Functional Gustatory Role of Chemoreceptors in Drosophila Wings

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Article

Cell Reports

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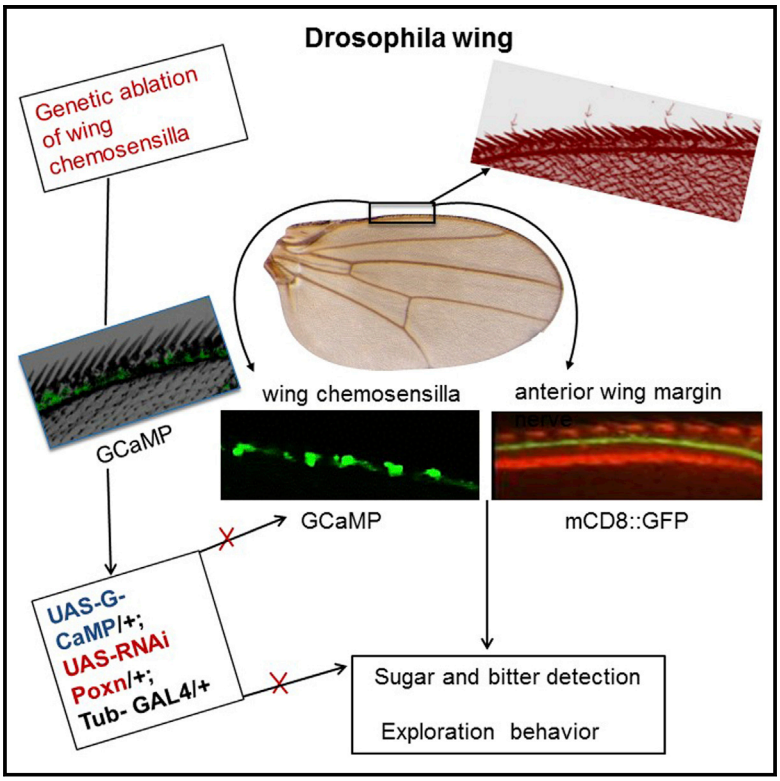
Graphical Abstract



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In Brief

The function of *Drosophila* wing chemosensilla is poorly understood. GFP gene reporter assays and electrophysiology are hampered by the nano-architecture of bristles and dense chitin. Raad et al. report that the wing taste organ responds to bitter and sugar stimuli and is critical for exploration of ecological niches.

Highlights

- Expressed GRs in *Drosophila* wing respond to sweet and bitter stimuli
- Genetic ablation of *Drosophila* wing chemosensilla abolishes taste signaling
- *Drosophila* wing chemosensilla constitute a functional taste organ
- *Drosophila* wing taste organ contributes to exploration of ecological niches

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Functional Gustatory Role of Chemoreceptors in *Drosophila* Wings

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SUMMARY

Neuroanatomical evidence argues for the presence of taste sensilla in *Drosophila* wings; however, the taste physiology of insect wings remains hypothetical, and a comprehensive link to mechanical functions, such as flight, wing flapping, and grooming, is lacking. Our data show that the sensilla of the *Drosophila* anterior wing margin respond to both sweet and bitter molecules through an increase in cytosolic Ca^{2+} levels. Conversely, genetically modified flies presenting a wing-specific reduction in chemosensory cells show severe defects in both wing taste signaling and the exploratory guidance associated with chemodetection. In *Drosophila*, the chemodetection machinery includes mechanical grooming, which facilitates the contact between tastants and wing chemoreceptors, and the vibrations of flapping wings that nebulize volatile molecules as carboxylic acids. Together, these data demonstrate that the *Drosophila* wing chemosensory sensilla are a functional taste organ and that they may have a role in the exploration of ecological niches.

INTRODUCTION

In *Drosophila melanogaster*, the anterior wing margin harbors two types of sensory bristles arranged in three rows: stout mechanosensory bristles and slender chemosensory hairs. The axons of all of the mechanoreceptor and chemoreceptor neurons housed in these bristles form intertwined bundles, constituting a unique nerve routed along the anterior wing margin toward the thoracic ganglion (Hartenstein and Posakony, 1989; Stocker, 1994; Couso et al., 1994). The chemosensory neurons in *Drosophila* wings are anatomically organized in spatially separated gustatory sensilla, mainly along the anterior wing margin. Their functional roles have not been documented (Hartenstein and Posakony, 1989; Stocker, 1994; Couso et al., 1994).

During flight, insect wings create air turbulence that allows sophisticated propulsion and trajectory guidance according to the physical principles of fluid dynamics (Dickinson et al., 1999; Dickinson, 2006). Amazingly, researchers have demonstrated that the local air vortex spirals off along the wing anterior margin, where the chain of mechano- and chemosensory bristles is precisely located (Dickinson et al., 1999; Dickinson, 2006). Moreover, this neuroanatomical feature likely facilitates the subsequent capture and detection of nonvolatile tastants brought by the legs during the grooming sequences and then which are dispersed to accessible receptors by the vortex created by wing flapping. Consequently, even if gustatory receptors (GRs) are dedicated to taste perception, their putative expression in the wing is still intriguing with regard to their flight-associated functions.

The genomes of insects, such as the fly *Drosophila melanogaster*, the honeybee *Apis mellifera*, and the aphid *Acyrtosiphon pisum*, have been sequenced (Hoskins et al., 2015; Honeybee Genome Sequencing Consortium, 2006; International Aphid Genomics Consortium, 2010). Taste receptors with seven transmembrane domains have been reported for these three species using algorithms for computational searching based on structural features and on genomic databases (Clyne et al., 2000; Kim et al., 2000). In *Drosophila*, 68 GRs have been identified, and for most of them, the respective patterns of expression in taste neurons of larvae, adult legs, and proboscis are presently known (Scott et al., 2001; Robertson et al., 2003; Chyb, 2004). In aphids, 77 taste receptors have been found using sequence homology (Smadja et al., 2009). For the honeybee, only ten GRs have been described (Robertson and Wanner, 2006). Here, we report the expression of GRs identified by PCR analysis in the wing of these three species, suggesting the universality of this sensory modality.

In mammalian models, sweet-sensitive neurons transduce signals relying on receptor binding via an increase in the second messengers cyclic AMP (cAMP) and/or Ins(1,4,5)P3 (Kinnamon, 2000; Lindemann, 2001; Margolskee, 2002). An increase in the cytosolic Ca^{2+} concentration occurs through both pathways, with calcium arriving either from the extracellular space through voltage-gated calcium channels controlled by cAMP levels or from intracellular stores opened by the binding of Ins(1,4,5)P3 to its receptors (Lindemann, 2001; Margolskee, 2002; Amrein and Bray, 2003; Zhang et al., 2003; Medler, 2010). However, the transduction cascade of bitter molecules involves G-protein-coupled receptors, some of which activate phospholipase C, thus leading to an Ins(1,4,5)P3 increase and to the subsequent opening of intracellular Ca^{2+} stores. The signaling pathways appear even more complicated because of the recent findings regarding the role of the cation channel TRPM5 in the taste

transduction cascade for all sweet, bitter, and umami tastes (Liman, 2007; Talavera et al., 2008).

In invertebrates, gustatory receptor transduction appears to be far more complex, and the mechanisms involved are still debated (Vosshall and Stocker, 2007; Nakagawa and Vosshall, 2009). Sweet and bitter receptors are likely expressed in distinct gustatory receptor neurons (GRNs) of taste sensilla to prevent interferences between signaling pathways (Montell, 2009; Weiss et al., 2011). *Drosophila* GRs clearly belong to a single family of seven transmembrane domain proteins but do not show sequence homology with the mammalian taste receptors (Clyne et al., 2000; Kim et al., 2000; Robertson et al., 2003). Compared with their mammalian homologs, insect olfactory receptors (ORs) and GRs appear to be a highly evolutionarily divergent class of molecules (Kent and Robertson, 2009). Transduction of taste signaling in insects appears to be related to ion channels (Murakami and Kijima, 2000; Sato et al., 2011; Benton et al., 2009) that include those associated with the *pickpocket* (*ppk*) genes belonging to the degenerin/epithelial sodium channel (DEG/ENaC) family and the transient receptor potential (TRP) channels (Weiss et al., 2011; Pikielny, 2012; Starostina et al., 2012). Tasting and sensing water in *Drosophila* appears to be orchestrated by the Ppk28 channel (Inoshita and Tanimura, 2006; Cameron et al., 2010; Waterson et al., 2014). Finally, recent work showed that a new clade of ionotropic receptors (the IR20a family) is involved in the process of taste and pheromone perception (Koh et al., 2014). In contrast to mammalian GRs, insect GRs do not have the binding domain for heterotrimeric G proteins (Benton et al., 2006; Ishimoto et al., 2005). At the mechanistic level, it is not yet clear whether insect GRs in some cases mediate signal transduction via an association with subunits bearing G-protein-binding domains (metabotropic model) or, in some other cases, associate with subunits to form ligand-gated ion channels (ionotropic model); however, in both cases, the channels are not activated until the early stage (Nakagawa and Vosshall, 2009). Furthermore, GRs are found in the hygroreceptive neurons of the arista, in oenocytes and in the auditory Johnston's organ, suggesting that they perform diverse nongustatory functions (Thorne and Amrein, 2008; Montell, 2009). This finding strongly implies that their associated functions might be unrelated to taste. Re-enforcing this hypothesis, researchers have reported that one GR is involved in CO_2 detection (Fischler et al., 2007).

