LPS perception through taste-induced reflex in *Drosophila melanogaster*

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**Abstract**

In flies, grooming serves several purposes, including protection against pathogens and parasites. Previously, we found *Escherichia coli* lipopolysaccharides (LPS) can induce grooming behavior via activation of contact chemoreceptors on *Drosophila* wing. This suggested that specific taste receptors may contribute to this detection. In this study, we examined the perception of commercially available LPS on *Drosophila* wing chemoreceptors in grooming reflex. Behavioral tests conducted with bitter, sweet and salty gustation such as caffeine, sucrose and salt, using flies carrying a defect in one of their taste receptors related to the detection of bitter molecules (Gr33a, Gr33a), sugars (Gr5a, Gr4f), or salt (IR76b). LPS and tastants of each category were applied to wing sensilla of these test defectflies and to wild-type Canton Special (CS) flies. Our results indicate that the grooming reflex induced by LPS requires a wide range of gustatory genes, and the inactivation of any of tested genes expressing cells causes a significant reduction of the behavior. This suggests that, while the grooming reflex is strongly regulated by cues perceived as aversive, other sapid cues traditionally related to sweet and salty tastes are also contributing to this behavior.

1. Introduction

The induction and modification of behavior in *Drosophila* is dependent on chemical cues from the environment (Depetris-Chauvin *et al*., 2015). Grooming reflexes can be elicited in decapitated flies by contacting them with *Escherichia coli* or lipopolysaccharides (LPS) (Yanagawa *et al*., 2014). Insects groom themselves for a number of purposes, including the maintenance of body integrity and the avoidance of noxious stimuli (Böröczky *et al*., 2013; Dethier, 1972; Newland, 1998; Page and Matheson, 2004). LPS is the principal component of the outer membrane of Gram-negative bacteria and it is considered as an endotoxin, which may elicit strong immune reactions in vertebrates (Rietschel and Brade, 1992) as well as in insects (Tanaka *et al*., 2009; Rao and Yu, 2010; Kazlauskas *et al*., 2016). In flies, the presence of peptidoglycans (PGN) but also of unknown impurities contained in sLPS extracts seem to be responsible for triggering grooming, as the response intensity to sLPS is higher than to PGN (Yanagawa *et al*., 2017). This behavior is quite efficient against microbes which require a physical contact to infect insects (Vega and Kaya, 2012). Hygienic behaviors such as grooming are efficient if they can also be triggered by volatiles or tastes which are a signature of the presence of such harmful microbes (Yanagawa and Shimizu, 2007; Yanagawa *et al*., 2009). In flies, specific olfactory receptors are devoted to the detection of chemicals from harmful microbes which modify the flies’ orientation and oviposition abilities (Stensmyr *et al*., 2012), and specific contact chemicals induce grooming and feeding avoidance (Yanagawa *et al*., 2014). However, *Drosophila* habitats contain a great variety of microbes (Rolf, 2005), which also trigger positive reactions such as feeding, oviposition, and courtship behavior (French *et al*., 2015; Hiroi *et al*., 2004; Joseph *et al*., 2009; Hu *et al*., 2015). Thus, although specific chemicals may trigger stereotyped aversive responses, microbial cues may elicit different responses depending on the context. In decapitated insects, the responses induced in these insects bypassed the downstream control normally exerted by higher-order nervous centers in intact animals. Using this approach allowed us to study the networks subtending grooming.

**Abbreviations:** Conc, concentration; sLPS, lipopolysaccharides extracts from *Escherichia coli* obtained from Sigma Corp

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behavior that involve local ganglia and not the brain. The observation of this simple neural network from input to output reflex is possible only with decapitated flies.

