Cellular identification of water gustatory receptor neurons and their central projection pattern in *Drosophila*

Tsuyoshi Inoshita and Teiichi Tanimura*

Department of Biology, Graduate School of Sciences, Kyushu University, Ropponmatsu, Fukuoka 810-8560, Japan

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Water perception is important for insects, because they are particularly vulnerable to water loss because their body size is small. In *Drosophila*, gustatory receptor neurons are located at the base of the taste sensilla on the labellum, tarsi, and wing margins. One of the gustatory receptor neurons in typical sensilla is known to respond to water. To reveal the neural mechanisms of water perception in *Drosophila*, it is necessary to identify water receptor neurons and their projection patterns. We used a Gal4 enhancer trap strain in which GAL4 is expressed in a single gustatory receptor neuron in each sensillum on the labellum. We investigated the function of these neurons by expressing the upstream activating sequence transgenes, *shibire^{ts1}*, tetanus toxin light chain, or diphtheria toxin A chain. Results from the proboscis extension reflex test and electrophysiological recordings indicated that the GAL4-expressing neurons respond to water. We show here that the water receptor neurons project to a specific region in the subesophageal ganglion, thus revealing the water taste sensory map in *Drosophila*.

taste | water receptor neuron

Chemical senses are necessary for all animals to perceive environmental chemical information. In insects, contact chemoreception plays a role in a variety of behaviors, including feeding, courtship, and oviposition site choice. Because sophisticated genetic tools can be used in *Drosophila*, this organism offers an excellent system to explore the neural and molecular mechanisms of taste (1). The chemosensory organs of *Drosophila* are composed of bristles called sensilla that are located on the labellum, tarsi, wing margins, and ovipositor. There are usually four gustatory receptor neurons and one mechanosensory neuron at the base of a typical sensillum (2, 3). Electrophysiological studies illustrate that these bipolar receptor neurons respond to different stimuli such as sugar, salt, water, and deterrent compounds (4–6). Although perception of water induces feeding behavior of flies when they are thirsty, there is less information about neural mechanisms of water perception. The axons of gustatory receptor neurons in the labellum project to the subesophageal ganglion (SOG), and those of the tarsi project to the thoracic–abdominal ganglion or to the SOG. Previous studies using horseradish peroxidase (HRP) as a marker reported projection patterns of gustatory receptor neurons on the labellum (7–10) and described seven types of projection patterns. Recently, the *Gr* family of putative gustatory receptor genes was identified. Understanding the function of this protein group may clarify the neural mechanism underlying the sensory coding of taste (11–13). *Grs* encode G protein-coupled receptors with seven transmembrane domains, such as the sweet and bitter taste receptors found in vertebrates (14–16). One of these *Grs*, *Gr5a*, is required for the sensory response of *Drosophila* to trehalose (17–19). The gene expression pattern of *Gr5a* was observed by using a *Gr5a* promoter–Gal4 strain (19–21). Recent studies using *Gr* promoter–Gal4 strains suggested that gustatory receptor neurons expressing one group of *Grs* might respond to deterrent compounds (6, 20, 21). Observing the GAL4 expression pattern

in the SOG of these *Gr-Gal4* strains provided vital information on the taste map that presumably corresponds to stimuli by sugar or deterrent compounds. To reveal a water sensory map in the brain, it is necessary to identify water receptor neurons at a cellular level and to observe their projection patterns.

In this study, we describe a Gal4 enhancer trap line (22, 23) in which GAL4 is expressed in only one of the four gustatory receptor neurons that are housed in taste sensilla on the labellum. Using the GAL4/upstream activating sequence (UAS) system, we expressed several genes that interfere with the function of the GAL4-expressing gustatory receptor neurons. Behavioral and electrophysiological data demonstrate that these neurons respond to water. Observation of the projection patterns of a single GAL4-expressing neuron in the SOG demonstrated that water receptor neurons project to a specific region of the SOG.

