyond sugar and starvation cues, including perhaps gustatory cues, such as bitter or salty, and signals of other modalities. In any case, our laser ablation experiments suggest that inputs that govern feeding responses probably pass through the single pair of Fdg neurons. The identification of these neurons here and the demonstration of their pivotal position in the feeding circuit open the door to systematic future experiments on their roles in sensory integration and its plasticity in fly feeding behaviour.

**METHODS SUMMARY**

In behavioural observation, the temperature was maintained within ± 1 °C of a set temperature. Immunohistochemistry was performed according to a protocol described previously with a modification for adult brains. Ca\(^{2+}\) imaging as well as laser activation and inactivation were performed using FLIES apparatus, which was designed to expose the brain of a fly for general purposes such as live imaging, electrophysiology and to keep the fly’s proboscis dry and free for movement. A sugar-free saline (1.5 mM Ca\(^{2+}\)) used previously for *Drosophila* electrophysiology was continuously perfused at 21 °C. The head capsule was opened by a tungsten ‘sword’, which was originally designed for dissection of a *Drosophila* embryo used in study of synaptic plasticity, and by ‘scissors’, which are forceps modified to act as scissors. An ultrathin, smooth, traditional Japanese Washi paper, Gampi-shi (Haibara), was used as a wick for sugar stimulus.

Full Methods and any associated references are available in the online version of the paper.

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**Author Contributions** M.Y., S.I., T.F.F. and M.G. designed the research. T.F.F. screened GAL4 lines under the supervision of K.I. and M.Y. T.F.F., S.I. and M.Y. performed behavioural analyses. S.I. performed analyses of ingestion and pump movement while M.Y. visualized the pump movement. M.G., S.I. and M.Y. performed neurological analyses; A. Taylor and R. Seeham for technical help; and N. Yoshihara for material information. This work was supported by National Institute of Mental Health Grant MH65958, and the Worcester Foundation (to M.Y.), the National Institute of Mental Health Intramural Research Program (B.W.), the summer program of the Japan Society for the Promotion of Science/National Science Foundation (to T.F.F.), and a Japan Science and Technology Agency CREST grant (to K.I.).

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METHODS

Immunohistochemistry. We performed immunolabelling according to a protocol described previously with a modification for adult brains (see Supplementary Methods for details).

Saline and dissection tools. A sugar-free saline used previously for *Drosophila* embryonic electrophysiology was also used in this study. The saline contained (in mM): NaCl, 140; KCl, 2; MgCl₂, 4.5; CaCl₂, 1.5; and HEPES-NaOH, 5 (pH 7.1). The head capsule was opened by a tungsten ‘sword’, which was originally designed for dissection of a *Drosophila* embryo used in study of synaptic plasticity, and by ‘scisceps’, which are forceps modified to act as scissors.

Fly strains. *Drosophila* crosses were performed at 21°C or 25°C according to standard protocols. Canton S was used as the wild-type control. Transgenic strains were balanced with FM7c, CyO, TM3 or TM68 chromosomes. UAS–TRPM8 has been previously described. UAS–TrpA1 (ref. 19) was obtained from P. Garrity. UAS–mCD8–mCherry was made by A. Sheehan, and generously provided by M. Freeman before publication. Mhc–gfp was from K. Gajewski, UAS–mCD8–gfp and a heat-shock-flippase (HS-FLP) strain were from T. Lee. Tubulin–GAL80 (in which ‘GT’ denotes a flip recombination target, FRT, sequence to be recognized by fly) was generously provided by M. Roshbash. Mhc–GAL80 was from S. Waddell, UAS–brp–gfp and UAS–nAChR–gfp. Mhc–GAL80 were from S. Sigrist, UAS–n-syb–gfp was from M. Ramaswami, GrSa–gfp–IREs–gfp–IREs–gfp was from K. Scott, UAS–Kir2.1 was from V. Budnik, tubP–GAL80 (temperature sensitive) was from S. Waddell, UAS–GCaMP3.0 (ref. 20) was from L. Looger and UAS–mCD8–rfp was from T. Awasaki.

We used two GAL4 strains that were established by the NP consortium. The NP883 line has a GAL4 insertion approximately 500 base pairs 5’ upstream of the untranslated region of *CytoP6* (ref. 42), a locus encoding a member of the cytochrome P450 family, which functions for electron transfer. Although none of the GAL4 lines screened showed temperature-induced behaviour similar to NP883, another NP line not included in the screen, NP5137, was later identified as having an insertion at a more proximal site to the coding region of *CytoP6* (ref. 42). This line exhibited a similar pattern of feeding behaviour (Fig. 1e, f) when driving TRP1A, and included Fdg neuron in its expression pattern common to NP883 (Supplementary Fig. 8c).