In our previous study (Yanagawa et al., 2014), only bitter and microbial substances seemed to be important for inducing hygienic behavior (i.e., the grooming reflex). This hypothesis was confirmed by Soldano et al. (2016), who reported that TRPα1 cation channels expressed in taste sensilla expressing Gr66α on labellum, regulate sLPS avoidance in D. melanogaster. Then, Raed et al. (2016) reported that Drosophila wings can detect sweet and bitter molecules with the corresponding Gr genes as on the proboscis. Here, to learn if microbial cues are attractive or aversive to Drosophila melanogaster, we assessed the role of gustatory receptors in eliciting grooming reflex either by using optogenetic stimulations of gustatory neurons or by monitoring flies’ reactions to chemical stimuli in individuals subjected to genetic ablation of one category of taste neurons. In our optogenetic experiments, we investigated Gr64f, considered as a co-receptor for the detection of sugars (Jiao et al., 2008), and Gr33a, which is required for the detection of bitter substances (Weiss et al., 2011). We further examined the roles of Gr5a, Gr66α, and IR76b which are assumed to be involved in the detection of sugars, bitter substances and salt, respectively (Jiao et al., 2008; Weiss et al., 2011; Zhang et al., 2013). Grooming induction by the best agonists of these genes was also tested: this included caffeine (bitter) for Gr66α, sodium chloride (NaCl; salty) for IR76b, and sucrose (sweet) for Gr5a (Zhang et al., 2013). Our observations indicate that the expression of grooming reflexes not only requires functional bitter receptor genes and neurons but also gustatory genes and neurons related to the detection of sugars and salt. Lastly, to confirm the involvement of tested gene, Gr and Ir expressions in wing chemoreceptor cells were observed with a confocal microscope using GAL4 constructs to drive the expression of a fluorescent protein. Together with the confocal observation, the chemical composition of wing chemosensilla was investigated using Raman spectroscopy to see if internal structure of the chemosensillum support its functionality. It is said that Raman information of chemical compositions such as the C=O stretching of amide I indicates the presence a lymph meniscus, whose structure is important to deliver chemical cues to chemoreceptor cells (Valmalette et al., 2015).

2. Materials and methods

2.1. Flies

D. melanogaster flies were maintained on the standard cornmeal agar medium at 20 °C and 80% humidity. Most experiments were conducted on Canton Special (CS) flies. We also used mutants and UAS-Gal4 constructs listed in a separate table (Table S1).

2.2. Chemicals

sLPS (L2630, Lipopolysaccharides from Escherichia coli 0111:B4, Sigma), sucrose, caffeine, NaCl, and potassium chloride originated from Sigma-Aldrich (France and Japan) and were dissolved in distilled water. Five concentrations were prepared for all chemicals, LPS: 0, 0.1, 1, 5, 10 mg/ml, caffeine: 0, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M, NaCl: 0, 10⁻³, 10⁻², 10⁻¹, 1 M and sucrose: 0, 10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴ M. The solutions with no chemical were used as control.

2.3. Optogenetic stimulation

For optogenetic experiments, we expressed a channel rhodopsin into Drosophila taste neurons in order to activate them with blue light. To accomplish this, we used the ubiquitous UAS-Gal4 system (Brand and Perrimon, 1993) to express channelrhodopsin2 (ChR2) (Nagel et al., 2003; Hornstein et al., 2009) into cells expressing either Gr33a, which encodes for a receptor essential for aversive taste (Weiss et al., 2011), or Gr64f, which encodes for a receptor essential for appetitive taste (Dahanukar et al., 2007). Flies were beheaded by a single cut made at the neck with micro-scissors. Micro-scissors were washed and wiped with 70% ethanol before and after use. Beheaded flies were placed in the upright position on a clean paper sheet and the body was allowed to stand-up. Flies were then exposed during 3 min to blue light (> 25 W, 480 nm, COO-pe-100F-WH1-20, CoolLED, UK). During stimulation, headless flies were placed on filter paper in the dark. The blue light was the only light source in the dark room, therefore, the observation time was arranged as the same duration as the previous behavioral test (Yanagawa et al., 2014). Since the light stimulus was delivered for 3 min, the intensity of the grooming response was more variable than that induced by a quick brush with contact chemicals. Therefore, we scaled the grooming response from 0 to 5 (score 1: grooming occurrence (1–2 brushes); score 2: grooming persistence of more than 10 sec but less than 20 s; score 3: grooming persistence of more than 20 s but less than 1 min; score 4: grooming persistence of more than 1 min but less than 2 min; score 5: strong grooming which persisted for more than 2 min). Four-day old flies were used in all tests. Note that because pooled data suggested a sex difference in the grooming responses (grooming: p < 0.001, χ² = 23.4, sex difference: p = 0.007, χ² = 7.36 in Gr33a-Gal4 × UAS-ChR2 and grooming: p < 0.001, χ² = 56.7, sex difference: p = 0.27, χ² = 1.22 in Gr64f-Gal4 × UAS-ChR2, logistic regression), the data from females and males were analyzed separately. Siblings, which did not carry the full construct, were employed as controls.