Results and Discussion

Electrophysiological studies showed that there are water-responding gustatory receptor neurons in the taste organs of flies (4, 24). It is believed that the receptor neurons might be detecting the change in osmolarity, although the molecular mechanism of water perception is not known. The cellular identification of water receptor neurons might help to elucidate the molecular mechanism of water reception and the neural mechanism of information processing of water perception.

Taste information is received by gustatory receptor neurons and is then transmitted to the brain. Axons of gustatory receptor neurons project to the SOG of the brain. To elucidate the neural mechanism underlying taste discrimination in *Drosophila*, the projection of four types of gustatory receptor neurons must be determined. To this end we screened the Gal4 enhancer trap strains using UAS-GFP to find strains in which GAL4 is expressed in gustatory receptor neurons in the labellum.

In one of the strains, NP1017, GFP-expressing neurons were observed mainly on the labellum and tarsi (Fig. 1). GFP-expressing neurons were also found in interpseudotracheal papillae on the inside of the labellum. There are 31 taste sensilla on each side of the labellum that are classified into three types: s-, l-, and i-type (5, 25). s- and l-type sensilla have four gustatory receptor neurons and one mechanoreceptor neuron, whereas i-type sensilla have only two gustatory receptor neurons and one mechanoreceptor neuron (5, 25). We found that a single GFP-positive neuron is innervated to all of the l- and s-type sensilla, whereas no GFP-positive neuron was found innervated to the i-type sensilla (Fig. 1B and D–F). Hiroi *et al.* (24) found that one

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Abbreviations: DTI, diphtheria toxin A chain; HRP, horseradish peroxidase; SOG, subesophageal ganglion; TNT, tetanus toxin light chain; UAS, upstream activating sequence; PER, proboscis extension reflex.

*To whom correspondence should be addressed. E-mail: tanimura@rc.kyushu-u.ac.jp.

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Fig. 2. Functional identification of the GAL4-expressing neuron. (A) Sugar responses (on the left) and salt aversion (on the right) in NP1017 carrying UAS-shibire^{ts1} flies. Open bars represent the percentage of responding flies at a permissive temperature (22°C), and solid bars represent the result at a restrictive temperature (30°C). (B) The percentage of water-responding flies was decreased in NP1017 carrying UAS-shibire^{ts1} flies. Water responses of parental control strains were not affected. (C) Water and sugar responses of taste sensilla on the labellum in NP1017 carrying UAS-TNT flies. Data are based on two to six experiments using 10–16 flies in one series of tests. Error bars indicate SE. *, $P < 0.01$.

DTI in water receptor neuron induced malfunction of water responses or induced cell death. These electrophysiological and nuclear staining data indicated that GAL4 is expressed in the water receptor neuron.

Projection Pattern of Water Receptor Neurons in the SOG. To identify the projection pattern of water receptor neurons, we observed

Fig. 3. Electrical and cytological identification of a water receptor neuron by ablation with UAS-DTI. (A) No spikes to water were recorded. (B) In a sensillum of other flies, spikes were recorded only at the initial phase after stimulation. (C) Normal water responses were recorded from most of taste sensilla. Arrowheads show the start of stimulation in A–C. (D and E) There are five neurons (arrows in E) in a taste sensillum, and there are four neurons (arrowheads in D) in an l4 sensillum from which no water responses were recorded, as shown in A. (Scale bar: 10 μ m.)

