Neuronal Projections and Putative Interaction of Multimodal Inputs in the Subesophageal Ganglion in the Blowfly, *Phormia regina*

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Abstract

In flies, the maxillary palp possesses olfactory sensilla housing olfactory receptor neurons (ORNs), which project to the primary olfactory center, the antennal lobes (ALs). The labellum possesses gustatory sensilla housing gustatory receptor neurons (GRNs), which project to the primary gustatory center, the subesophageal ganglion (SOG). Using an anterograde staining method, we investigated the axonal projections of sensory receptor neurons from the maxillary palp and labellum to the SOG or other parts of brain in the blowfly, *Phormia regina*. We show that maxillary mechanoreceptor neurons and some maxillary ORNs project to the SOG where they establish synapses, whereas other maxillary ORNs terminate in the ipsi- and contralateral ALs. The labellar GRNs project to the SOG, and some of these neural projections partially overlap with ORN terminals from the maxillary palp. Based on these anterograde staining data and 3D models of the observed axonal projections, we suggest that interactions occur between GRNs from the labellum and ORNs from the maxillary palp. These observations strongly suggest that olfactory information from the maxillary palp directly interacts with the processing of gustatory information within the SOG of flies.

Key words: blowfly, gustatory receptor neuron, mechanoreceptor neuron, neural projection, olfactory receptor neuron, subesophageal ganglion

Introduction

Taste and olfaction are crucial sensory modalities for insects to distinguish between nutritious foods and harmful poisons, and thus regulate many aspects of feeding behavior. Traditionally, olfaction is studied for its role in orientation, whereas taste is considered for its role in regulating ingestion. However, insects use both modalities to analyze multisensory information from foods and can learn to associate an odor with a taste reward. For example, the honeybee, *Apis mellifera*, and the fruit fly, *Drosophila melanogaster*, can be conditioned to a certain odor with a sugar reward (Bitterman et al. 1983; Menzel et al. 2001; Watanabe et al. 2003; Davis 2005; Giurfa and Sandoz 2012; Gruber et al. 2013; Perry and Barron 2013). The feeding threshold in *Phormia regina* can be influenced by prior feeding experience with a non-appetitive odor, D-limonene, and an appetitive odor, dithiothreitol (Nisimura et al. 2005). Recently, Shiraiwa (2008) presented evidences that odors detected by the maxillary palps (but not by the antennae) enhance phagostimulant taste in *D. melanogaster*, suggesting a close integration of gustatory and olfactory information in the central nervous system. However, little is known about the neural mechanisms underlying the integration of taste and odor in the brain of insects.

Flies use their antennae and maxillary palps as primary and secondary olfactory organs, respectively. Their antennae and maxillary palps bear sensilla basiconica, which are
characterized with multiple wall pores. In *D. melanogaster*, each of these sensilla houses 2–4 olfactory receptor neurons (ORNs), and their distribution, their sensitivity, and their olfactory receptor genes (Ors) they express have been precisely reported (Stocker 1994; de Bruyne et al. 1999, 2001; Vosshall 2000, 2001; Hallem et al. 2004). Each ORN expresses only 1 Or (Vosshall et al. 1999) and projects to the antennal lobe (AL), where individual glomerulus concentrates the projections from neurons expressing the same Or (Gao et al. 2000). The olfactory information from both the antenna and the maxillary palps converges thus into a single structure of the brain, AL, which is modality specific.