Flies carrying a Gr-Gal4 construct were crossed with flies carrying UAS-channel rhodopsin (UAS-CHR2) (Bloomington Drosophila Stock Center, stock no. 28995). The genotype was Gr33a-Gal41/CyO;Dr/TM3, Sb, Ser for the Gr33a-Gal4 construct, Gr64f-Gal4 – 5/Cyo;MRKS/TM2(UBX) for the Gr33a-Gal4 construct, where siblings expressed a curling wing phenotype and progenies displayed normal wings and UAS-H134R-CHR2 for UAS-channel rhodopsin. During development, larvae were fed on normal medium supplemented with 1 mM trans-retinal (Hornstein et al., 2009).

2.4. Role of chemoreceptor genes in the induction of grooming

In order to examine Gr gene involvement in the detection of LPS, we used flies deprived of the gustatory cells expressing the tested gene (i.e. using a promoter of that gene driving the expression of GAL4). Gr33- and Gr66a-GAL4 were chosen to test bitter perception, Gr64f- and Gr5a-GAL4 were chosen to test sweet perception, and IR76b-GAL4 was chosen to test salt detection. The Gal4 lines were crossed with UAS-diphtheria toxin (UAS-DTI) flies (Wang et al., 2004). Thus, in the progeny, all cells expressing GAL4 express the toxin, causing the death of the cell. We could thus select in the progeny individuals which expressed the construct (called progeny) and others (siblings) which did not express the phenotype. For Gr33a, the construct was Gr33a-Gal41/CyO; Dr/TM3, Sb, Ser × UAS-DTI/TM6b, Tb, where the siblings expressed the tubby and the curl wing phenotype and the progenies had a normal body size and straight wings. For Gr66a, the construct was w63; Gr66a-Gal4 (II) × UAS-DTI/TM6b, Tb, where the siblings expressed the tubby phenotype and the progenies had a normal body size. For Gr64f, the construct was Gr64f-Gal4 – 5/Cyo; MRKS/TM2(UBX) × UAS-DTI/ TM6b, Tb, where the siblings expressed the tubby and the curl wing phenotype and the progenies had a normal body size. For Gr5a, the construct was pGr5a-Gal4/CyO; TM2/TM6b × UAS-DTI/TM6b, Tb, where the siblings expressed the tubby and the curl wing phenotype and the progenies had a normal body size. For IR76b, the construct was SP/CyO; IR76b-Gal4/TM3, Sb × UAS-DTI/TM6b, Tb, where the siblings expressed the tubby and the curl wing phenotype and the progenies had a normal body size and body color. As an additional control, no involvement of water perception on grooming reflex was confirmed. In order to generate flies deprived of water cells, we crossed ppp28-Gal4 flies with UAS-DTI flies (Wang et al., 2004). ppp28 mediates the cellular
and behavioral response to water (Cameron et al., 2010). Since the balancer chromosome of this construction was TM6b, Tb, we could examine the progeny and select individuals which expressed the construction (called progeny) and others (siblings) which did not express the phenotype. For ppk28, the construction was CyO/Bl; ppk28-Gal4 × UAS-DTI / TM6b, Tb, where the siblings expressed the tubby phenotype and the progenies had a normal body size.