The anterior wing margin of *Drosophila* is known to harbor chemosensory sensilla that are gustatory organs (Stocker, 1994). However, transcript analysis of GRs in wings is lacking, likely due to the scarcity of RNA in this tissue. Basically, the physiological functions and the behavioral roles of GRs in insect wings remain largely understudied and presently unknown. This situation also suggests that the wing location of taste cells expressing the same or different GRs as elsewhere (proboscis, legs, and abdomen) might be associated with specialized functions and/or behaviors (Dunipace et al., 2001).

To date, taste perception in insect wings has been understudied because of technical difficulties in performing electrophysiological experiments in this tissue. In addition, the robust and dense chitin matrix of the insect wings forms an effective barrier that considerably limits fluorescence experiments by causing poor resolution or weak signals. In this report, we used a transgenic fly expressing a hybrid GFP/calmodulin (CaM)/M13 (GCaMP) molecule in which the fluorescence drastically increases on calcium binding (Nakai et al., 2001; Akerboom et al., 2009). This calcium sensor was driven by a strong promoter to allow the light to pass through the chitin barrier, showing that *Drosophila* wings can detect sweet and bitter molecules in a manner similar to the proboscis. Moreover, flies exposed to air-pulverized microdroplets of water/sugar showed a strong aggregation response, which was impaired in flies with wing-specific taste sensilla knockdown. In parallel, a Bayesian behavioral experiment performed with wild-type flies (*CS*) demonstrated that wing grooming at a food spot in the conditioning step sequentially influenced/guided the choice for a new ecological niche. In contrast, the calcium imaging and the behavioral skills were abolished or significantly affected in genetically engineered flies in which gustatory sensilla were specifically altered in the wing.

RESULTS

A Strong Increase in Cytosolic Ca^{2+} Is Elicited by Sweet and Bitter Tastants in *Drosophila* Wing Sensilla

To investigate the functions of wing chemosensory organs, we used a *D. melanogaster* transgenic strain carrying a calcium-sensitive GFP sensor (GCaMP) that allowed us to follow the signal transduction of GRs in the wing (see the Experimental Procedures). Using this tool, we evaluated the ability of food-related chemosensory stimuli to provoke a calcium peak in dissected wings. Confocal microscopy observations coupled to kinetic measures of fluorescence were performed in *UAS-GCaMP*/+; *Tub-GAL4*/+ flies (Figures 1, 2, and 3). Briefly, we used *Tub-GAL4* instead of *GR-GAL4s* in the experiments for the following reasons: (1) the use of the same promoter for the calcium sensor and for the investigated GR is a potential source of misleading results in the context of allelic exclusion, (2) the GR promoters are unlikely to overcome the strong promoters driving calmodulin or other calcium-binding proteins, which are endogenous molecules, (3) we found that the *GR-GAL4s* that are efficient for localization studies are not appropriate for calcium imaging in the wing tissue, and (4) movies clearly show that only chemosensory sensilla respond to tastants using *Tub-GAL4*. The Experimental Procedures also describe our arguments for using the *Tub-GAL4* driver. As expected, when these dissected wings were examined in dry conditions, GCaMP emitted weak fluorescence in the absence of calcium release. These wings also showed a faint basal level of fluorescence when they were immersed in water as control (Figure S1A). We tested several sweet-tasting molecules, including the monosaccharides glucose, fructose, and trehalose (Figures 1, 3, and S2). We also assayed the effect of some glycosylic conjugates, such as hesperidin (flavonone glycoside), arbutin (a phenol linked to glucose), and steviol glycoside (a sweetener), some of which are the hydrophilic precursors of fruit and flower aroma (Loughrin et al., 1992). None of the glycosylic compounds or any tested odorant (benzaldehyde, ethyl acetate, and ethanol) could trigger detectable calcium responses.

Conversely, all tested monosaccharides triggered some rapid flashes of light, which diffused at the base of the slender hairs and

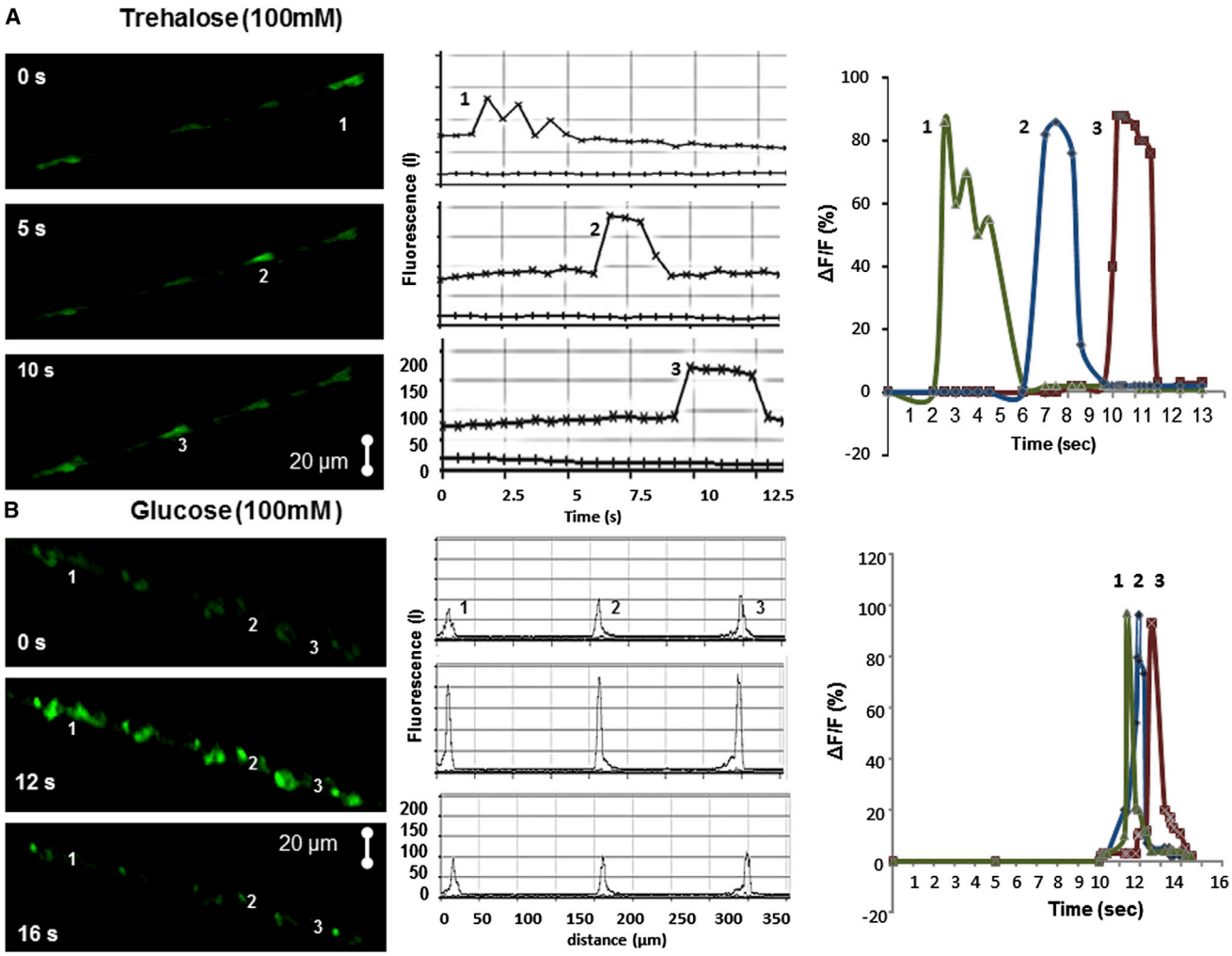

Figure 1. Increase in the Calcium Levels Elicited by Sweet Tastants in the Anterior Wing Margin Nerve

(A and B) The GCaMP calcium sensor was used to monitor the calcium peaks using the GFP fluorescence in dissected wings of *UAS-GCaMP/+*; *Tub-GAL4/+* flies stimulated with 100 mM trehalose (top) or 100 mM glucose (bottom). The three photographs shown for each series represent sequential steps during the observation period (between 0 and 20 s) after stimulation. Statistical analysis of the fluorescence peaks is presented in Figure 3. Movies S1 and S2 allow direct monitoring of the kinetics.

(A) Fluorescence changes in response to 100 mM trehalose (left). The fluorescence of three sensilla was independently measured for a 12.5-s period (middle). ΔF/F° is presented for these three sensilla over the same period (right). We observed successive flashes from one gustatory sensilla to the next.