Flies detect tastants through gustatory sensilla located on the labella at the distal end of proboscis, on the legs’ tarsi, on the anterior wing margins, and on the ovipositor. These sensilla have all a single pore at the tip (Nayak and Singh 1983; Stocker 1994; Ozaki and Tominaga 1999). Each sensillum contains at most 5 functionally distinct sensory neurons in a cuticular apparatus, 1 mechanoreceptor neuron (MRN) and other gustatory receptor neurons (GRNs). The large gustatory sensilla (200–300 μm in length), 11 pairs of which symmetrically line the outer margin of labellar lobes in *P. regina*, are called LL-type sensilla. Each LL-type sensillum houses 4 GRNs, and they adequately respond to water, salts, bitter or noxious substances, and sugars or phagostimulative substances, respectively (Dethier 1976; Liscia and Solari 2000; Ahamed et al. 2001; Ozaki et al. 2003; Murata et al. 2006; Ozaki and Nakamura 2009; Masala et al. 2009; Solari et al. 2010). GRNs of each appendage project first to the ipsilateral ganglion and then to the subesophageal ganglion ([SOG], de Bruyne and Warr 2006). In several insect species, GRNs from the mouth parts and from the antennae project directly to the SOG (Stocker and Schorderet 1981; Nayak and Singh 1985; Bräunig et al. 2004; Jørgensen et al. 2006), thus the SOG is considered to be the primary gustatory center (Rajashekhar and Singh 1994; Miyazaki and Ito 2010).

The olfactory and gustatory systems are thus dealing with chemical information within separate structures, raising the question on how and where multimodal integration occurs. The dominant view to understand integration between olfactory and gustatory inputs is that interneurons are providing the neural substrate for integration occurring in higher brain centers, such as the mushroom bodies. In the honeybee, specific interneurons such as VUMm1x1, a large, multipolar neuron with dendrites receiving input in the SOG and multiple axonal branches extending into the ALs and mushroom body calyces, connects the gustatory and olfactory systems and further higher brain centers (Hammer 1997). The VUMm1x1 plays a critical role via octopamine in appetitive olfactory learning, that is, a paradigm in which odors associated with a sucrose reward can drive the proboscis extension reflex (PER). Such multimodal interneurons are found also in *D. melanogaster* (Busch and Tanimoto 2010).

Nevertheless, we considered that if flies had an additional shortcut neuronal rout that is not involved in learning and can directly integrate information between olfaction and taste without interneurons like VUMm1x1, flies should use this rout for immediate evaluation of encountered foods. Because flies should have a basic neuronal rout from the taste sensory input to the PER behavioral output, the PER sensitivity might be increased, if “phagostimulative” GRN could receive activating input coming from “appetitive” ORNs within the SOG. In this work, we examined the hypothesis that neural integration could also occur by direct integration of olfactory, gustatory, and mechanosensory information within the SOG, using *P. regina*.

In *P. regina*, many reports about gustatory system and feeding behavior have appeared (Dethier 1976; Ozaki and Nakamura 2009), but little is known about sensory system and information processing of olfactory input or about integration mechanism between olfactory and gustatory inputs. Coexistence of multiple receptor sites in the sugar receptor neuron was predicted in *P. regina* earlier than in *D. melanogaster* (Hara 1983), but gustatory or olfactory receptor genes have neither been identified nor utilized for in situ hybridization or receptor-GAL4/UAS-GFP visualization. Thus, the neuroanatomy of *P. regina* sensory systems has been less extensively characterized, when compared with *D. melanogaster*.

Because genetic dissection techniques using Or or Gr sequences are not applicable to *P. regina*, we conducted manual dissection for anterograde staining of the MRN from single sensillum trichodea and ORNs from sensilla basiconica on the maxillary palp, and GRNs from single gustatory sensilla on the labellum. Compared with *D. melanogaster*, *P. regina* has a larger body size amenable for fine-surgery ablations of either a single or few sensilla, thereby allowing us to follow a small number of receptor neuron projections through anterograde staining.