2.5. Grooming in response to a chemical stimulation

Grooming induction was assayed using the method in Yanagawa et al. (2014, 2017), which reported together with movie files. Briefly, decapitated flies were placed on a filter paper at room temperature (22°–25 °C). We used a sharpened tooth pick previously soaked into the test solution, to gently touch their wings, margin (MR) III/IV. Their grooming activity was monitored by eyes and scored up to 3 min after the stimulation. 4-day old CS flies were tested with LPS, sucrose, caffeine and NaCl diluted in water. The concentrations of all compounds were physiological (Moon et al., 2006; Yanagawa et al., 2014) and the highest was set as that starts inducing the significantly strong reflex by the pilot test. Controls were performed by stimulating flies with distilled water. Each chemical was tested on 20 female and 20 male flies. Since the duration of single grooming differs by its intensity, a scoring system was employed to estimate the behavior: score 1: grooming occurrence (1 to 2 brush: < 10 s), score 2: grooming persistence more than 10 s but less than 20 s, score 3: grooming persistence more than 20 s but less than 1 min. The scoring had been simpler since the stimulus was applied with single contact, while the scoring in the blue light has higher level as the blue light stimulus was given continuously for 3 min.

2.6. Raman spectra of Drosophila chemosensory wing hair

Valmalette et al. (2015) reported that the Raman spectra can indicate the presence a lymph meniscus. Therefore the composition of wing chemosensory sensilla were examined with those spectra at 1350 cm<sup>-1</sup>, 1540 cm<sup>-1</sup> and 1610 cm<sup>-1</sup>. Sensilla at three locations on the wing margin were chosen to observe Raman spectra, and these values were then compared to that of small taste bristles on the leg (Sensillum S1, Fig. 6A), whose architecture is already known (Stocker, 1994). The yellow triangle on the linear wing vein pictured in Fig. 6B indicates the three assessed sensilla. Sensillum S2 is on the wing margin 2 region, which is reported that the 1350 cm<sup>-1</sup> bands resulting from the combination of N=O stretching and N−H bending of aromatic amino acids, which is consistent with the expected presence of tryptophan, phenylalanine and tyrosine. 1540 cm<sup>-1</sup> band corresponds to the position of the amide II bands resulting from the combination of N−H bending and C−N stretching, and a mode at 1610 cm<sup>-1</sup> could be assigned to the C=O stretching of amide I, and these bands were deduced as the bands indicating a lymph meniscus (Valmalette et al., 2015).

A Renishaw’s inVia Raman microscope and its Windows-based Raman Environment (WiRE2.0) software were used to obtain Raman spectra from wing taste sensilla. Raman spectral analysis used a wavelength of 514.5 nm (UV ready). Raman spectra were acquired using the method reported by Valmalette et al. (2015). Briefly, laser power was set at 5%, the spectrum range was 720−2400 cm<sup>-1</sup>, and acquisition time was 30 s. Drosophila wings from live specimen were removed at the wing/thorax muscle junction and immediately placed on a glass slide. Raman spectra were obtained from the apical and basal area of the wing sensillum.

2.7. Confocal microscopy

In order to confirm gustatory gene expression on wing taste sensillum, RFP expression in taste cells encoding each tested Gr gene were observed using a confocal microscope (LSM-700; Carl Zeiss, Jena, Germany). Gal4 lines of Gr64f, Gr5a, Gr33a, Gr66a, and IR76b were crossed with an UAS-RFP line. Gr gene expression on the proboscis was used as a positive control, and siblings and CS flies were observed as negative controls. The expression pattern of ppk28 was also visualized to confirm Grs, as ppk28 is known to co-assemble with chemoreceptor cells (Hiroi et al, 2004; Cameron et al., 2010; Chen et al., 2010). Fly wings were removed from bodies and carefully mounted onto glass slides with a drop of Vectashield (Vector Laboratories, USA) to stabilize the fluorescence. Samples were scanned within one or two days after mounting or stored at 4 °C for longer latency. Observations were made with a water immersion objective (20× plan apochromat; 1.0NA) and RFP was excited with a 555 nm solid-state laser. Images were acquired with a resolution of 1024×1024 pixels, with 1 µm interval (z) between each optical section.