(B) Fluorescence changes in response to 100 mM glucose over a 20-s period (left). The numbers indicate the corresponding sensilla on the photograph for which the fluorescence intensity was measured at the same specified times for the three consecutive sequences. The distance, which was measured in a linear manner (middle), refers to the space between sensilla. ΔF/F° is presented for these three sensilla over a 20-s period (right). A dose-response curve (concentration versus fluorescence intensity) and controls are presented in additional Figures S1A and S1B. See also Movies S1 and S2 and Figure S2 for additional analysis of the glucose and fructose activation.

See also Figures S1 and S2 and Movies S1 and S2.

then in the anterior wing margin nerve (Figures 1 and 3; Movies S1 and S2). Our experiments revealed three general patterns of light flashes. First, some cells lighted up/turned off with apparent temporal and spatial synchrony. Second, the fluorescence emission oscillated in some sensilla. Third, a regular and strong fluorescence signal that was first found in the chemosensory sensilla diffused extremely rapidly along the anterior wing margin nerve before fading homogenously. The latter pattern was the most common among the numerous wings tested. We often observed that sweet (and also bitter, see below) stimuli provoked a bimodal calcium response: a very brief fluorescent flash followed by an intense and sustained fluorescent peak. Fixed photographic representation and quantification turned out to be difficult to achieve with our technical set up; thus, the observations can be better estimated using Movies S1 and S2.

The effects of the two bitter compounds quinine and denatonium were also measured (Figures 2 and 3). Both stimuli induced a series of strong fluorescent flashes along the anterior wing margin, highlighting the calcium diffusion in axons (see Movies S3 and S4). Two successive calcium peaks—a very brief one,

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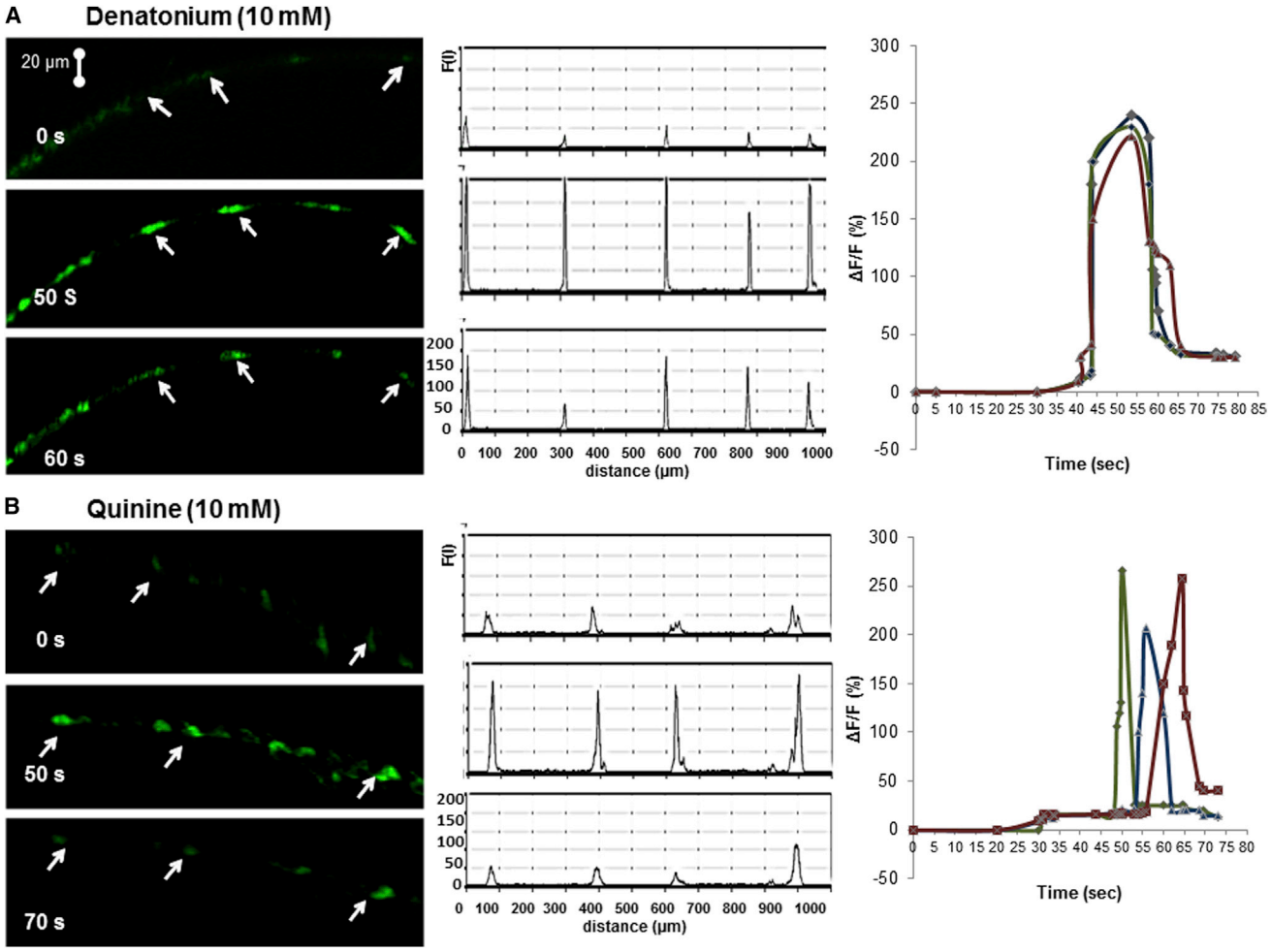

Figure 2. Increase in the Calcium Levels Induced by Bitter Tastants in the Anterior Wing Margin Nerve

(A and B) A protocol similar to that in Figure 2 was used to measure the response of wing chemosensory sensilla to 10 mM denatonium (top) or quinine (bottom). For each bitter substance, three consecutive photographs were taken during the time course of the experiment (0 to 70 s). The statistical analysis of the temporal onset of fluorescence is presented in Figure 3. Movies S3 and S4 allow for direct monitoring of the kinetics. The white arrows indicate the corresponding sensilla that are the same between the three consecutive photographic sequences (left). The fluorescence intensity of five sensilla displayed in the photographic sequences is shown (middle). These fluorescence peaks highlight that the sensilla are activated by bitter molecules. $\Delta F/F^{\circ}$ is presented (right) for the three sensilla marked by the white arrows in the photographs. A dose-response curve (concentration versus fluorescence intensity) and controls are shown in Figure S1 for denatonium. See also Figure S1 and Movies S3 and S4.

followed by a second more intense and sustained peak—were consistently observed. These strong calcium signals propagated along the wing margin nerve (Movies S3 and S4). The bitter stimuli tested here elicited stronger signals compared with those obtained with sugars, suggesting a predominant component for bitter detection in the wings. Due to technical limitations that were difficult to overcome, the delayed fading of the fluorescent peaks and the sustained fluorescence emission might be magnified due to a strong in vivo affinity between calcium and the overexpressed GCaMP. This possibility could explain the apparent slow return of calcium to its basic level. The strong and persistent concentration of sweet/bitter stimuli in situ may also enhance the intensity and the width of the fluorescent peaks. Note that the latency between the addition of tastants and the fluorescent peaks varied, likely due to the physical properties of the tastant solutions (density, viscosity, and temperature) and, more importantly, the variable capability of molecules to penetrate the sensilla and bind to receptors. A single pore at the top of the hair is the unique passage for accessing the chemoreceptors, and an air layer separates the pore and the lymph that must be disrupted to reach the receptors (Valmalette et al., 2015). Therefore, the latency observed here may not be representative of the natural situation: in the lab, the dissected wing was immersed in diverse solutions and was kept flat and fixed, whereas in nature, the flapping wing creates vibrations and an air vortex, which could be an essential process for molecules to access wing receptors and for wing tasting. In any case, the general functionality of dissected wings and particularly their ability to respond to tastants are preserved in our experimental protocol. Despite these technical limitations, a minimal dose response of tastant