GFP expression patterns in the SOG of NP1017 carrying UAS-GFP flies. The GFP-expressing neurons in the labellum project to two specific regions in the SOG. One group of neurons projects to the lateral–anterior region of the SOG (Fig. 4 B, D, and F, arrowheads), and the other group projects to the central region (Fig. 4 B, C, and F, arrows). The lateral–anterior projection is tortuous and revealed less arborization (Fig. 4 B, D, and F, arrowheads), whereas the central projection showed sparse arborizations in its synaptic region (Fig. 4 B, C, and F, arrow). In the labellum, GAL4 is expressed in receptor neurons of both taste sensilla and interpseudotracheal papillae. To know the projection pattern originated from the water receptor neurons in the taste sensilla, we used the mosaic analysis with a repressible cell marker system and observed a projection from a single GAL4-expressing neuron (Fig. 5). We studied 24 flies in which GFP was expressed in a single GAL4-expressing neuron in a taste sensillum on the labellum. In half of them a GFP-expressing neuron was connected with an s-type sensillum; in the remaining half, a GFP-expressing neuron was connected with an l-type sensillum. The axon of a GFP-expressing neuron in a taste sensillum projected to the central region in the SOG. In >10 flies, GFP was expressed in interpseudotracheal papillae and the axons of the GFP-expressing neurons projected to the lateral–anterior region in the SOG. Our results show that all of the water receptor neurons in the taste sensilla project to the central SOG region and that the lateral–anterior projection pattern originates from the GAL4-expressing neurons in the interpseudotracheal papillae. All of the water receptor neurons in the s-type sensilla project to the central SOG region and exhibited arborization in

Fig. 4. Central projection of the water receptor neurons. (A and E) Diagrams of the frontal (A) and sagittal (E) views of the head and brain. Axons of gustatory receptor neurons in taste sensilla on the labellum project to the SOG. (B) A frontal view of the SOG of an adult brain in NP1017 carrying UAS-GFP flies. Two projection patterns were observed in the SOG (arrows and arrowheads). (C and D) One group of axons is winding and has less arborization (arrowheads in B and D), and another had narrow arborizations in the synaptic region (arrows in B and C). (F) A sagittal view of the projection pattern of water receptor neurons in the SOG. Less-arborized fibers (arrowhead) projected to the anterior region of the SOG, and narrow arborized axons (arrow) projected to the central region of the SOG. Left is anterior. (Scale bars: 50 μ m.)

the synaptic region (Fig. 5C). In some cases, the axons associated with the water receptor neurons in the l-type sensilla were diverged in the SOG (data not shown). In summary, water receptor neurons in s- and l-type sensilla projected to a same region in the SOG (Fig. 5F).

Previous studies using cobalt filling or HRP uptake on the labellum taste sensilla suggested that there are seven types of projection patterns in the SOG (8, 10). Shanbhag and Singh (10) used HRP combined with water, sugar, or salt to identify the projection patterns of each receptor neuron. Although different projection patterns were observed by HRP uptake in combination with the different compounds, it is uncertain whether the gustatory receptor neurons that absorbed the HRP/compound mixture responded to any of the three compounds. In recent studies using the *Gr* promoter-*Gal4* strain (20, 21), the GAL4 expression patterns observed among several *Gr-Gal4* strains (such as *Gr32a-Gal4*, *G47a-Gal4*, and *Gr66a-Gal4*) revealed that seven *Gr* genes are coexpressed in one of four gustatory receptor neurons. Results from their behavioral tests suggested that the *Gr5a*-expressing neurons respond to sugar and that the *Gr66a*-expressing neurons may respond to deterrent compounds (20, 21). The axons of the

Fig. 5. Projection pattern of a single GAL4-expressing neuron. Images of projection pattern (shown in C–F) were taken from one side of SOG (a box of broken lines in A). (B and C) There is one GFP-expressing neuron in one side of the labellum. This GFP-expressing neuron is connected with an s-type sensillum (arrow in B), and the axon of this neuron projects to the central region and has arborizations in the synaptic region (C). (D) A GFP-expressing neuron connecting with intersegmental papillae projects to the lateral-anterior region in the SOG. (E) Axons of two GAL4-expressing neurons, one connected with a taste sensillum and the other connected with intersegmental papillae, project to different regions in the SOG. (F) Axons of GAL4-expressing neurons in one s-type sensillum and two l-type sensilla project to same region in the SOG. (Scale bars: 50 μ m in B and 20 μ m in C–F.)