In order to confirm each Gr, IR and ppk gene expression on wing taste sensillum, RFP expressions in taste cells was observed. The progeny individuals which expressed the construct were found by its RFP expression. The gene construct was as follow: Gr33a-Gal4<sup>Gal4</sup>/CyO; Dr/TM3, 5b, Ser × b y1w DbGFP(x); UAS-RFP(2), w<sup>2</sup>; Gr66a-Gal4<sup>II</sup> × b y1w DbGFP(x); UAS-RFP(2), w<sup>2</sup>; Gr64f-Gal4<sup>(1)</sup>/TM3.ser × b y1w DbGFP(x); UAS-RFP(2), Gr5a-Gal4<sup>Cyo</sup>/CyO; TM2/TM6B × b y1w DbGFP(x); UAS-RFP(2), IR76b-Gal4<sup>CyO</sup>/CyO; Dr/TM3 × Cy-x9D-GFP; UAS-RFP and CyO/Bl; ppk28-Gal4<sup>4</sup> × b y1w DbGFP(x); UAS-RFP(2).

2.8. Statistical analysis

To examine concentration-dependent increases in grooming behavior in headless flies with respect to sex, chemical, and fly strain, a multiple logistic regression (JMP 10.0 software, SAS) was applied using the least square method. The independent variable (y) was the grooming score (ranking scale), and dependent variables (xi) were physiological (Moon et al., 2006; Yanagawa et al., 2014) and the highest was set as that starts inducing the significant response by the pilot test. Controls were performed by stimulating flies with distilled water. Each chemical was tested on 20 female and 20 male flies. Since the duration of single grooming differs by its intensity, a scoring system was employed to estimate the behavior: score 1: grooming occurrence (1 to 2 brush: < 10 s), score 2: grooming persistence more than 10 s but less than 20 s, score 3: grooming persistence more than 20 s but less than 1 min. The scoring had been simpler since the stimulus was applied with single contact, while the scoring in the blue light has higher level as the blue light stimulus was given continuously for 3 min.

3. Results

3.1. Optogenetic activation of taste neurons and grooming

To determine if grooming can be elicited by perception of aversive or attractive stimuli, we used UAS-Gal4 constructs to drive the expression of channel rhodopsin receptors sensitive to blue light (ChR2) in neurons, which express either Gr33a or Gr64f. First we confirmed that blue light does not affect grooming behavior in decapitated flies (grooming in females: p = 0.32, χ<sup>2</sup> = 1.0 and grooming in males: p = 0.25, χ<sup>2</sup> = 1.34, Fig. 1A). As expected from previous data (Yanagawa et al., 2014), the activation of bitter-sensitive (Gr33a) neurons induced significant grooming (Fig. 1B, females: p = 0.044, χ<sup>2</sup> = 4.07, Mann-Whitney test, males: p < 0.001, χ<sup>2</sup> = 24.4, males: p < 0.001, χ<sup>2</sup> = 15.5, Mann-Whitney test).

During our observations, we noted that decapitated flies could use their forelegs to brush other legs and the thorax, while they used their hind legs to clean their abdomen and wings. We also noted that use of
3.2. Responses to sLPS in taste-ablated females in response to optogenetic activation (Fig. S1). Observations, males tended to groom their bodies and wings more than females following activation of Gr64f neurons (Fig. S1). Throughout these observations, males tended to groom their bodies and wings more than females in response to optogenetic activation (Fig. S1).