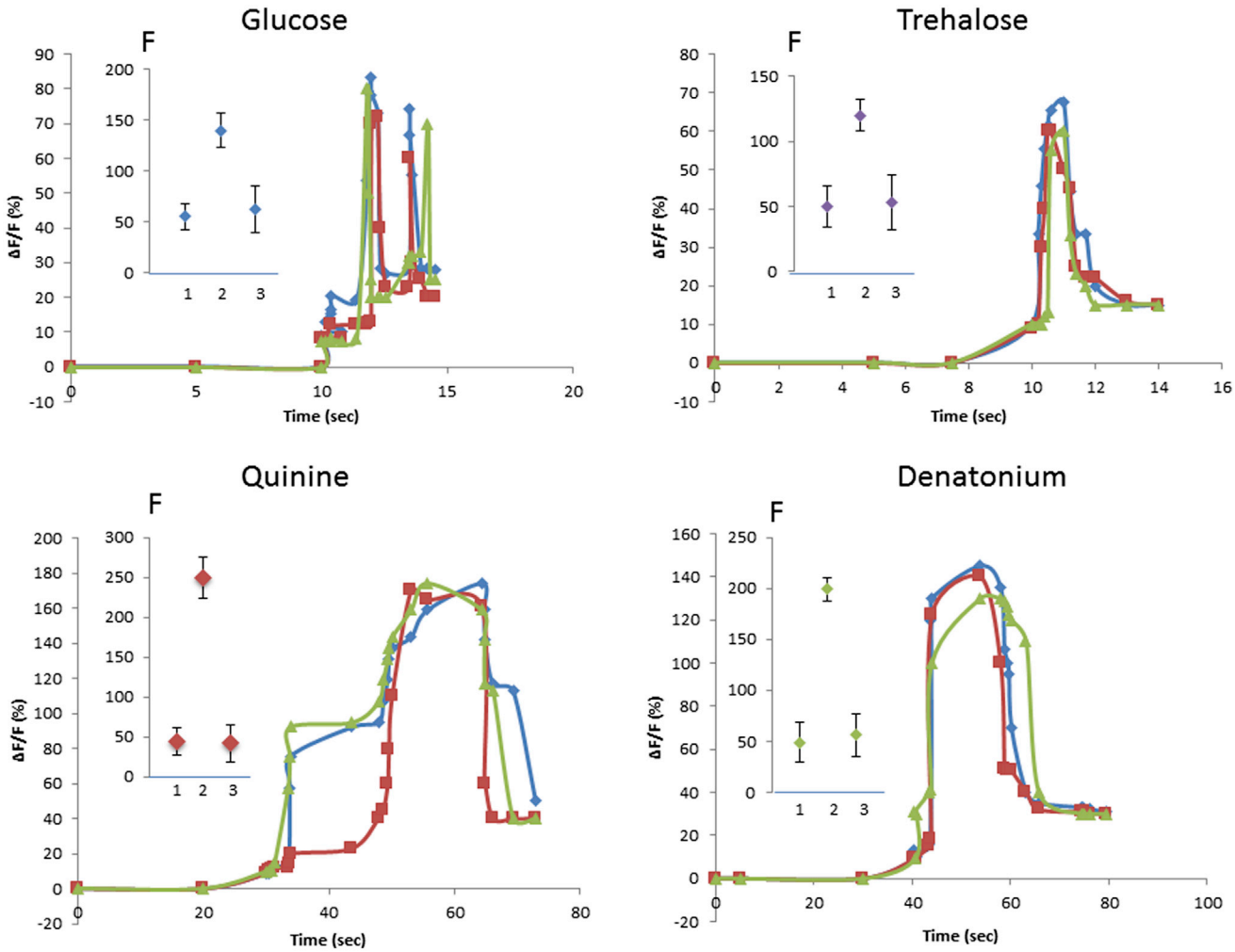

Figure 3. Time-Lapse Monitoring of the Fluorescence Produced by Bitter and Sweet Tastants in Individual Wing Sensilla

The dissected wings of *UAS-GCaMP/+*; *Tub-GAL4/+* were stimulated by tastants, and the kinetics of calcium release was monitored via the fluorescence emitted by the calcium/GCaMP complex. The variation in fluorescence was measured according to the equation $F(t) - F_0/F_0$, and each graph represents the kinetics over the time period indicated for each tastant. Three sensilla, one each from three different flies but localized at the same place, were analyzed and are represented by different colors (blue, green, and red). As explained in the Results regarding the variable latency between the addition of tastants and the fluorescence flashes, the statistical time-lapse analysis is not presented. Instead, the statistical analysis of the fluorescence of 50 sensilla is shown. In the insets, 1, 2, and 3 represent the relative fluorescence at t = 0, the peak fluorescence intensity at stimulation and the 20 s after stimulation, respectively. Ten wings from different flies and five sensilla for each at the same location were analyzed. Signs are the mean ± SE, n = 50, obtained with 100 mM glucose, trehalose along with 10 mM quinine or 1 mM denatonium. For more details, see Movies S1, S2, S3, and S4. Statistical analyses were performed with the paired Student's test. No significant difference was found between 1 and 3. A comparison between the groups 1and 2 and the groups 2 and 3 gave a p value < 0.001.
See also Figures S1 and S2 and Movies S1, S2, S3, and S4.

concentration/calcium signal (concentration of tastants versus fluorescence intensity) was found. The dose response with three concentrations of glucose and denatonium is presented in an additional figure (Figure S1B). The statistical analysis of the fluorescence intensity induced by tastants on 50 sensilla at the same location is reported in the insets of Figure 3.

Transcripts of Taste Receptor Genes Are Detected in the *Drosophila* Wing

Specific sequences of identified chemosensory receptors in insects for which genome databases are available were used to design primers for RT-PCR analysis. Our first goal was to detect the presence of RNAs coding GR proteins in the wings of three phylogenetically divergent insects, *D. melanogaster*, *A. mellifera*, and *A. pisum*, which were used as key models for the Diptera, Hymenoptera, and Hemiptera orders, respectively. The gene organization (introns/exons) of the tested GRs is presented in the Supplemental Results, along with the sequences and locations of primers. Due to the scarcity of RNA and the limited quantity of tissue (except for honeybee wings), a few hundred wings were dissected, collected, and frozen before the extraction procedures. Figures 4A and S4C show that receptors

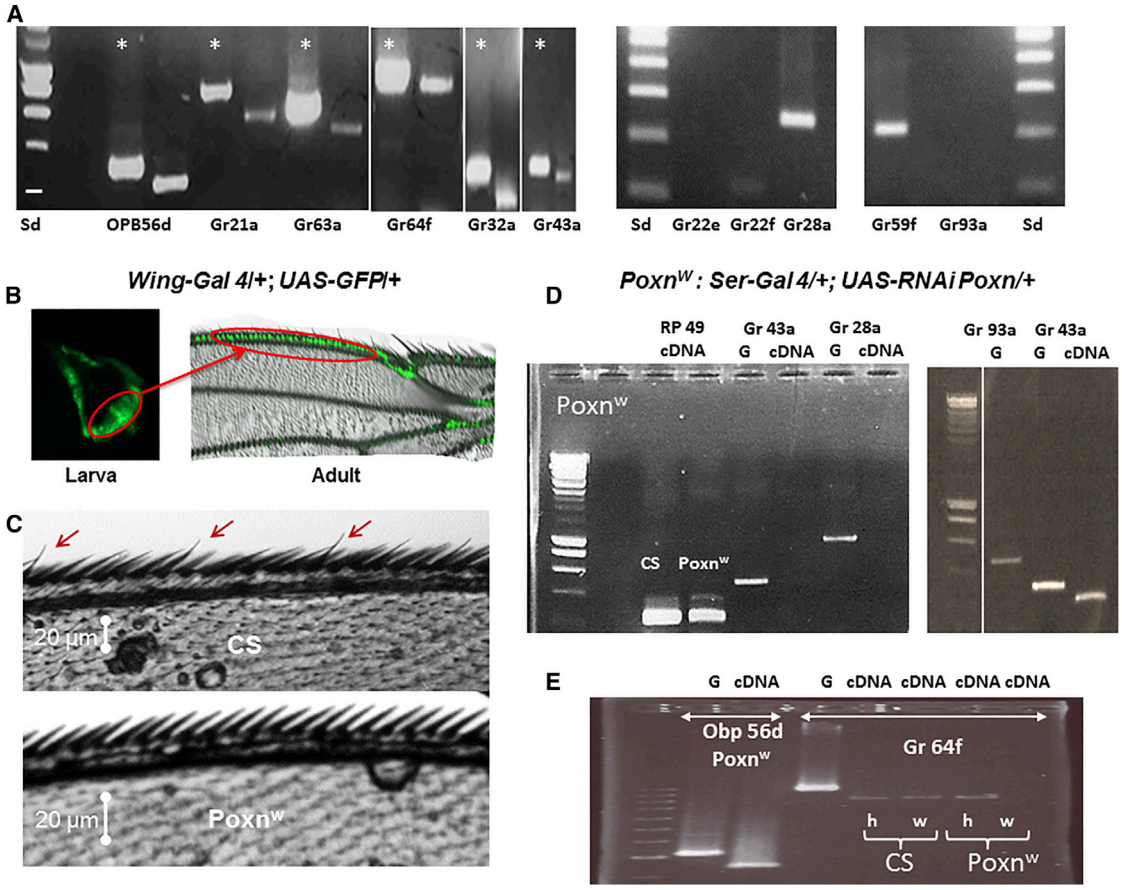

Figure 4. Genetic Targeting of Chemosensory Tissues in the Adult *Drosophila* Anterior Wing Margin

(A) The presence of RNAs coding GR proteins was assessed by RT-PCR in *Drosophila* wings. Total RNA was extracted from wings, and cDNA was synthesized prior to the PCR analysis. When two bands are shown for the same gene, they correspond to the genomic amplification (*, left) and to the cDNA (right). When only one band per gene is shown, it corresponds to the cDNA without ambiguity regarding a possible genomic contaminant. See also Figure S3 for the honeybee and aphid analysis.

(B) The *Ser-GAL4* transgene drives *GFP* expression in the wing imaginal disc of the third-instar larva and in the nerve of the anterior wing margin of the newly emerged adult.

(C) Anterior wing-margin bristles in wild-type (*CS*) control flies and in *Ser-GAL4* > *UAS-RNAi Poxn* ($Poxn^W$) manipulated flies. In the WT (*CS*) wing, stout mechanoreceptor bristles alternate with slender hairs housing chemoreceptors (red arrows). In $Poxn^W$, all slender hairs of the anterior wing margin are transformed to stout bristles.