If a putative interaction between maxillary ORNs responsible for appetitive odor reception and labellar GRNs responsible for phagostimulative tastant reception can be demonstrated, it would suggest that an appetitive olfactory input via maxillary ORN can increase attractive feeding motivation. Our anatomical data and its 3D model constructed with DeltaViewer demonstrated that a subset of ORNs directly projects into the SOG, in the same area as neurons originating from labellar gustatory sensilla. Thus, we performed the PER test to sucrose stimulation in the absence or the presence of an appetitive odor, in order to measure the feeding motivation change by odor. After surveying natural food odors and floral scents by preliminary PER tests, we selected 1-octen-3-ol, given its involvement in stimulating flower nectar feeding in *P. regina*. In order to determine receptor organ for 1-octen-3-ol, we conducted behavioral experiments not only in intact animals but also in the maxillary palps-ablated and antennae-ablated flies. Based on these anatomical and behavioral data, we discuss
possibilities of direct interaction between ORNs and GRNs in the SOG.

Materials and methods

Flies

Blowfly, *P. regina*, which were generously donated by Prof. H. Morita, Kyushu University, were reared in our laboratory under a 12:12 h light:dark cycle at 21 ± 2 °C. Larvae were fed on chicken livers and yeast bait (Oriental Yeast). Adults were provided with water and 100 mM sucrose solution in separate cups. To harvest eggs, flies older than 7 days after emergence were reared in a separate cage and were provided with chicken liver in addition to water and sucrose. Egg masses laid on the chicken liver were collected every morning, and newly hatched larvae were transferred into glass bottles with chicken livers and yeast bait.

Newly emerged adult flies derived from the same egg masses were collected in separate plastic cages (22 × 15 × 13 cm) and provided with water and 100 mM sucrose solution in separate cups. To harvest eggs, flies older than 7 days after emergence were reared in a separate cage and were provided with chicken liver in addition to water and sucrose. Egg masses laid on the chicken liver were collected every morning, and newly hatched larvae were transferred into glass bottles with chicken livers and yeast bait.

Newly emerged adult flies derived from the same egg masses were collected in separate plastic cages (22 × 15 × 13 cm) and placed in an environmental chamber with a 18:6 h light:dark cycle at 21 ± 2 °C until use. Four-day-old flies were used in all experiments.

Scanning electron microscopy

The heads of flies were cut with microscissors (WPI; Sarasota) and prepared for scanning electron microscopic observation. The isolated heads were dehydrated in an ethanol concentration series of 70%, 80%, 90%, and 95% for 30 min each, followed by 100% for 2 h. Samples were then stored in fresh 100% ethanol at 4 °C or air dried for observation under a scanning electron microscope S-2150N (Hitachi Ltd).

Anterograde staining with a fluorescent dye

A test fly immobilized using an aluminum washing pin was set under a stereomicroscope (SZX-9; Olympus). The proboscis was fixed in an extended position, and the movement of the neck and legs were limited using dental wax (GC Co.). A single mechanosensory sensilla or a small number of olfactory sensilla on a maxillary palp was cut at the base and a drop of 0.1% EDTA and 2Na was placed over the lesion. Immediately after the EDTA and 2Na solution was removed, a small crystal of dextran-Alexa488 or dextran-tetramethylrhodamine (Invitrogen-Molecular Probes) was placed on the lesion. For the staining of sensilla of the maxillary palp, we used dextran-cascade blue and dextran-tetramethylrhodamine (Invitrogen-Molecular Probes).

We stained GRNs in the single LL-type sensilla on the labellum by forming a pit around the base of each sensillum with dental wax, filling the pit with 0.1% EDTA and 2Na, and cutting the sensillum in the middle of its shaft with microscissors. Immediately after the EDTA and 2Na solution was removed, we filled the inside of the pit with 1% dextran-Alexa488 or dextran-tetramethylrhodamine dissolved in distilled water and made a lid with dental wax. The flies were placed in a wet chamber and kept in the dark for 72 h at 4 °C.

Fly brains were then isolated in phosphate-buffered saline (PBS; 128 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM Na₂HPO₄, 0.34 mM KH₂PO₄, 1.83 mM CaCl₂, and 25 mM glucose), fixed in 4% paraformaldehyde in PBS overnight at 4 °C, washed with PBS, and dehydrated in an ethanol series consisting of 50%, 70%, 80%, 90%, 95%, and 100% (twice) each for 10 min. Finally, the brains were soaked in methyl salicylate and observed under a confocal laser scanning microscope (A1RMP and A1R DU4-GaAsP; Nikon).