Observation of fly behaviour. For observing TRP1A-induced behaviour, we used a custom-built plastic chamber (Supplementary Fig. 2), which enabled the temperature gradient to be maintained within ±1°C from its floor to ceiling (height 4 mm) at experimental temperatures. The chamber was designed to fit snugly into a Nunc 35-mm plastic dish, and temperature was regulated by a TS-4 SPD Controller (Physitemp) and monitored with an IT-23 probe connected to a microprobe thermometer, BAT-10 (Physitemp) (Supplementary Fig. 2). The inside of the fly chamber was cleaned after each use. We observed fly behaviour by the usual techniques (see Supplementary Methods).

For observing labellar movement with immobilized proboscis (Supplementary Fig. 5), we anaesthetized a fly in a 15-ml plastic tube immersed in ice, and placed the fly in a Pipetman tip with its tip cut to expose its head. The rostrum and haustellum of the fly’s proboscis were fixed using light-curing glue (Tetric EvoFlow, Ivoclar Vivadent). The fly held in the Pipetman tip was videotaped for 1 min at 21°C. Then, the Pipetman tip holding the fly was placed in the temperature-controlled chamber pre-warmed to 31–32°C. One minute later, when the temperature monitored by the temperature probe reached 31–32°C, we videotaped the labellar lobes for 1 min. After that, we took the Pipetman tip holding the fly out from the chamber and placed it in the room at 21°C, and we videotaped labellar lobes for 1 min.

Visualization and quantification of pump movement and quantification of dye ingestion amount. For visualization of pump movement, a fly with Mhc–gfp was constrained in a Pipetman tip as stated above. For natural feeding, an aqueous 100 mM sucrose solution with 0.03 mg/ml Brilliant Blue FCF (Acros Organics) was provided to a 24-h-starved fly through a hypodermic needle. For visualizing dye ingestion amount, we noticed that, in a partially satiated wild-type flies and sometimes in NP883 > TrpA1 flies, contractions of m12-1 and m12-2 were not necessarily associated with those of m11 (Supplementary Video 3), causing backward flow from the spherical lumen (Supplementary Video 3). To quantify ingestion directly, we therefore measured the net amount of ingested fluid as follows.

For quantification of pump movement and ingestion amount, a tethered fly on its back was provided with the dye solution through a glass capillary tube, which was loaded on a manipulator and connected with an injector as stated above. The fly was allowed to move for 1 to 2 min and videotaped in the 2.5 mm × 10 mm (2 mm height) space through the top glass (3.5 mm × 10 mm) at each time point for every genotype. Proboscis extension that reached the food was counted for 30 s, 1–1.5 min after placing the fly into the chamber, and proboscis extension per minute was calculated.
Calcium imaging with observation of proboscis extension response. Ca$^{2+}$ imaging as well as laser activation and ablation were performed using FLIES, which was designed to expose the brain of a fly for general purposes such as live imaging, electrophysiology and to keep the fly’s proboscis dry and free for movement. Ca$^{2+}$ imaging was performed by a method modified from that previously reported. An adult fly was anesthetized in a 15-ml plastic tube standing on ice and set in a tube attached to a FLIES apparatus. Light-curing glue was used to seal the proximally adjacent part of the rostrum to the inner edge of the chamber’s hole. To minimize movements of the fly, we immobilized the proboscis, which we kept half extended to prevent the pump unit from bumping into and from occluding the SEG, with light-curing glue leaving only labelar lobes free to move. In the saline described above, the head capsule was opened by the tungsten ‘sword’, and by the ‘scisceps’ to better clip the cuticle and trachea and expose the SEG. The oesophagus, muscle 16 (ref. 12) and the antennal nerves were removed, and air sacks were stretched to the side to expose a Fdg neuron’s cell body and to avoid movements that could add noise to the Ca$^{2+}$ signal. Ca$^{2+}$ imaging was performed following a previous report. We scanned the cell body of an Fdg neuron through a 40× water immersion lens (0.80 numerical aperture), using the spinning disk confocal laser system, CSU X1 (Improvision/Yokogawa) using Velocity software, v.4.3, on a BX51W1 microscope (Olympus). mCD8–RFP was co-expressed to check movement artefact, and GCaMP3.0 and mCD8–RFP were labelled at the same time. GCaMP3.0 signal was imaged with an exposure time of 300 ms of 491-nm laser for detection, and mCD8–RFP fluorescence was imaged with a 535-nm laser with an exposure time of 100 ms every 1.4 s. GCaMP3.0 fluorescence and mCD8–RFP fluorescence at the cell body of a Fdg neuron were quantified at a region of interest using the Velocity software (Improvision). Identification of a Fdg neuron by its location was confirmed by immunolabelling with anti-GFP antibody recognizing GCaMP3.0 after Ca$^{2+}$ imaging experiments. Throughout the experiments, saline was slowly (one drop per second) perfused. Perfusion dramatically reduced the spontaneous movement of a proboscis, which is one key source of movement artefact. The proboscis was stimulated by an aqueous sucrose solution in the same manner as the PER experiments with Kir suppression. Labellar bristles (Supplementary Fig. 3a) sensed the sucrose and the proboscis extended reproducibly if flies were starved for 24 h immediately before PER experiments (Supplementary Video 7). PER behaviour was monitored and recorded through a CCD camera attached to a dissection microscope (SMZ-800, Nikon) supported by a swing arm while the targeted cell body (Fdg neuron or LPE neuron) was set in the FLIES setup at 21°C. All preparation for the Fdg neuron was set into the FLIES apparatus stated above, but the proboscis extension was monitored and recorded through a CCD camera attached to a dissection microscope (SMZ-800, Nikon) supported by a swing arm while the targeted cell body (Fdg neuron or LPE neuron) was set in the FLIES setup at 21°C. All preparation for the Fdg neuron was set into the FLIES apparatus stated above, but the proboscis was kept half extended and monitored for movement artefact even after several attempts to reduce movement artefact. Before and after ablation, we tested PER with 400 mM sucrose stimulation. We perfused saline chilled to 21°C during all experiments. After ablation of a neuron, we waited for 15 min until ablation effect appeared on PER. Numbers of samples were: 5 (Fdg neuron), 5 (LPE neuron). All Fdg neuron ablation gave consistent results with those in Fig. 4e and Supplementary Video 9, whereas ablation of LPE neuron showed no recognizable effect on proboscis extension. For assessing asymmetry both in laser activation and in laser ablation, we analysed movie frames, and judged asymmetry if the midline of the labella extended beyond 5% of the distance between the midline and the lateral edge of the fly’s head.