(D and E) RT-PCR analysis of transcripts to assess the knockdown of the wing chemosensory receptors.

(D) cDNA bands in $Poxn^W$ adult wings indicate that *GR43a* and *GR28a* are not expressed in $Poxn^W$ wings (left). A control *CS* for Gr43a is shown (right, a complete gel is provided in Figure S4E). G, the genomic bands for the corresponding GRs.

(E) *GR64f* and *Obp56d* cDNA bands from the head (h) and wing (w) of WT (*CS*) and $Poxn^W$ specimens. The control ubiquitous gene (*Obp56d*) is shown only for the wing of $Poxn^W$ to assess the cDNA quality. The gels were run in the same conditions and cropped for representation. See also Figure S4 for additional analysis of the silencing of the *Poxn* gene in embryo and adult. The 100-bp DNA ladder and the 1-kb DNA ladder were used depending on the DNA size.

See also Figures S3 and S4

for sugars (trehalose, fructose, and glucose: *GR64a* and *GR43a*), CO_2 (*GR21a* and *GR63a*), and bitter molecules (*GR32a*, *GR28a*, and *GR59f*) are expressed in adult *D. melanogaster* wings. Some GRs (mostly sweet and bitter receptors) were also found to be expressed in the adult wings of the aphid *A. pisum* (Figure S3). Similarly, the expression of several GRs (*GR2(ii)*, *GR3*, *GR10(i)*, and *GR10(ii)*) was also detected in the honeybee wings (see Figure S3). This finding suggests that the wings of these three insects can potentially detect tastant cues from the environment.

Genetic Manipulation of Wing Chemosensory Organs

RNAi silencing of *Pox neuro* (*Poxn*), a gene involved in determining the type of sensory sensilla during late larval development, was performed in the anterior wing margin without affecting the rest of the body (Nottebohm et al., 1992; Boll and Noll, 2002). GFP under the control of a wing-specific GAL4 driver transgene (Bloomington #6791, hereafter named *Ser-GAL4*) appeared to be specifically expressed in the wing imaginal disc of the third-instar larvae and in the anterior wing margin of pupae and emerging adults (Figure 4B). This *GAL4* driver transgene

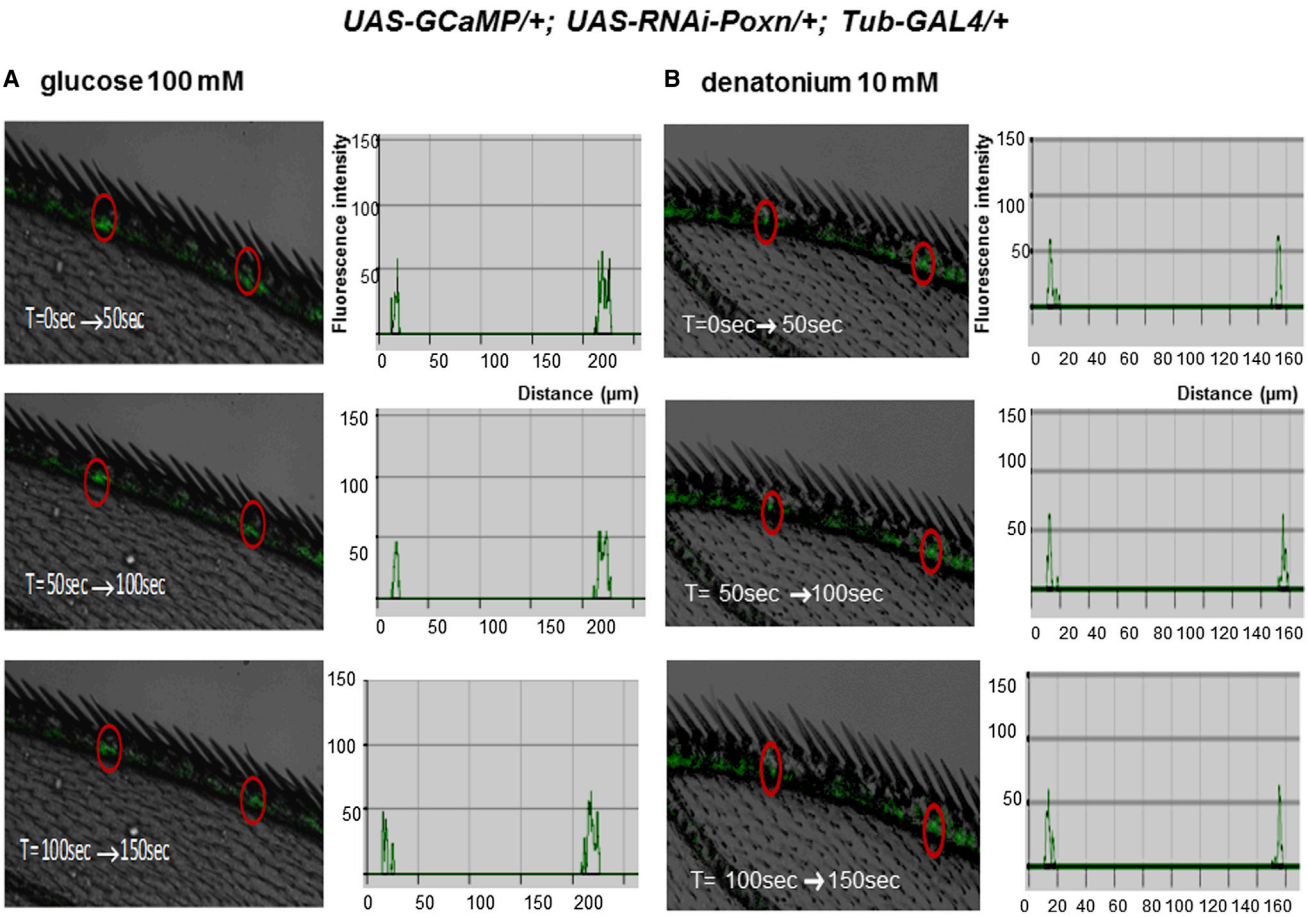

Figure 5. Effect of Bitter and Sweet Molecules on the Calcium Levels in the Wing Margin Nerve of *UAS-GCaMP/+; UAS-RNAi-Poxn/+; Tub-GAL4/+* Flies

(A and B) The calcium signal was measured in $Poxn^{W}$ transgenic flies with altered wing chemosensory organs following stimulation with 100 mM glucose (A) and 10 mM denatonium (B). The photos represent three sequential steps (between 0 to 150 s) during the confocal microscopy recording. The intensity of light quantified in the individual spots in the photographs (red circles) is presented in the corresponding graphs. No increase in the fluorescence signal was observed relative to the background in $Poxn^{W}$ wings during the 150-s recording. See also Table S1.

contains a minimal promoter of *Serrate* (*Ser*), which explains why this *GAL4* expression appears to be restrained compared with that of the *Ser* gene. This minimal promoter appears to be inactive at the embryonic stage (see the Experimental Procedures; Figure S4). With this GAL4 driver, the targeted silencing of *Poxn* in *Ser-GAL4/+; UAS-RNAi-Poxn/+* flies (hereafter indicated as $Poxn^{W}$) caused the expected morphological consequences: the transformation of the chemosensory cells into mechanosensory cells, as shown by the replacement of slender chemosensory hairs by stout mechanoreceptor bristles (Nottebohm et al., 1992; Boll and Noll, 2002) (Figure 4C). We also verified that the $Poxn^{W}$ manipulation altered the expression of taste-related genes, such as the gustatory receptors *GR43a and GR28a* (Figure 4D). Moreover, we showed that *GR64f* in $Poxn^{w}$ disappears in the targeted wing cells, but not in the proboscis (Figure 4E). The expression of a major odorant-binding protein gene (*Obp56d*) used as a control due to its ubiquitous expression (Arya et al., 2010) was not affected in $Poxn^{W}$ wings (Figure 4E). Together, these findings indicate that $Poxn^{W}$ is suitable for a specific manipulation of the wing margin to study its behavioral effects.