Confocal images of optical sections from SOG and/or ALs, to which the observing sensory neurons filled with fluorescent dyes projected, were recorded and manually aligned. Each image was imported into DeltaViewer 2.1.1 and reconstructed into 3D images. DeltaViewer is an application program originally developed for Apple Macintosh by Prof. Masaaki Wada at Osaka University and can make use of OpenGL graphic library (http://delta.math.sci.osaka-u.ac.jp/DeltaViewer/index.html).

PER test

A population of 4-day-old flies derived from the same egg mass was divided into 3 groups: intact, maxillary palps-ablated, and antennae-ablated. The ablation was performed with microscissors. The flies were then starved (but provided with water) for 24 h. Prior to the PER test, flies were immobilized by securing their wings with aluminum washing pins. Under a stereomicroscope (SZX-9; Olympus Co.), they were provided with distilled water to satiation. For gustatory stimuli, 6 steps of sucrose concentrations were prepared by 4-fold serial dilutions with distilled water starting from 1 M. The labellar gustatory sensilla were stimulated with each concentration of sucrose in a yellow micropipette tip, starting with the lowest concentration at 5-min intervals, preventing flies from ingesting the stimulus solution during the test. In the intact, maxillary palps-ablated, and antennae-ablated fly groups, we conducted the PER test by stimulating their labellar sensilla with a series of sucrose concentrations in the absence and the presence of 1-octen-3-ol odor. The test odorant, 1-octen-3-ol (50 μL in a small glass bottle) was placed 2 cm below the test flies.

We purchased sucrose (Nacalai Tesque Inc.) and 1-octen-3-ol (95+%; Wako Pure Chemical Industries Ltd) for the PER test use.

Results

Sensilla on the maxillary palps

Scanning electron microscopic observations of the maxillary palps in both male and female adult flies showed a single
type of sensilla trichodea (100–500 μm in length), sensilla basiconica (5.2 ± 0.3 μm in length, average ± standard error of the mean [SEM], n = 30), and spinules (Figure 1). The sensilla trichodea, which were morphologically categorized as mechanosensory sensilla, exhibited a characteristic socket structure at the basement (Figure 1A) but neither wall pores nor a pore at their tip (Figure 1C). The sensilla basiconica were characterized as olfactory sensilla as they bear multiple pores on the cuticular walls of the sensillar shafts (Figure 1B). We found no gustatory sensilla, which would be characterized by a single taste pore at the tip of the cuticular shaft. Many spinules, sometimes called microthri- cia, were also found (Figure 1D). However, these spinules are generally considered simple cuticular expansions without innervation and likely do not play a direct role in olfactory or gustatory chemosensing (Singh and Nayak 1985; Smallegange et al. 2008).

Figure 2 shows the ventral and dorsal surfaces of a maxillary palp in a female, where triangles and circles represent sensilla trichodea and sensilla basiconica, respectively. Both types of sensilla were symmetrically distributed in the left and right maxillary palps. Table 1 summarizes the distribution of sensilla on the maxillary palps (average number of sensilla ± SEM, n = 5). There was a significantly larger number of sensilla basiconica and trichodea in the distal half than the proximal half of the maxillary palp (P < 0.01, Student’s t-test, n = 5). Sensilla trichodea were located across the entire surface of the maxillary palp, whereas sensilla basiconica were clearly concentrated in the distal half. Females had a significantly larger number of sensilla basiconica than males (P < 0.01, Student’s t-test, n = 5), but their number of sensilla trichodea was similar to those found in males (P > 0.05, Student’s t-test, n = 5).
Projection of MRNs and ORNs from the maxillary palps to the brain

When the receptor neuron derived from a single maxillary mechanosensory sensillum was filled with dextran-Alexa488, a distinct axon projecting to the SOG was stained (Figure 3). The staining pattern observed was similar to that shown in Figure 3 in 9 preparations out of 27 attempts (the staining was not complete in 18 preparations, see Table 2). As reported in D. melanogaster, each maxillary mechanosensory sensilla has 1 MRN.