3.2. Responses to sLPS in taste-ablated flies

Since decapitated flies are capable of self-grooming following optogenetic or direct contact stimulation with specific chemicals (Yanagawa et al., 2014), we asked which taste neurons are necessary to induce grooming with sLPS. Water perception was confirmed not to be involved in sLPS inducing grooming reflex using ppk28-Gal4 × UAS-DTI (Fig. 2). In order to address this, we expressed a diphtheria toxin (DTI) (Wang et al, 2004) into cells that express gustatory receptors by using specific GAL4 constructs: Gr64f and Gr5a (sweet), Gr33a and Gr66a (bitter), or IR76b (salt). When such flies are crossed with those carrying a UAS-DTI construct, in the progeny of these flies, all cells expressing Grs/IR76b express Gal4, which in turn drives the expression of DTI, thus killing these cells. In order to stimulate these flies, we gently brushed the margin of their wing with the tip of a toothpick that had been dipped in a sLPS solution at increasing concentrations, from 0.1 to 10 mg/mL. While sLPS induced a clear grooming response in control flies at high sLPS concentration, such response was absent in Gr64f, Gr5a, Gr33a, Gr66a, and IR76b-ablated flies (Fig. 3, Table S1).

3.3. Responses to sLPS and other tastants in Canton S flies

The data presented above suggest that other Gr genes than those linked to bitter detection (Yanagawa et al., 2014) may also be involved in the expression of grooming reactions. In the next experiment, we thus evaluated the response of normal flies (Canton S: CS) to increasing concentrations of sucrose, caffeine and sodium chloride. The grooming induction pattern induced by each chemical was compared to that induced by the microbial surface compound, sLPS. With the exception of sucrose, all contact chemicals induced concentration-dependent grooming responses ($p \leq 0.01$ Table S2). Moreover, a slight sex difference appeared in the responses to sLPS, NaCl, and sucrose (Table S2 and Fig. 4). The contact chemical which induced the clearest concentration-dependent grooming response was NaCl. Contrary to quinine tested previously (Yanagawa et al., 2014), which induces intense grooming in both females and males, grooming induction by caffeine was significant in females but not in males (females: $p = 0.003$, $\chi^2 = 4.24$, males: $p = 0.24$, $\chi^2 = 1.41$, logistic regression, Fig. 4). As for sucrose, a significant peak in grooming behavior appeared in males following application of $10^{-6}$ M sucrose ($p = 0.002$, Dunnet’s test, Fig. 4B) but not at higher concentration.
3.4. Wing Gr gene expression

In order to see which of the gustatory genes studied above are expressed in wing sensilla, we drove the expression of a red fluorescent protein (RFP) in whole body using a UAS construct (Fig. 5). We found cells clearly marked along the wing margin only for IR76b. RFP expression patterns in Grs and its control: ppk28 constructs were unspecific (Fig. S2). We checked the validity of all genetic constructs by monitoring RFP marking in the taste sensilla of the proboscis and confirmed that all coded genes were indeed expressed on the proboscis.

3.5. Raman spectra of Drosophila chemosensory wing hairs

We next addressed the structure of wing sensilla by measuring Raman spectra to complement the difficulties in RFP monitoring. According to Valmalette et al. (2015), Raman spectra can indicate the functionality of wing chemoreceptors together with its signature of internal structure. We successfully obtained the targeted molecular motifs of bands at 1350 cm\(^{-1}\), 1540 cm\(^{-1}\), and 1610 cm\(^{-1}\) (Fig. S3D–G). Leg sensillum was taken for a comparison with wing sensilla. The pattern of Raman spectrum varied depending on locations of sensilla. We classified sensilla into four groups according to the Raman spectra-determined modes (Fig. S3D–G), and found that the molecular...
motifs of chemosensilla on the wing margin varied depending on the location (Fig. 6H). Clear bands consistently appeared in sensilla located on the legs (sensillum S1) and posterior wing margin (sensilla S3-1 and S3-2), but not on the anterior wing margin (sensillum S2). The data suggest that the chemical composition of the sensillum shaft of taste sensilla in marginal region III resemble that of taste sensilla on the leg, but different from that of sensilla in region II.