The Calcium Signaling Stimulated by Sweet and Bitter Molecules in Wing Chemosensory Sensilla Is Abolished in the Transgenic *UAS-GCaMP/+; UAS-RNAi-Poxn/+; Tub-GAL4/+* Fly

To investigate the taste functionality of the anterior wing margin sensilla, we examined the calcium levels in the wings of *UAS-GCaMP/+*; *UAS-RNAi-Poxn/+*; *Tub-GAL4/+* flies (see Table S1 for the genetic strategy for obtaining this transgenic strain), which lack chemosensory sensilla. To measure the residual calcium peaks in the manipulated wing taste cells, we used the same ubiquitous *Tub-GAL4* driver as in the previous analysis (Figures 1 and 2). The calcium peaks normally triggered by glucose or denatonium stimuli were not detected in these wings; thus, the basal level of fluorescence remained unaffected (Figure 5). This finding supports the role of gustatory receptors in the tastant-induced calcium peaks and allows us to rule out

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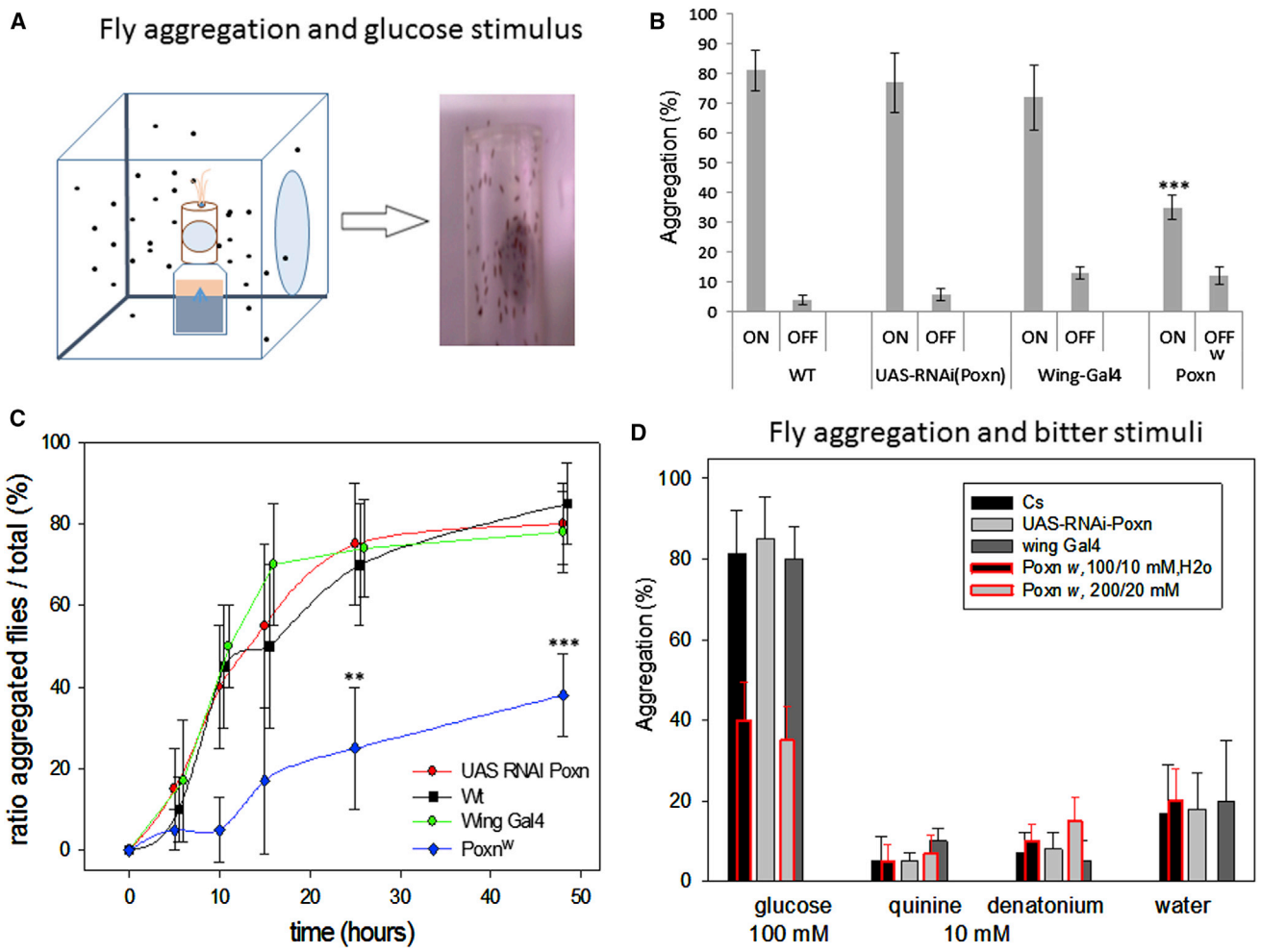

Figure 6. Role of Wing Chemosensory Receptors in Aggregation Behavior

(A–D) Aggregation rates were compared between *CS*, *UAS-RNAi Poxn*, *Ser-GAL4*, and *Ser-GAL4>UAS-RNAi-Poxn* ($Poxn^{w}$). Wing-GAL4 stands for *Ser-GAL4*.

(A) For the nebulization/aggregation experiment, 100 5-day-old female and male flies were released in a chamber containing a device consisting of a pulverizator (blue rectangle) attached to a small cylinder with a hole at the top and a mesh on the side. Solutions of glucose (100 mM) or quinine/denatonium (10 mM) were nebulized. To avoid dehydration, a water-soaked paper towel was placed on the chamber floor.

(B) Flies were placed in the chamber, and the accumulation in the cylinder was measured 2 days after the start of the experiment. Gray bars represent the mean (±SEM, n = 10) percentage of flies that entered the small cylinder. Experiments were conducted with (on) or without (off) the pulverized sugar solution. A significant difference as determined using a Tukey non-parametric test (***$p < 0.001$) indicates that $Poxn^{W}$ flies diverged from the three control genotypes.

(C) A kinetic study was performed between 0 and 48 hr for the four genotypes (data represent the mean ± SE, n = 10, Tukey non-parametric test: ***$p < 0.001$, **$p < 0.01$ for $Poxn^{W}$ *versus CS* flies).

(D) The four strains were tested for their accumulation in the cylinder, as stimulated by the nebulization of three substances (100 mM glucose, 10 mM quinine/10 mM denatonium, and water control). $Poxn^{W}$ flies were also tested with 200 mM glucose and 20 mM quinine/denatonium. Bars represent the percentage of flies that entered the small cylinder (±SEM, n = 10). The Tukey non-parametric test was performed: ***$p < 0.001$ for glucose versus quinine, denatonium, or water. For lethality controls, see the Experimental Procedures.

See also Figure S5.

the possibility of an artifact and/or a side effect generated during the course of the experimental procedure.

Aggregation Behavior Elicited by Water/Sugar Pulverization

Next, the functional role of wing chemoreceptors was investigated in a chemo-induced behavior. To validate the chemosensory function of the *Drosophila* wing in an ecological context, we nebulized microdroplets of either water (control substance) or water mixed with glucose, quinine, or denatonium, and we compared the aggregation pattern caused by the different stimuli (Figure 6). Aggregation is a subsocial behavior in which flies gather at food spots for mating and egg laying. Therefore, the pulverization method allowed us to test the behavioral response triggered by the taste detection of nonvolatile nebulized hydrosoluble compounds. The intensity of the effect produced by each stimulus was determined as the number of flies aggregating on the source during 2 days. A control without

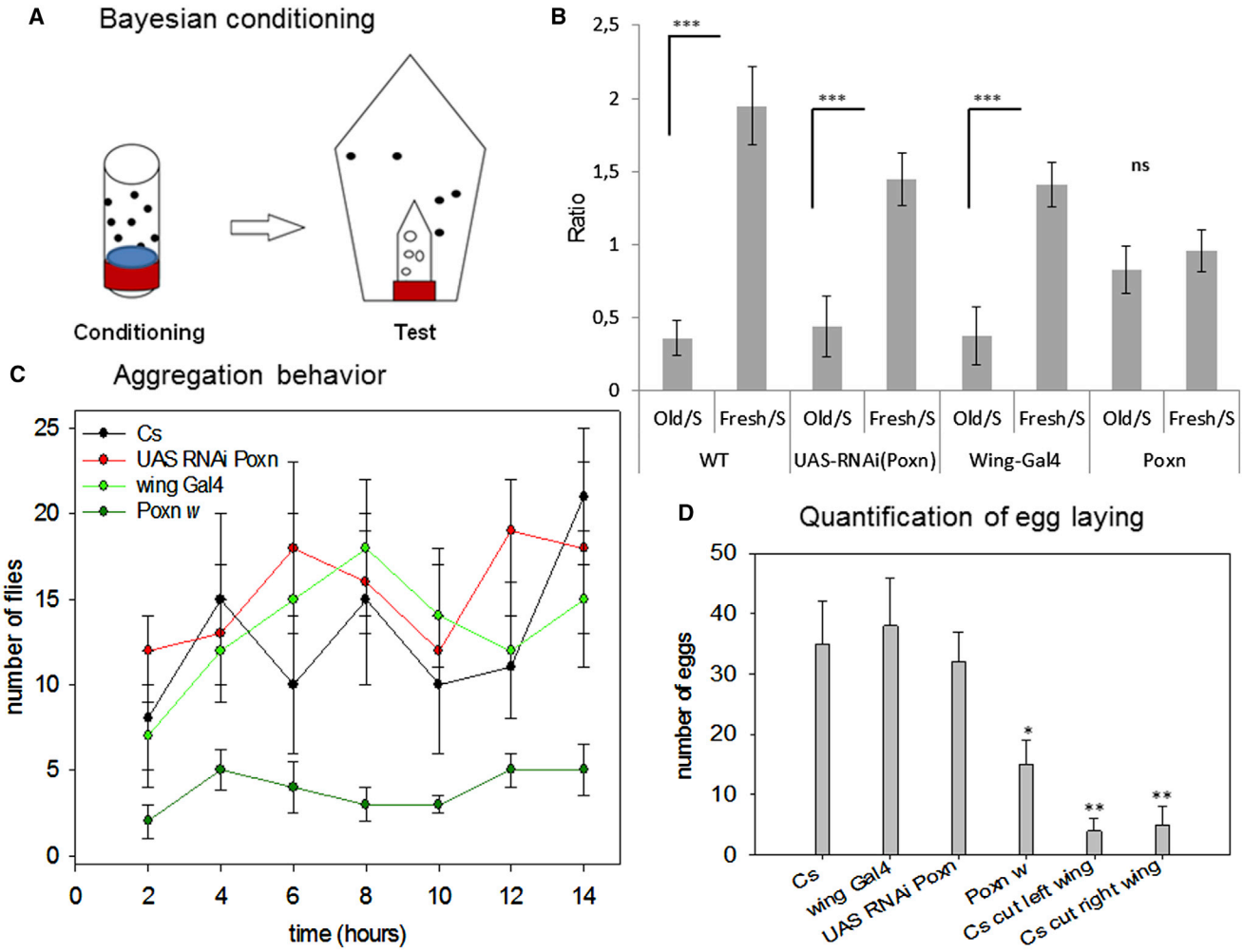

Figure 7. Role of Wing Chemosensory Receptors in Bayesian Food Conditioning, and Effect of a Cut of the Anterior Wing Nerve