### Laser activation and laser ablation of an Fdg neuron.

The FLIES apparatus was used, and experiments were performed under the Zeiss two-photon microscope, LSM 7 MP. The fly was set into the FLIES apparatus stated above, but the proboscis was left free for observation of its movement, especially for testing asymmetry of proboscis extension. Dissection was done in the same manner as in the Ca$^{2+}$ imaging experiments. We used the same saline as that used for Ca$^{2+}$ imaging in this section for laser activation and ablation experiments.

For laser activation, we first briefly imaged a saturated NP883 > mCD8–gfp fly and identified an Fdg neuron and an LPE neuron (control), limiting infrared illumination as much as possible to avoid triggering activity (Supplementary Fig. 16). Then we set a 15.4-μm (55 pixel at 3× zoomed condition; pixel size, 0.28 μm) diameter region of interest surrounding the cell body (Fdg neuron or LPE neuron). We set the circle so that its diameter was twice the diameter of the cell, so as not to miss the cell body even after small movements, which were inevitable because the proboscis was moving freely. Using the ‘test bleaching’ program of the LSM 7 MP system’s Zen software, we scanned the area of the circle for 120 ms total (four iterations) with 20% power at a laser setting of 870 nm. We set the scan speed at 10 ms as total (five iterations) with 30% power at 870 nm. The strong laser made a damaged-looking cell body (arrowhead in Supplementary Fig. 16) to confirm that the cell was ablated. In some cases, we could observe a small, transient bubble, which shrank and disappeared in a few seconds, then ended up with the aforementioned damaged look. Before and after ablation, we tested PER with 400 mM sucrose stimulation. We perfused saline chilled to 21°C during all experiments. After ablation of a neuron, we waited for 15 min until ablation effect appeared on PER. Numbers of samples were: 5 (Fdg neuron), 5 (LPE neuron). All Fdg neuron ablation gave consistent results with those in Fig. 4e and Supplementary Video 9, whereas ablation of LPE neuron showed no recognizable effect on proboscis extension.

For assessing asymmetry both in laser activation and in laser ablation, we analysed movie frames, and judged asymmetry if the midline of the labella extended beyond 5% of the distance between the midline and the lateral edge of the fly’s head.

### Statistics.

All statistical analyses were performed according to standard methods using Prism, v.5.0a (GraphPad Software) and Excel (Microsoft).

For statistics in Fig. 1c, the six groups were analysed with the Kruskal–Wallis test using a one-way analysis of variance by ranks, and the significant difference between groups was found with P<0.0001. * denotes P<0.001 by Dunn’s post-hoc multiple comparison test between progeny from this cross: NP883 × UAS–TrpA1 (NP883 > TrpA1), compared to these crosses NP883 × wild type, wild type × UAS–TrpA1, or wild type × wild type. The same post-hoc analysis was performed for the NP1537 line.

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