Gr5a- and *Gr66a*-expressing neurons project to different specific regions in the SOG (20). In the *Gr32a-Gal4*, *Gr47a-Gal4*, and *Gr66a-Gal4* strains, the axons of the GAL4-expressing neurons in the each side of the labellum project to the medial SOG, and water receptor neurons in one side of the labellum did not send axons to the medial SOG (Figs. 4B and 5C). We crossed NP1017 carrying UAS-DsRed flies to *Gr47a-GFP* flies and observed the projection pattern in the SOG (Fig. 6A–C, which is published as supporting information on the PNAS web site). The projection region of the water receptor neurons differs from that of the *Gr47a*-expressing neurons. We could not confirm whether the projection region of the water receptor neurons and that of the *Gr5a*-expressing neurons were distinct. GAL4 is expressed in two or three receptor neurons in some of the taste sensilla on the labellum in the *Gr5a-Gal4* strain (20, 21). It is not known whether all of the *Gr5a*-expressing neurons respond to sugar. A part of the projection region comprising the water receptor neurons may overlap with that of the *Gr5a*-expressing neurons (Fig. 6D). Perception

of water, sugar, and low concentrations of salt induces feeding, whereas perception of high concentrations of salt and deterrent compounds inhibits feeding. Our studies support the view that phagostimulatory and aversive information are diverged in the SOG (20, 21). Our studies also revealed that the projections from interseodotracheal papillae terminate in a region different from those from labellar chemosensilla. Probably the sensory input from the interseodotracheal papillae is involved in the control of sucking behavior, whereas the input from outer chemosensilla has a role in the initiation of feeding; thus, they are connecting to separate pathways. The taste map of *Drosophila* provides a simple model system to understand the neural mechanisms underlying feeding behavior.

Materials and Methods

Flies. Flies were reared on cornmeal–glucose–yeast medium at 25°C. The *Gr47a*-GFP strain was a gift from K. Scott (University of California, Berkeley). The UAS-DTI strain was a gift from R. L. Davis (Baylor College of Medicine, Houston). The UAS-DsRed.S197Y strain was a gift from K. Ito (University of Tokyo, Tokyo). T. Kitamoto (University of Iowa, Iowa City) provided the UAS-*shibire*^{ts1} strain. UAS-TNT strains and UAS-IMPTNT (inactive TNT) strains were gifts from C. O’Kane (University of Cambridge, Cambridge, U.K.). The UAS-GFP.S65T, FRT-UAS-mCD8::GFP, FRT-*Gal80*, and *hs-FLP* strains were from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington). The NP Consortium in Japan has established a large collection of Gal4 enhancer trap strains (23). We crossed NP Gal4 strains to a UAS-GFP strain and checked the GFP expression patterning on the labellum and tarsi under a dissecting microscope equipped with epifluorescence. We identified a number of Gal4 lines in which GFP expression was observed in gustatory receptor neurons. Herein we describe one of these lines, NP1017, in which a P [Gal4] transposon is inserted in the X chromosome.

Imaging. Dissected whole-mount labella, tarsi, brains, and thoracic–abdominal ganglions were fixed in 4% formaldehyde in PBS at room temperature for 3 h. The tissues were washed in PBS and mounted in 60% glycerol. GFP images were captured by using an LSM510 confocal microscope (Zeiss).

Immunostaining. Whole-mount labella were fixed in 4% formaldehyde in PBS for 3 h at room temperature. They were washed in PBS-T (PBS containing 5% Triton X-100) and treated in 0.3% H₂O₂ in methanol for 30 min at room temperature to kill endogenous peroxidase activity. Immunostaining was carried out by using standard techniques. A rabbit primary anti-GFP antibody (1:250, Sigma) and a HRP-labeled secondary antibody from Envision+ (DAKO) were used. HRP activity was visualized in the presence of 1 mg/ml diaminobenzidine in 0.01% H₂O₂.