(A) Scheme for the Bayesian conditioning. Flies were mass conditioned with fresh grape juice, spoiled juice, or water plus sugar for 3 hr and subsequently released in a cage in which their aggregation in a food vial containing fresh grape juice was measured after 24 hr. 15 replicate experiments were carried out for the following genotypes: *CS*, *UAS-RNAi Poxn*/+, and *Ser-GAL4*/+ control flies and *Ser-GAL4*/+ > *UAS-Poxn*/+ ($Poxn^{W}$) silenced flies. Wing-GAL4, *Ser-GAL4*.

(B) Mean ratio ± SEM of the number of aggregated flies conditioned by either spoiled or fresh grape juice, divided by the number of flies aggregating in the water/glucose conditioning experiment used as a control. The difference in aggregation rates observed in the wild-type and the transgenic controls was abolished in $Poxn^{W}$ flies. Bars are the mean ± SE, n = 15. Statistical analysis was performed with a Tukey non-parametric test: ***$p < 0.001$.

(C and D) Effect of the knockdown of wing taste sensilla on aggregation and egg-laying behavior. 100 7-day-old *CS* (50 female, 50 male), transgenic controls and $Poxn^{W}$ flies were released in a chamber, and the number of flies on the two food spots was counted (C). Eggs laid on food spots were counted at the end of the experiment (14 hr) (D). Ten independent experiments were conducted, and the numbers (C) or bars (D) represent the mean ± SE (n = 10). Flies presenting a unilateral cut of the anterior wing margin nerve were also tested only for egg laying (n = 3). Statistical analysis was performed with a Student's t test: *$p < 0.01$ and **$p < 0.001$.

See also Figure S5.

pulverization showed equal lethality after 2 days between the tested strains (approximately 12%). In such conditions, the solution of glucose (Figures 6B and 6C), but not the solution of bitter molecules (Figure 6D), triggered aggregation behavior in wild-type flies. The number of flies accumulating around the glucose source was high for all control genotypes, except for $Poxn^{W}$ flies, where it was significantly decreased. Doubling the amount of glucose and quinine/denatonium did not modify the pattern of aggregation for the mutant $Poxn^{w}$ (Figure 6D). As shown in Figure S5, motility and velocity were not affected in $Poxn^{W}$ flies, as indicated by trajectory analysis using olfactory grape juice stimuli. This finding suggests that taste-guided aggregation behavior, at least partly, depends on wing chemosensory detection.

To validate these observations, we used another protocol to score the aggregation of flies in a new environment after they had previously been subjected to a chemosensory experience (Collett, 2012; Naug and Arathi, 2007; Biernaskie et al., 2009) (Figure 7A; see the Experimental Procedures). Each group of flies was first kept in contact with either two types of grape juice (fresh

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or spoiled) or with a control solution (water plus sugar) for 3 hr (the conditioning phase), during which the flies groomed their wings with their legs. Each group was then transferred to a test chamber to measure the number of flies that aggregated on a fresh grape juice source after 24 hr. The number of accumulated flies was standardized relative to the number of sugar-conditioned flies. A strong behavioral difference was found in wild-type flies (*CS*) and in genetic background flies (*UAS-RNAi-Poxn* and *Ser-GAL4*) between the two modes of conditioning: either fresh or spoiled grape juice (Figure 7B). This difference, detected in both transgenic controls, was totally abolished in $Poxn^{W}$ flies, suggesting that the Bayesian exploratory task involves wing-mediated chemoperception. Finally, the importance of the wing margin nerve for the exploration of an ecological niche and egg laying was assessed using *CS*, *UAS-RNAi-Poxn*, *Ser-GAL4*, and *Ser-GAL4>UAS-RNAi-Poxn* ($Poxn^{W}$). A unilateral surgical cut of the wing nerve was also performed as a negative control (see Figures 7C and 7D). The results showed that taste sensilla knockdown in the wing abolishes aggregation and significantly reduces the number of spotted eggs laid.

DISCUSSION

We combined biochemical, genetic, imaging, and behavioral approaches to study the effects of sweet and bitter molecules on *Drosophila* wing chemosensory organs. Several studies have reported the potential presence of GRs in wings, raising the possibility of taste ability in these appendages, similar to that in the proboscis and legs (Stocker, 1994; Montell, 2009). However, these studies did not reveal the functionality and the role of wing taste sensory cells. Indeed, the intriguing co-existence of GRs with mechanoreceptors in two organs associated with vibrations (Johnston's organ for hearing and the anterior wing margin nerve for flight) led to the hypothesis that GRs might not be always related to taste function (Montell, 2009). We think that wing vibrations occurring at a relatively high frequency (50–2,000 Hz depending on the insect species) might be involved in the process of chemical detection by this organ. Furthermore, the air vortex produced by flapping wings could facilitate the nebulization of environmental compounds and their subsequent accessibility to the wing GRs. Unlike the situation for the proboscis and legs, no convincing electrophysiological studies on insect wing neurons are available. Technical limitations explaining this failure include the rigidity of the chitin layer that wraps the wing hairs and protects internal sensory neurons against the mechanical stress generated by the high-frequency wing beat. The nano-architecture of the hairs and the presence of an air layer below the pore preventing electrode contact might be another reason for this failure (Valmalette et al., 2015). The taste ability of wings may be enhanced when wings vibrate, thus explaining the delayed response of dissected wings to tastants. Here, we show that wing chemosensory sensilla respond to diverse monosaccharides and bitter molecules through Ca^{2+} signaling. Importantly, insect GRs lack the canonical structural elements observed in G-protein-coupled receptors, particularly the G-protein-binding domain, leading to the hypothesis that GRs act as ion channels (Nakagawa and Vosshall, 2009). Our data obtained with *Drosophila* wings fit the known role of calcium in the activation of GRs in the proboscis (Montell, 2009). However, the biochemistry of transduction with all the associated subunits underlying insect responses to sugar and bitter molecules remain elusive, particularly in wings.

For this report, we used the *Ser-GAL4* transgenic driver to globally target the wing margin chemosensory organs without affecting the GR receptors in other locations. The behavioral data obtained using this approach suggest that these wing sensory organs are required for the exploration and subsequent aggregation on food sources. The orientation/exploration response could rely on a sophisticated coincident system, such as the wing margin nerve integrating the inputs of both mechano- and chemosensory neurons. Although the two types of neurons are spatially separated along the wing margin, they may burst in synchrony following mutual stimulation by air friction and chemical capture. The absence of the vibratory component in the dissected wing may partly affect the calcium diffusion in the wing axons after chemo-stimulation, leading to an underestimation of its effects compared with the diffusion in a flapping wing.

We hypothesize that the air vortex created by wing vibrations during flight and/or grooming facilitates the access of hydrosoluble molecules inside the gustatory wing hairs. Wing flapping at a high frequency (200 Hz in *Drosophila*) would nebulize and/or spray microdroplets and dust particles that could be engulfed in the "leading edge vortex" that spirals off along the anterior wing margin, where chemoreceptors are located (Dickinson et al., 1999; Dickinson, 2006). In this case, pollinator insects would sample food sources using their wings, allowing them to detect sugar and bitter molecules without the need to land. This would facilitate exploration and also prevent the digestive intoxication caused by noxious molecules, which would occur when the food is tasted by proboscis contact. The wing taste system, as an initial chemical sensor orchestrating the cycles of exploration/aggregation in *Drosophila*, appears to be distinct from the proboscis taste system and appears to act as a contact sensor for evaluating food before ingestion.