On the maxillary palp, there is an area where only olfactory sensilla are found (see the dotted circle in Figure 2). When we ablated sensilla in this area and dextran-Alexa488 was placed over the lesions, we found axonal fibers projecting to both the ipsi- and contralateral ALs through the ventro-medial edges of ALs. Figure 4 shows a case where we could follow the axonal staining from a single maxillary olfactory sensillum that houses 2 or 3 ORNs in P. regina (Maeda T, unpublished data) and Musca domestica (Smallegange et al. 2008). This axonal staining looks like a single string, but it likely consists of 2 axons of 2 separate ORNs packed together at the basement of a sensillum (Figure 4, right panel) because they project to 2 pairs of ventro-posterior bilateral glomeruli in ALs (Figure 4, left panel). If we ablated a wider region in the olfactory sensilla-rich area, we found more fluorescent staining into the ALs or into the ALs plus the SOG (see Figures 7 and 8; n = 24 out of 50 samples, see Table 2). Occasionally, we also found ORNs projecting only to the SOG and not to the ALs as shown in Figure 5 (n = 9 out of 50 samples and staining was not fully succeeded in 17 of 50 samples, see Table 2). Figure 6 shows anterograde staining of ORNs from the olfactory sensilla-rich area on the ventral (A) and the dorsal surfaces of a maxillary palp (B). Both the ventral and dorsal surfaces sides of the ORNs direct to the SOG and the projection patterns looked similar to each other. We also confirmed that no staining in the brain was observed in 50 control samples, in which the same anterograde staining procedure was performed on flies without ablated olfactory sensilla (0% staining efficiency, see Table 2). This strongly suggested that the dye could not seep through fissures in the wax and transverse pores of intact sensilla.

| Table 1  Distribution of sensilla on the maxillary palp |
|------------------------|------------------------|------------------------|
| Type of sensilla      | Number of sensilla     |
| Distal    | Proximal   | Distal    | Proximal   |
| Sensilla basiconica (olfactory sensilla) | Male | 83.6±4.3 | 0.8±0.3 | 65.8±1.2 | 0.8±0.5 | 151.0±5.0 |
|          | Female    | 92.8±3.9 | 1.8±0.5 | 89.0±4.2 | 0.6±0.2 | 184.2±7.7 |
| Sensillum trichodea (mechanosensilla) | Male | 20.8±1.2 | 10.8±0.8 | 12.6±0.5 | 7.4±0.7 | 51.6±1.3 |
|          | Female    | 21.0±1.0 | 10.4±1.2 | 12.0±1.6 | 10.2±0.8 | 53.6±2.3 |

Figure 3  Axonal projection of single maxillary MRN to SOG. Triple arrows show anterior (A), dorsal (D), and medial directions (M) in Figures 3–10. ES, esophagus in Figures 3–7. Bar = 50 μm.

| Table 2  Summary of observations for single labeling |
|------------------------|------------------------|------------------------|
| Fluorescent dye introduction | Total number of samples | Number of samples | Staining efficiencies (%) |
| Distal    | Proximal   | Distal    | Proximal   | AL | SOG | AL + SOG |
| Olfactory sensilla | 50 | 1 | 9 | 23 | 66.0 |
| Mechanosensilla | 27 | 0 | 9 | 0 | 33.3 |
| No ablation | 50 | 0 | 0 | 0 | 0.0 |