PER Test. The PER test was performed as described by Kimura *et al.* (35). Briefly, we starved flies for 14 h and allowed them free access to water at 22°C. We then fixed each fly with myristyl alcohol on a glass slide. To test their responses to sugars and salts, we incubated the fixed flies for 2 h in a moist chamber. The flies were satiated with water until they stop responding to it. To measure the sugar responses, we used a 200 mM glucose solution as a stimulus. To measure salt responses, one prothoracic leg was stimulated with a 200 mM glucose solution, and the other leg was stimulated with a 125 mM NaCl solution simultaneously. The PER test was initially performed at 22°C. Next, the flies were placed for 20 min in a moist chamber on a temperature-controlled warming plate set at 30°C. We carried out the second PER test at this temperature, and then flies were reintroduced to the 22°C

environment for 20 min before being tested for the third time. To test the water responses, fixed flies were placed in a dry chamber over silica gel for 2 h. Water responses were then tested in a dry silica gel free chamber with water as a stimulus. NP1017 carrying UAS-*shibire*^{ts1} flies were used to test sugar, salt, and water responses by stimulating the prothoracic leg of each subject. After carrying out the first test at 22°C, the test flies were placed on the warming plate at the 30°C and incubated for 10 min before the second test. The third test at 22°C was performed 10 min after incubation at 22°C. To test the NP1017 carrying UAS-TNT flies, we examined PER to water or sugar by directly stimulating the labellum sensillum.

Electrophysiology. Flies were electrically grounded by using a glass capillary tube filled with *Drosophila* Ringer’s solution inserted into the abdomen. The recording and stimulating electrode (glass capillary with tip diameter of 20 μm) was connected to a TasteProbe amplifier (SYNTECH, Hilversum, The Netherlands). Electric signals were amplified and filtered (CyberAmp 320, Axon Instruments, Union City, CA; gain, 1,000; eighth order Bessel pass-band filter, 1–2,800 Hz). Contacting a taste sensillum with the stimulus electrode triggered a data acquisition sequence (sampling rate, 10 kHz, 12 bits; DT2821 Data Translation), which was stored as an individual file on a computer. Data were then analyzed with AWAWE software (36). Spikes were detected and analyzed by using interactive procedures from a custom-made software package, DBWAWE.

To express DTI (31–33) in a limited number of GAL4-expressing neurons in the labellum of NP1017, we used the mosaic analysis with a repressible cell marker system (34). To induce mitotic recombination, pupae at 6 h after puparium formation were heat-shocked at 37°C for 30 min. We used a strain with the following genotypes: *NP1017; FRT-UAS-mCD8::GFP/FRT-tubP-Gal80; UAS-DTI/hs-FLP*. We recorded the electrophysiological responses to sugar, salt, and water on an I-type sensillum that was confirmed to have only one GAL4-expressing neuron on the labellum in NP1017. Solutions of 1 mM KCl, 100 mM sucrose, and 200 mM NaCl were used to stimulate the water-, sugar-, and salt-responding neurons, respectively. Two kinds of spikes with different amplitude, originating from an L1 and an L2 cell, are recorded by the stimulation with 200 mM NaCl.

Nuclear Staining. To observe the number of neurons in each taste sensillum, we performed nuclear staining with propidium iodide (Sigma). Whole-mount labella were fixed in 4% formaldehyde in PBS for 3 h at room temperature. They were washed in PBS-T and treated in propidium iodide (1 μg/μl) in PBS-T after RNase (10 μg/μl, Roche Diagnostics) treatment for 30 min.

Single-Neuron Labeling. To label a single GAL4-expressing neuron, we used the mosaic analysis with a repressible cell marker system (34). To induce mitotic recombination, pupae at 6 h after puparium formation were heat-shocked at 37°C for 30 min. Flies with the following genotype were used; *NP1017; FRT-UAS-mCD8::GFP/FRT-tubP-Gal80; hs-FLP*.

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