EXPERIMENTAL PROCEDURES

Drosophila Strains and Genetic Constructs

The maintenance of the *Drosophila* stock is in accordance to the institutional guidelines (french government agency for safety of transgenic animals related to *Drosophila* model). The *Ser-GAL4* line corresponds to w(*); P{w(+mC) = Ser-GAL4.GF}1 P{Ser-GAL4.GF}2 (Bloomington Stock Center #6791). The RNAi line to inactivate *Poxn* (V10844) was obtained from the Vienna *Drosophila* RNAi Center. Tub-GAL4 is Bloomington stock number 5138. The *UAS-GCaMP* flies were a kind gift from Leslie Vosshall. The newer GCaMP calcium sensor (Bloomington stock no. 42037) was also used and gave the same results as the previous one. *Canton S* (*CS*) is the wild-type control strain used for all experiments. A wild-type strain freshly collected in the south of France was used for the surgery experiment. The genetic strategy for building a fly strain in which GRs were specifically silenced in the wings where the calcium sensor GCaMP was expressed is summarized in Table S1. *Ser* expression shows two peaks during development (early embryos and then third-instar larvae/pupae/emerged adult). We therefore determined whether *Ser-GAL4* was active in $Poxn^{W}$ (*Ser-GAL4>UAS-RNAi-Poxn*) embryos. We found that *Poxn* expression is only slightly affected in $Poxn^{W}$ embryos (Figure S4A), but is

abolished in the wings of newly emerged $Poxn^{W}$ adults (Figure S4E). Thus, this construct allowed us to downregulate the *Poxn* transcript in the wing imaginal disc during development (between the third-instar larva and the newly emerged adult) without significantly affecting *Poxn* embryonic expression. This system allowed us to specifically manipulate the wing neurosensory cells and avoid the undesired developmental effects associated with the lack of *Poxn* embryonic expression. As a consequence, GR expression was abolished in $Poxn^{w}$ wings (Figures 4D and S4D), and not in the proboscis (Figures 4E and S4B).

PCR Analysis of Wing Extracts

300 pairs of wings from *Drosophila* and aphids and 50 pairs of wings from honeybees were dissected and immersed in liquid nitrogen. The material was ground in a mortar with a pestle until evaporation. The powder was then extracted using an RNeasy Micro Kit (QIAGEN) to determine the mRNA content. This material was used as the template for cDNA synthesis with a Superscript II RNase H Reverse Transcriptase Kit (Invitrogen). PCR reactions were performed using a Thermal Cycler Eppendorf. Amplification was carried out with 10 ng of DNA in a final volume of 50 μl with 1 U of UPitherm DNA polymerase (Uptima), 5 μl of 10× buffer reaction, and 20 μM dinucleotide triphosphate master mix with the following steps: denaturation of DNA at 94°C for 2 min, 35 cycles with a denaturation step of 30 s, hybridization for 45 s at the T_m appropriate for each pair of oligonucleotides, elongation for 1 min and 30 s at 72°C, and a final elongation for 8 min and 30 s. After migration in 1% agarose in 0.5× Tris-borate-EDTA buffer, bands were made visible under UV. The primers used in this study are described in the Supplemental Information (Data S1). For the *OBP56d* control, the 5′-ATTGTCCTCTCCG TCATTTTGGCCATTTCGGCTGCTGA-3′ (F) and 5′-GAGGTCCAGCCCGATG -3′ (R) oligonucleotides were used. For gel analysis of DNA fragments, a 100-bp DNA ladder (no. 15628-050, ThermoFisher Scientific) or 1-kb DNA HyperLadder (no. BIO-33026, Bioline) was used as a standard.

Confocal Microscopy Analysis and Calcium Kinetics

The GCaMP protein is the fusion of a modified GFP, calmodulin (Ca), and a myosin light-chain kinase domain. The GCaMP system was introduced through a transgene (*UAS-GCaMP*) for which the expression was driven by the ubiquitous *Tub-GAL4* driver line. Many *GAL4* lines, particularly all of the tested *GR-GAL4s*, induce a weak or null fluorescence signal through *UAS-GFP* or *UAS-GCaMP* in adult wings, although these drivers were found to be fully active in larvae and/or in the adult proboscis using *UAS-mCD8::GFP* (GFP attached to the membrane). In contrast, the *Tub-GAL4* driver elicits a strong fluorescence signal in the wing neuronal cells with no background noise in the other wing structures. We found that *GR-GAL4s* crossed with UAS-GFP worked well for localization in larval tissue and adult proboscis, but were inefficient for monitoring the calcium signaling in the wing. The reason likely resides in that weak *GR* promoters are not able to overcome the massive endogenous expression of calmodulin or other calcium-binding proteins for the capture of calcium. As a consequence, the GCaMP expressed at low levels failed to provide significant fluorescence signals in wings. Moreover, we have observed that the wing chitin barrier strongly hampered the excitation/emission of light for fluorescence study in the GR-GAL4s > UAS-mCD8::GFP strain. Conversely, *Tub-GAL4* worked well because the GCaMP expression obviously competes favorably with endogenous calmodulin and/or other calcium-binding proteins and because this promoter is strong enough to overcome the chitin barrier. The wings of 5-day-old *UAS-GCaMP*/+; *Tub-GAL4*/+ flies were used for our studies. Briefly, wings were dissected with a razor blade and immediately mounted in water between a glass slide and a coverslip for observation with a ZEISS LSM 510 META confocal microscope (20× objective). A drop of the sugar solution (1 mM to 1 M in water) or bitter molecules (10 μM to 20 mM in water) was deposited at the edge of the coverslip, and the time course of the fluorescence variation was recorded in real time. A 900-s-long movie was obtained for each experiment. The figures shown represent a few consecutive sequences within each movie.

Pulverization of Sugar/Water Solution and Quinine or Denatonium/Water Solutions

A nebulizer device was used to create fog inside a chamber (50 × 50 × 75 cm). A paper towel soaked in water was placed in the chamber. For the control without nebulization and the experiments with quinine and denatonium, the paper towel was soaked in water enriched with 50 μM glucose. A solution of water/sweet or bitter molecules (100 mM glucose or 10 mM denatonium or quinine) was placed inside the reservoir. An open plastic cage with a hole at the top and a mesh on the side was fixed above the nebulizer to allow flies to aggregate inside the device. 100 (5-day-old) flies of both sexes were released in the chamber, and the number of flies aggregating inside the cage was counted during the course of the 2-day experiment. The lethality analysis was only conducted for the control without nebulization and was found to be null after 24 hr for all the tested strains and reached the same value for all strains at 48 hr: a mean ± SE of 12% ±5.

Food Conditioning

We designed a behavioral protocol based on a Bayesian paradigm (a first-step sensory experience subsequently influences/guides the exploration/choice in a second step in a new environment) (Collett, 2012; Naug and Arathi, 2007; Biernaskie et al., 2009). This protocol consists of two consecutive steps: (1) a conditioning period (3 hr) during which flies were allowed to groom and spread molecules from the environment to their wings and (2) a test period (24 hr) during which their ability to aggregate on a food source in another context was measured. More specifically, 40 male and female flies (7 to 8 days old) were placed in 30-ml vials (three vials per genotype and per experiment) to be conditioned by physical contact with a filter paper that was soaked with a liquid stimulus: (1) fresh grape juice, (2) spoiled grape juice (1 month after opening and presenting strong bacterial and fungal contaminations), and (3) water plus sugar (used as a control stimulus). Then, 100 conditioned flies were immediately transferred to a cage (50 × 50 × 75 cm) containing a pierced plastic tube containing fresh grape juice, in darkness. A water-soaked paper towel was placed in this chamber. No significant lethality in the different strains was observed after 24 hr. To standardize our results, we showed the ratio of the number of accumulated flies conditioned either with the fresh or the spoiled grape juice against the number of accumulated control flies (conditioned by the water plus sugar solution) (n = 15).

Aggregation and Egg-Laying Activity

100 7-day-old *CS*, *UAS-RNAi-Poxn*, *Ser-GAL4*, and $Poxn^{W}$ flies (female and male) were released in a cage (50 × 50 × 75 cm) in which two grape juice/agar spots were placed. The aggregation was examined every 2 hr for 14 hr, and the number of eggs was counted at the end. An additional control was performed with *D. melanogaster* flies that had been newly captured in the south of France; in these flies, the right or left wing had been subjected to a surgical unilateral cut of the margin nerve. These flies were kept at 4°C for 20 min and then were immobilized with a small paint brush in order to cut the margin nerve with a razor halfway through the wing.

Statistical Analysis

Paired Student's t test was used to compare two groups. For analyses presenting more than two groups, one-way ANOVA was conducted, followed by additional statistical tests. Thus, for the analysis of behavioral experiments (Bayesian conditioning, trajectomotry analysis of the chemo-oriented behavior using Noldus technology), the Tukey non-parametric test and the Wilcoxon-Mann-Whitney test were performed. Statistical significance was determined by the p value indicated in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, five figures, one table, four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.040.

AUTHOR CONTRIBUTIONS

H.R. conceived the experimental design and performed the following experimental work: genetic constructs, fluorescence experiments, behavioral studies, molecular work, and data analysis. J.-F.F. participated in the experimental design and manuscript drafting. N.L. performed the molecular biology

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work. M.C. wrote the manuscript, conceived of the experimental genetic design, and analyzed the data. A.R. designed experiments, analyzed experiments, and wrote the manuscript.

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