Overlapping projection of the maxillary ORNs and labellar GRNs

We observed that some maxillary ORNs extended their axons to the SOG, and therefore, we examined whether maxillary ORNs interact with labellar GRNs in the SOG. With this method, we conducted anterograde double staining from a small number of olfactory sensilla on 1 side of maxillary palp and from an LL-type sensillum at the 4th
position from the most anterior LL sensillar site on the ipsilateral labellar lobe. We obtained staining patterns similar to that shown in Figure 7 (n = 23 out of 250 samples and the remaining 227 samples showed incomplete staining of either olfactory or gustatory sensilla, see Table 3). In Figure 7, the projection regions of the green-labeled maxillary ORNs and red-labeled labellar GRNs are seen. Figure 8 shows the same preparation as Figure 7 but magnified to show that they are partially overlapped both in the x-y plane and along the z axis. Some loci where interactions can occur between the maxillary ORNs and labellar GRN(s) are indicated by arrowheads “a–d.” Figure 9 shows serial optical slices for one of these putative loci (arrowhead “a” in Figure 8; see Supplementary Figures S1–S3 for other putative loci corresponding to arrowheads “b–d” in Figure 8). These slices were taken every 0.14 μm along the z axis (slice numbers 1–15 are ordered from ventral to dorsal side). When the green-labeled ORNs and red-labeled GRN(s) are merged, several yellowish points are found in a few subsequent slices, suggesting putative interactions between maxillary ORNs and a labellar GRN(s). Yellowish color turns to reddish and greenish in the ventral and the dorsal sides, respectively. Based on confocal data of Figure 8, a 3D model was constructed (Figure 10). A putative interactive locus (arrowhead “a” in Figure 8) is indicated by an arrowhead in Figure 10.

Facilitation of PER to sugar by an appetitive odor input via the maxillary palps

Because flies have 2 olfactory organs, the antenna and the maxillary palp, we examined which organ is responsible for the detection of 1-octen-3-ol that is known as an attractive odorant in some Diptera species (Takken and Kline 1989) and for the enhancement of the PER response.
to sucrose. We prepared 3 groups of 20 flies, which were intact or in which we ablated either the antennae or the maxillary palps. The PER tests were performed in the absence or the presence of odor of 1-octen-3-ol. Figure 11 shows that in intact flies (A) and antennae-ablated flies (B), the number of flies showing PER to $2^{-8}$ M and higher concentrations of sucrose is significantly increased in the presence of 1-octen-3-ol odor (Wilcoxon signed-rank test, $P < 0.05$, $n = 5$) so that the sucrose concentration-PER curves are shifted to the left. On the contrary, in maxillary palps-ablated flies (C), 1-octen-3-ol odor did not influence the PER responses to sucrose. This clearly indicates that the maxillary palps are the main organ by which 1-octen-3-ol facilitation of the PER occurs, whereas the antennae play no role in this.

### Discussion

**Multimodal sensory neuronal projections from maxillary palp and labellum to SOG**

In the maxillary palp of *P. regina*, olfactory sensilla are mainly found on the distal half and mechanosensory sensilla are distributed all over the surface. However, there are no gustatory sensilla (Figure 1). Even in starved flies, we never observed food-sucking movement of their labellum when the maxillary palps were stimulated with sucrose ($n = 10$). Furthermore, standard PER assays cannot be tested on the maxillary palps because the maxillary palps can be approached only when the fly extends its proboscis. Thus, the neurons we found projecting from the maxillary palps to the SOG are suggested not to be GRNs. The distribution of olfactory and mechanosensory sensilla facilitated our anatomical study, allowing us to conduct anterograde staining separately in these different sensilla. We found 1 area on the surface of the maxillary palp, where olfactory sensilla but no mechanosensory sensilla exist (dotted circle in Figure 2). In this area, we could perform fine surgery for the introduction of fluorescent dyes into olfactory sensilla. There was an olfactory sensilla-free region in the proximal half of the maxillary palp, where mechanosensory sensilla were distributed, hence we could also conduct fine surgery on single mechanosensory sensilla.