4. Discussion

4.1. sLPS perception through gutatory genes

In this study, we attempted to address the question of how gustatory genes are involved in the grooming reflex induced by the taste perception at wing chemoreceptors. Our results suggest that the detection of sLPS involves cells expressing bitter and salt receptors. Additionally, we found a dependency of grooming induction on cells expressing Gr64f, which are considered as sensitive to sugars (Jiao et al., 2008; Dahanukar et al., 2007; Slone et al., 2007; Jiao et al., 2007). We confirmed this by examining the grooming reactions induced by sLPS following the ablation of cells expressing different Gr genes. Simultaneous excitation of cells expressing Gr64f, Gr33a and IR76b could be important for eliciting the grooming reflex because if any of the cells expressing them is ablated, a reduction of grooming is observed. We also tested the responses to different tastants and to sLPS in control flies. The intensity of the grooming response induced by bitter substances was different between caffeine and quinine (Fig. 4). This may be because fruit flies would encounter less caffeine than quinine in their life. We found that chemoreceptors expressing Gr64f were important for expressing a grooming response to sLPS but not to sucrose (Figs. 3, 4). This is intriguing since Gr64f is expressed in most taste neurons responding to sugar on the proboscis (Jiao et al., 2008; Dahanukar et al., 2007). Taken together with our previous study, sLPS is indeed perceived by the receptor cells of aversive chemicals to flies but it might actually stimulate simultaneously variety of gustatory neurons expressing cells.
Fig. 6. Raman spectra of Drosophila chemosensory wing hair under 514.5 nm laser irradiation. A: Taste sensillum S1 on leg. B: sensillu, S3-1 on wing margin 2 region, which is at five sensilla away from sensillum S3-1. Sensillum S3-1 locates right at the landscape. Sensillum S3-2 is again at five sensilla away from sensillum S3-1 to the direction on wing margin 3 region. Yellow triangle on linear wing vein was a landscape for three sensillum as in B. D-G: Raman band pattern at apical/basal area of each sensillum S1–S3-2. □: White part indicates the sensilla, which had clear spectra both at apical/basal area. ■: Dotted part indicate the sensilla, which had clear spectra only at apical. ▱: Grey part indicates the sensilla, which had clear spectra only at basal area. ■: Black parts indicates the sensilla, which had no spectra both at apical/basal area. H: The percentage of each sensillum that show each kind of spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
4.2. Responses to sLPS

Microbes contain various compounds on their surface including a mixture of proteins, nucleic acids, and, to a lesser extent, lipids and polysaccharides (Butt et al., 2016). In addition, insects have immune-receptors to microbial secondary metabolites (Salton and Kim, 1996). Chemical compounds indicating the presence of microbe can be also a cue for food source or for oviposition site. Therefore, understanding how insects perceive microbes requires a step-by-step approach. LPS consists of hydrophilic polysaccharide and lipophilic lipid moieties (Butt et al., 2016). The polysaccharide component comprises two distinct portions: a core oligosaccharide and a polysaccharide chain consisting of several repeating oligosaccharide units. The presence of phosphates, fatty acids, and acidic sugars in LPS makes it a unique anionic polyelectrolyte (Panda and Chakraborty, 1998). Electro-dialyzed lipopolysaccharides could be rendered soluble by neutralizing them with alkali or with a basic amine (Galanos and Lüderitz, 1975).

Though the water cells encoded by ppk28 do not seem to play a role in the grooming reflex (Fig. 2) (Yanagawa et al., 2014), water molecules separate a large proportion of cations and basic amines from lipopolysaccharides where they neutralize negatively charged groups of the molecule. It is said that removal of these water molecules leads to acidic salt forms by neutralization with a given base (Galanos and Lüderitz, 1975). Our current findings—specifically that IR76c seemed important to perceive LPS on wing chemosensory sensilla—are in line with these aspects. IR76c has been reported not only to encode a receptor for NaCl (Zhang et al., 2013), but also a receptor detecting amino acids (Croset et al., 2016; Ganguly et al., 2017) and sourness in collaboration with IR25a (Chen and Amrein, 2017). Additionally, although the expression patterns of Grs on the wings were not clear, the expression was amenable with the RNA sequence data on Drosophila wing (Agnel et al., 2017). Supportively, we recorded similar Raman spectrum profiles from sensilla on the leg and on the wing marginal region III, suggesting that these sensilla are functional (Valmalette et al., 2015). On the other hand, we could not get the same spectrum from the Drosophila hemolymph. Though the Raman spectrum seems to indicate some internal structure of the chemoreceptor sensillum, to determine the molecular structure, which it can indicate, more investigations are required.