<table>
<thead>
<tr>
<th>Fluorescent dye introduction</th>
<th>Total number of samples</th>
<th>Number of samples</th>
<th>Double-staining efficiencies (%)</th>
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<tr>
<td></td>
<td>AL SOG AL + SOG</td>
<td></td>
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<tr>
<td>Olfactory sensilla</td>
<td>250</td>
<td>0 12 10</td>
<td>8.8</td>
</tr>
<tr>
<td>Gustatory sensilla</td>
<td>0</td>
<td>22 0</td>
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In *D. melanogaster*, it is known that the nerve bundles from maxillary palps bilaterally project to 6 distinct pairs of glomeruli (VA4, VC1, VC2, VM7, VA7l, and 1 in the ALs; Rajashekhar and Shamprasad 2004; Couto et al. 2005; Fishilevich and Vosshall 2005; Laissue and Vosshall 2008). The targeted glomeruli of the maxillary palp ORNs in Figure 7 appear to be included in similar regions of the *P. regina* ALs. A limited number of morphological papers using anterograde staining method has mentioned axonal projections from the maxillary palps to the SOG in *D. melanogaster* (Singh and Nayak 1985). In *P. regina*, we found that some ORNs of the maxillary palps project to the SOG (Figures 5 and 6) as well as MRNs (Figure 3). By comparing the thickness of the nerve bundle running to the ALs with that to the SOG in Figure 7, we expected that less maxillary ORNs project to the SOG than to the ALs. In this work, we found that roughly one-fifth of the projection neurons go to the SOG, whereas the other four-fifth terminate in AL structures. This finding suggested that these ORNs perform different roles (Bhandawat et al. 2007; Olsen et al. 2010).

The gustatory sensilla investigated here have been studied by cellular electrophysiology (Dethier 1976; Ozaki and Nakamura 2009), by patch-clamp electrophysiology (Kan et al. 2008, 2011), and using Ca^{2+} imaging (Murata et al. 2006). These sensilla house 4 functionally distinct GRNs, which extend their dendritic processes to the tip of the sensillum. Each sensillum further house 1 MRN, with a tubular body at the basement of each sensillum (Dethier 1976). This is fully consistent with our finding that when an LL-type sensillum was cut in the middle of the sensillar shaft, up to 4 axons were stained with the introduced fluorescent dye. However, at the present time, we cannot distinguish the 4 GRNs by their projection patterns and the projections could correspond to any one or several of the 4 taste modalities (salt, sugar, bitter, or water).
Putative interaction between maxillary ORNs and labellar GRNs for feeding regulation

As mentioned previously, a limited population of maxillary ORNs could interact with labellar GRN(s) in the SOG (Figure 8). ORNs and GRNs project, respectively, 50–90 and 40–70 μm deep from the most ventral surface of the SOG along the z axis, which means that these projections overlap in the 50–70-μm range. Maxillary MRNs project more dorsally than the ORNs and have little chance to interact with labellar GRNs. Hence, we focused on putative contacts between the maxillary ORNs and labellar GRNs (see Figures 7–10). Verifying optical sections layer by layer, we observed that maxillary ORNs (green) and labellar GRNs (red) overlap (yellow) in a restricted range (Figure 9). These serial optical sections suggest that close interactions occur between maxillary ORNs and labellar GRNs at several sites. 3D modeling of the optical confocal slices (Figure 10) shows a button-like structure (arrowhead), which suggests that the maxillary ORN and labellar GRN may be close enough to interact via synapse.

The anterograde staining from a single LL sensillum in Figure 8 shows 2 axonal projections (red). Only one of these GRNs was likely to interact with maxillary ORNs (green) at several putative loci (arrowheads “a–d”). This suggests that interactions of the maxillary ORNs take place only with 1 of the 4 GRNs because we found only 1 GRN interacting with ORNs in 22 out of 23 double staining samples (see Supplementary Figure S4). In the remaining 1 sample, we could not judge whether only 1 GRN interacts with ORNs. However, because the double staining efficiency in our samples was fairly low (Table 3), we cannot rule out the possibility that we missed double staining samples including such a case that 2–4 GRNs interact with ORNs. In D. melanogaster, axonal projection patterns from the sugar receptor neuron and other GRNs of the labellum have been mapped into the SOG (Wang et al. 2004; Miyazaki and Ito 2010). However, this mapping does not help us to estimate which GRNs interact with the maxillary ORNs in P. regina, as the mapping in P. regina and D. melanogaster might not be the same.