4.3. Role of sweet and salty tastants in grooming reflex

In contrast to previous results, sugar cells turned out to be involved in eliciting grooming reflexes. Though the response induced by sucrose was low, the responses observed following optogenetic activation of cells expressing Gr64f and the decreased responses observed following the ablation of cells expressing Gr64a/Gr5a indicate the involvement of cells usually considered as allowing sweet perception. Moreover, supportively, we checked the involvement of sucrose perception in the grooming reflex by re-activating Gr64a cells from ΔGr64a flies, and found that the re-activation of these cells recovered the response to LPS (Fig. S4). Nevertheless, it has been reported that taste sensilla on the proboscis and legs respond to sugars in the range of 1 mM to 1 M (Hiroi et al., 2002; Meunier et al., 2003). Our previous study implied that 100 mM sucrose already induced burst firing from wing sensilla (Yanagawa et al., 2014). In this study, male response at the surprisingly low concentration of sucrose implies that the grooming reflex induced by sLPS occurs in the consequence of the interactions from both bitter and sweet related cell activations. An alternative hypothesis could be that cells expressing Gr64f not only respond to sugars but that they could be activated by the lipophilic part of sLPS, in analogy with recent observations that indicate that Gr64e is involved in the detection of fatty acids (Masek and Keene, 2013; Tauber et al., 2017; Kim et al., 2018). As for IR76b, the expression pattern seemed quite similar to that of IR52a (Köh et al., 2014). Ionotropic receptor (IR) localization is known to be different from that of gustatory receptors (Grs) on the proboscis (Benton et al., 2009) and this also seems to be the case for the wing. The concentration, which starts inducing the significant grooming reflex was higher in bitter and unexpectedly lower in sugar from those that flies can sense with proboscis. The concentration that flies start to perceive the chemicals would be lower. Further work is needed to clarify the localization and function of chemoreceptor cells on the wing margin.

4.4. Gustatory receptor cells on wing, proboscis and legs

The variations of grooming reflex to sucrose and bitter or salty compounds remains unclear (Figs 4 and S2). Taste receptors on the legs seemed to perceive sLPS aversive as those on wings (Yanagawa et al., 2014). On the other hand, in our pilot test, flies drank the solution containing sLPS as much as they did water (t test, p > 0.1, Fig. S5). Together with the report that 1 mg LPS in 1 mL of 100 mM sucrose was negative in proboscis extension assay (Soldano et al., 2016), our behavioral assays suggest three possibilities: one is that insects regulate their reaction to microbes according to their concentration; the second is that sensilla on different body parts may play different roles in obtaining information from the surrounding world and the third is that LPS might inhibit sucrose perception. In conclusion, our study showed that aversive chemicals, and Grs related to the perception of aversive compounds are indeed crucial to inducing the grooming reflex; however, the behavioral response seemed to involve also taste cells and receptors implicated in the detection of tastants, such as salt and unexpectedly low concentration sugars. Further studies are necessary to better understand how flies detect and analyze the complex chemicals which are the signature of the many microbes which they encounter in their environment.

Author contributions

Aya Yanagawa: Conducting experiments, writing manuscript
Antoine Couto: helping confocal microscope observation, writing manuscript
Jean-Christophe Sandoz: supervising the confocal microscope observation, writing manuscript
Toshimutsu Hata: supervising the investigation with Raman spectroscopy
Aniruddha Mitra: helping the genetic experiments and writing manuscript
Moutaz Ali Agha: helping the genetic/behavioral experiments
Frédéric Marion-Poll: Supervising, validation of all data, writing manuscript

Competing interests

No competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://
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