Our study showed axonal projection of ORNs to SOG in P. regina, raising the question on why such a maxillary ORN projection to the SOG has not been observed in D. melanogaster since Singh and Nayak (1985). One putative explanation is that maxillary ORN projection to the SOG is species specific, hence it was found in P. regina but not in D. melanogaster. Otherwise, this discrepancy might come from methodological differences between anterograde staining and genetic dissection using OR-GAL4 tools. Prior to this study, we have attempted anterograde staining in D. melanogaster and observed neuronal projections from the maxillary palp to the SOG, which consisted with the report by Singh and Nayak (1985). The low staining efficiency in P. regina suggests that any positive results are likely true, non-artifacts.
of SOG targeting, whereas the small size of D. melanogaster suggests that SOG targeting from maxillary palp projection neurons could be artifacts from accidentally trimming MRNs on the smaller preparation. However, we could not exclude the possibility in D. melanogaster that the maxillary ORNs project to the SOG. With regard to studies using OR-GAL4 tools to visualize maxillary palp ORNs, it is interesting that recent studies have not observed maxillary palp projection to the ORN in D. melanogaster. One possible explanation is staining strength by the OR-GAL4 technique is gene expression level dependent, hence Or gene expression level of the maxillary ORNs projecting to the SOG might not be high enough to allow those ORNs to be detected. On the other hand, in the anterograde staining, the staining efficiency in target ORNs is independent of gene expression. Now, we can use sensitive fluorescent dyes and high-spectrope models of confocal laser scanning microscope. This experimental setup allows us a higher level of sensitivity in axonal imaging that may not be available in certain OR-GAL4 lines with variable gene expression levels. Additionally, the anterograde staining method was further facilitated by recent improvements in microscope imaging utilized in our laboratory. We used 2 microscopes, A1RMP and A1R DU4-GaAsP, for Figures 7 and 8, respectively. Especially for Figure 8, we used GaAsP detector attached to the AIR-DU4 highly sensitive microscope, in order to obtain sharp images enough to construct reliable 3D model, in which noise level was decreased as low as possible even at the expense of brightness.

Our behavioral observations (Figure 11) clearly show that 1-octen-3-ol enhanced PER to sucrose when flies have functional maxillary palps but not when they are ablated. This suggests that maxillary ORNs responding to 1-octen-3-ol are directly involved in this facilitation. This observation is consistent with a previous report in D. melanogaster by Shiraiwa (2008). Particular odorants that enhance feeding behavior of flies may thus facilitate the responses of sugar receptor neurons by a direct interaction with these maxillary ORNs. In D. melanogaster, stimulation with 1-octen-3-ol induced an olfactory response from the maxillary palp, and it was suggested that 7 Ors expressed in the maxillary ORNs (Or33c, Or42a, Or46aA, Or59c, Or71a, Or85d, and Or85e) can contribute to the response (de Bruyne et al. 1999; Hallem et al. 2004; Couto et al. 2005; Ray et al. 2008). If some of these ORNs detecting 1-octen-3-ol send their axons to the SOG, they could increase the excitability of gustatory neurons responding to sugar and increase the PER sensitivity as shown in Figure 11. Usually, the maxillary palps are tightly folded against the head capsule. When the fly extends its proboscis toward food, the maxillary palps become erected and are thus exposed to food odors. Thus, the maxillary palps are likely to play a special role in detecting food odors and recognizing edible foods. To confirm this, future functional studies should clarify the underlying neuronal mechanism of the integration of olfactory and gustatory information triggering feeding in flies.

Supplementary material
Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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